ISSN 1927-0887 (Print)
ISSN 1927-0895 (Online)

Journal of Food Research

Vol. 4, No. 5 October 2015

Canadian Center of Science and Education®

Editorial Board

Contents

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Comparative Studies on the Physicochemical and Sensory Properties of Watermelon (*Citrullus lanatus*) and Melon (*Citrullus vulgaris*) Seed Flours Used in "EGUSI" Soup Preparation

Monday O. Akusu[1] & David B. Kiin-Kabari[1]

[1] Department of Food Science and Technology, Rivers State University of Science and Technology, Port Harcourt, Nigeria

Correspondence: David B. Kiin-Kabari, Department of Food Science and Technology, Rivers State University of Science and Technology, Port Harcourt, Nigeria. Tel: 234-803-775-7473. E-mail: kabaridavid@yahoo.com

Received: April 26, 2015 Accepted: June 10, 2015 Online Published: June 12, 2015

doi:10.5539/jfr.v4n5p1 URL: http://dx.doi.org/10.5539/jfr.v4n5p1

Abstract

A comparative study on the physicochemical and sensory properties of watermelon (*Citrullus lanatus*) and melon (*Citrullus vulgaris*) seed flours in food preparation were investigated. A composite flour containing equal parts of watermelon seed flour and melon seed flour were prepared. Egusi soups were prepared from the melon seed flour; watermelon seed flour and a combination of the two flours in equal proportions. Sensory properties of the three soups were evaluated. The results of the investigation showed that the equal proportions of watermelon/melon seed flours had higher crude protein of 27.73% and crude fat of 47.85% than the water melon seed and melon seed flours. There was no significant difference ($P>0.05$) in water absorption, foam capacity, viscosity and least gelation properties of the melon seed flour compared to the 50:50 flour sample. The sensory properties showed no significant difference ($P>0.05$) in appearance, taste, thickness and overall acceptability of egusi soup from melon seed flour and 50:50 flour sample. Therefore watermelon seed flour can be used to replace 50% melon seed flour in the preparation of egusi soup

Keywords: melon seeds, watermelon seed, egusi soup, comparative studies

1. Introduction

Melon (*Citrullus vulgaris*) is a member of the cucurbitaceous family and is the biological ancestor of water melon (*Citrullus lanatus*). It is a large plant family which includes many economic species such as melon, various gourds, pumpkins and cucumbers (Oyeleke et al., 2012). Melon (*Citrullus vulgaris*) is grown widely in the tropics and its seeds popularly known as "egusi" in Nigeria is consumed in various forms such as egusi soup, melon ball snacks and ogiri fermented melon seed (King & Onuora, 1983; Achinewhu, 1987).

Melon seeds have nutritive and caloric values which make them necessary in diets. Ojieh et al. (2008) reported that melon seeds (egusi) contain 3.7% ash, 45.7% ether extract, 23% crude protein, 12% crude fibre and 10% total carbohydrate. Similar proximate composition had been reported by Kiin-Kabari and Akusu (2014) on watermelon seed flour using different processing methods. The oils from melon seed and watermelon had been characterized by other researchers Oresanya et al. (2000), Ebuehi and Avwobobe (2006) and they observed that melon seed oil contained more of unsaturated fatty acids than watermelon seed oils.

The consumption of watermelon fruit in Nigeria had increased tremendously in recent years due to the increased awareness on the health benefits. The watermelon juice contains important carotenoids such as β-carotene, carotene and Lycopene which are important in neutralizing free radicals in the body (Oseni & Okoye, 2013; Penuel et al., 2013). In Nigeria, the utilization of watermelon fruits is limited to the direct consumption of the fresh fruits. The fruits contain seeds which are un-utilized fruit by-products. The seeds are discarded and not eaten but are consumed in other parts of the world either roasted and served as snacks or milled into flour for incorporation into wheat flour and baked into bread (El-Adway & Taha, 2011). Watermelon seeds are high in proteins and fats and can find applications as a protein source in various food formulations and preparation (El-Adway & Taha, 2011). Shadrach and Oyebiodun (1999) reported that the ultimate success of utilizing plant proteins as ingredients largely depends upon the beneficial qualities they impact to foods which also depend largely on their nutritional and functional properties.

Melon seeds (egusi) is used traditionally as the basis for a number of soup preparation especially the popular "egusi" soup in Nigeria, were the melon seed act as a thickener in the soup.

Water absorption, viscosity and the least gelation concentration are all important functional properties in egusi – like soup preparation (Kiin-Kabari & Akusu, 2014). One of the major sensory attributes of the egusi soup is the thickening properties of the soup, whether it is prepared from melon seeds flour or water melon seed flour.

However, melon (egusi) seeds are becoming very expensive in Nigeria whereas water melon seeds are discarded after the consumption of the water melon fresh fruits.

Improvement in the utilization of both melon seeds and water melon seeds can be achieved if we understand their proximate and functional behaviour of the seed flour in food preparation.

The functional properties are important in determining the organoleptic properties of "egusi" and "egusi-like" soup preparation. Combining melon seeds and watermelon seeds in "egusi-like" soup preparation may be of interest in reducing the costs of "egusi" soup preparation.

This study was aimed at evaluating the potential food uses of water melon seeds by comparing its proximate, functional and sensory properties with that of melon (egusi) seed in soup preparation as well as evaluating the functional impact on the organoleptic/ acceptability of "egusi-like" soup prepared from a 50:50 melon: Water melon seed flour blends.

2. Materials and Methods

2.1 Materials

Watermelon fruits (*Citrullus lanatus*) were purchased from a local market in Port Harcourt and transported to the Department of Food, Science and Technology laboratory for processing. All chemicals used were of the analytical grades, products of BDH chemical Ltd pool, England.

2.2 Methods

2.2.1 Preparation of Watermelon Seed Flour

Watermelon seeds were removed from the pod, washed, pre-boiled for 5 min and sun-dried at 34 °C for 3 days. The sun-dried seeds were dehaulled and milled into flour as shown in Figure 1a.

2.2.2 Preparation of Melon Seed Flour

Five (5) kg of melon seed was shelled, sorted, cleaned and oven dried (50 °C, 24 h) in a hot – air fan circulating oven (model QUB,305010G, Gallenkamp, U.K), ground to pass through a 0.25 mm British standard sieve (Model B5410, Endecoths Lt, London, UK), as shown in Figure 1b.

Both watermelon seed flour and melon seed flour were divided separately into fifteen lots for each; stored in air tight containers in a refrigerator. A 50:50 ratio was used based on the preliminary study on various blends of melon/watermelon seed flour that was most acceptable.

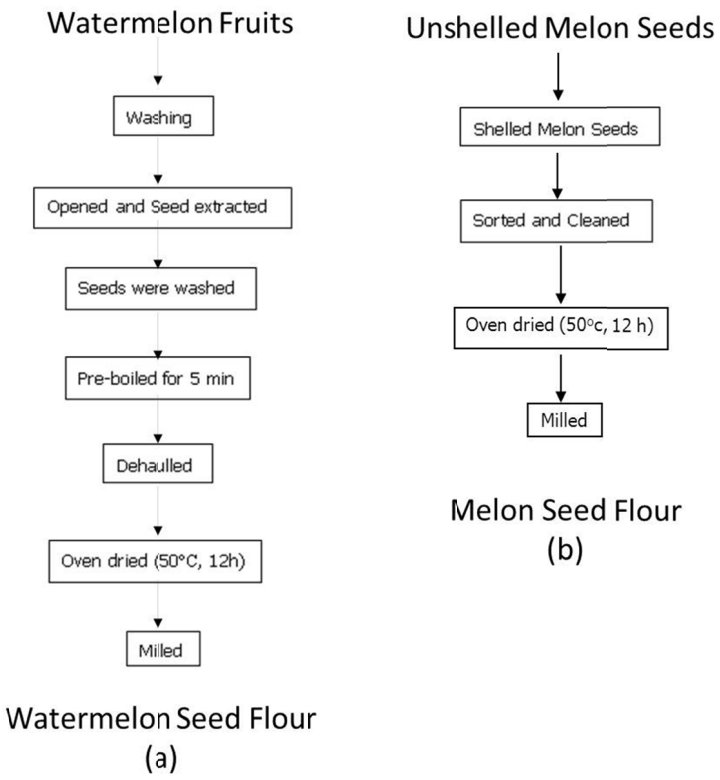


Figure 1. Flow chart for production of (a) Watermelon seed flour (b) Melon seed flour

2.2.3 Proximate Analysis of Watermelon/Melon Seed Flours

Moisture content (method 14.004), total ash (method 14.006), crude fiber (method 7.070), either extract (method 7.062) and crude protein (method 2.057) were determined according to AOAC (2006) procedures. The conversion factor N × 6.25 was used for conversion of nitrogen to crude protein. Carbohydrate content was determined using the method of clegg Anthrone reported by Osborne and Voogt (1978).

2.2.4 Physicochemical Properties

Some functional properties were determined on the melon seed flour, watermelon seed flour and the 50:50 melon/watermelon seed flour samples. Water and oil absorption capacities were determined according to the method of Beuchart (1977). The least gelation concentration and viscosity were determined according to the methods of Coffman and Garcia (1977) and Fleming et al. (1975).

Table 1. Common recipe for "egusi" soup

Ingredients	**Weight/volume**
Ground watermelon or melon seed flour	200 gm
Palm oil	60 ml
Water	700 ml
Onion	20 gm
Maggi cube	3.5 gm
Pepper	1.5 gm
Salt	0.2 gm

Source: Authors computation.

2.2.5 Preparation of Egusi Soups

Palm oil was heated in a pot for 2 min then chopped onion was added and finally watermelon or melon seed and watermelon/melon seeds blend was added and stirred for 15 min. 700ml water was added, maggi, pepper and salt was added to taste. The soup was allowed to cooked for 25 min as shown in Figure 2.

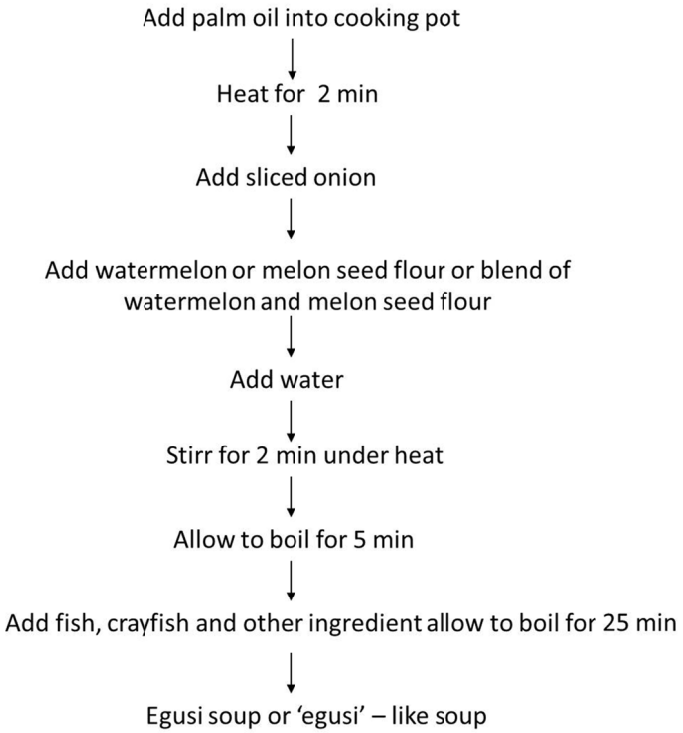


Figure 2. Flow chart for the preparation of traditional egusi soups

2.2.6 Sensory Evaluation

A panel of 20 people who are used to egusi soup were used for the sensory evaluation of the soup prepared with watermelon seed flour, melon seed flour and melon seed flour.

A 9-point hedonic scale with 9 = lie extremely, 5 = neither like nor dislike and 1 = dislike extremely was used for the evaluation of the prepared soups for colour, appearance, thickness, taste, aroma and overall acceptability. The panelists were served the "egusi" like soup and the egusi soup in the food and nutrition laboratory of the food science and technology department at room temperature (28 ± 2 °C).

Three soups were prepared: watermelon seed flour soup, melon seed flour soup (egusi soup) as control and a mixture of 50% water melon seed flour and 50% melon seed flour as shown in Table 1.

2.2.7 Statistical Analysis

The data obtained were subjected to analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS) version 20.0 software 2011. All analysis was done in triplicate and means separated using Duncan Multiple Range Test.

3. Results and Discussion

Table 2 shows the proximate composition of watermelon seed flour, melon seed flour and a 50:50 watermelon: melon seed flour blend. The 50:50 blended samples had a higher crude protein of 27.73% and crude fat of 47.85%, watermelon seed flour crude protein of 25.33% and crude fat of 45.66%. Melon seed flour had crude fat of 42.89% and crude protein of 25.36%, respectively. There was no significant difference in the moisture content and crude fibre of the three flour samples. These values are within the range reported by Akobundu et al. (1982), Akubor (2005) and Oyeleke et al. (2012).

Table 2. Proximate composition of watermelon and melon seed flour

Parameters (%)	**Watermelon seed flour**	**Melon seed flour**	**Watermelon flour and melon seed flour (50:50 blend)**
Moisture	9.59^{a}	9.30^{a}	9.47^{a}
Ash	3.36^{ab}	3.33^{b}	3.71^{a}
Crude fat	45.66^{b}	42.89^{c}	47.85^{a}
Crude protein	25.33^{b}	25.36^{b}	27.73^{a}
Crude fibre	4.20^{a}	3.83^{a}	4.30^{a}
Carbohydrate	11.86^{b}	15.31^{a}	6.54^{c}

abc= means bearing the same superscript within the same row do not differ significantly (P>0.05).

The selected functional properties of watermelon seed flour, melon seed flour and the 50:50 blended sample are shown in Table 3. There was no significant difference (P>0.05) in water absorption capacity, foam capacity, viscosity and least gelation concentration between melon seed flour and the 50:50 blended sample; however, watermelon seed flour gave lower values for these functional properties. Ige et al. (1984) reported that proteins are linked to some functional properties such as foaming, water absorption, viscosity and gelation. Matti (1970) reported that the desirability of carrying out functional test is to predict how the plant proteins will affect the food system in which they are incorporated.

Table 3. Selected functional properties of watermelon seed flour and melon seed flour

Functional properties	Watermelon seed flour	Melon seed flour	50:50 blend of watermelon and melon flour
Water absorption (g/g)	0.96[b]	1.47[a]	1,39[a]
Oil absorption (g/g)	1.13[b]	1.45[a]	1.10[b]
Foam capacity (ml/s)	6.50[c]	9.56[a]	8.95[a]
Viscosity (Pa.s)	0.87[b]	1.42[a]	1.40[a]
Least gelation Concentration (%)	0.95[b]	0.65[a]	0.70[a]

abc= means bearing the same superscript within the same row do not differ significantly (P>0.05) means of triplicate determinations.

The sensory properties of "egusi" soups prepared with watermelon seed flour, melon seed flour and the 50:50 blended sample is shown in Table 4. The result revealed that there was no significant difference (P>0.05) between melon seed flour soup and the 50:50 blended sample soup in terms of appearance, taste, flavour, thickness and overall acceptability. The viscosity of the melon seed flour is higher which may affect the thickness and functional properties such as foaming capacity, water absorption capacity and least gelation concentrations. This is similar to the observation made by Akusu and Kiin-Kabari (2013) on the relationship between viscosity and other functional properties of watermelon seed flour.

Table 4. Means sensory evaluation of watermelon seed flour soup, melon seed flour soup and 50:50 water melon seed flour: melon seed flour soup

Samples	Appearance	Colour	Taste	Flavour	Thickness	Overall acceptability
Melon seed flour soup (control)	5.2[a]	5.1[a]	4.6[a]	4.3[a]	5.5[a]	5.7[a]
Water melon seed flour soup	4.3[b]	4.3[b]	3.6[b]	3.4[b]	3.9[b]	4.5[b]
Watermelon/melon seed flour soup 50:50	5.3[a]	4.5[a]	4.4[a]	4.4[a]	5.2[a]	5.5[a]

abc = means with the same superscript within the same column do not differ significantly (p>0.05) values are the mean scores.

4. Conclusion

Equal proportions of melon/watermelon seed flours compared favourably well in viscosity, water absorption, foam capacity and least gelation concentration when compared to melon seed flour alone.

Similar patterns were observed in sensory properties of appearance, taste and overall acceptability of egusi soups. Thus the cost of egusi preparation can be reduced by substituting upto 50% of melon seed flour with watermelon seed flour which before now is un-utilized.

References

Achinewhu, S. C (1987). Carbohydrate and fatty acid composition of fermented melon seeds (*Citrullus Vulgaris*) *J. Ed. Science and Technology, 24*, 16-19.

Akobundu, E. N. T., Cheery, J. P., & Simmons, J. G. (1982). Chemical, functional and nutritional properties of "egusi" (*Colocynthis Citrullus L.*) Seed Protein Product. *J Food Science, 47*, 829-835. http://dx.doi.org/10.1111/j.1365-2621.1982.tb12725.x

Akubor, P. I. (2005). Influence of processing methods on the chemical and functional properties of melon seed. *J of Food Science and Technology, 41*(2), 181-185.

Akusu, M. O., & Kiin-Kabari, D. B. (2013). Effect of storage period on selected functionaol chemical stability and sensory properties of bush mango (*Irvingia Gabonensis*) seed flour. *African Journal of Food Science and Technology, 4*(6), 136-140.

AOAC. (2006). *Official Method of Analysis* (18th ed.) Washington, D.C: Association of Official Analytical Chemist.

Beuchat, L. R. (1977). Functional and electrophoretic characteristics of succinylated peanut flour proteins. *J. Agric Food Chemistry, 25*, 258-261. http://dx.doi.org/10.1021/jf60210a044

Coffman, C. N., & Garcia, V. V. (1977). Functional properties and amino acid content of a protein isolate from mung bean flour. *F Food Technology, 12*, 473-484. http://dx.doi.org/10.1111/j.1365-2621.1977.tb00132.x

Ebuehi, O. A. T. & Avwobobe, O. K. (2006). Physico-chemical and fatty acid composition of watermelon (*Citrullus lanatus*) and melon (*Cococynthis citrullus*) seed oils. *Nigerian Food Journal,* 24(1), 25-33.

EL-Adaway, T. A., & Taha, K. M. (2011). Characteristics and composition of different seed oil and flours. *Food Chemistry, 74*, 47-54. http://dx.doi.org/10.1016/S0308-8146(00)00337-X

Fleming, S. C., Sosulski, F. N., & Hamin, N. W. (1975). Gelation and thickening phenomena protein. *J. Food Science, 40*, 805-807. http://dx.doi.org/10.1111/j.1365-2621.1975.tb00562.x

Ige, M. M., Ogunsua, A. O., & Oke, O. L. (1984). Functional properties of the proteins of some Nigeria oil seeds: Casophor seeds and three varieties of some Nigerian oil seeds. *Food Chemistry, 32*, 822-825. http://dx.doi.org/10.1021/jf00124a031

Kiin-Kabari, D. B., & Akusu, O. M. (2014). Effect of processing on the proximate composition, functional properties and storage stability of water melon (*Citrullus Lanatus*) seed flour. *International Journal of Biotechnology and Food Science, 2*(7), 143-148.

King, R. D., & Onuora, O. J. (1983). Aspects of Melon Seed Protein Characteristics. *Food Chemistry, 14*, 65-67. http://dx.doi.org/10.1016/0308-8146(84)90019-0

Mattil, K. F. (1970). Considerations for choosing the right proteins. *Food Product Development, 7*(6), 40-43.

Oseni, O. A., & Okoye, V. I. (2013). Studies of phytochemical and Antioxidant properties of fruit of water melon (*Citrullus Lanatus*) *J. Pharma. Biomed. Sci, 27*(27), 508-514.

Oyeleke, G., Olagunju, E. O., & Ojo, A. (2012). Functional and physiochemical properties of watermelon (*Citrullus Lanatus*) seed and oil. *Journal of Applied Chemisty, 2*(2), 29-31.

Ojieh, G. C., Oluba, O. M., Ogunlowo, Y. R., Adebisi, K. E., & Eidangbe, G.O. (2008). Compositional Studies of Citrullus Lanatus Seed; *Internet Journal of Nutrition and Wellness, 6*(1), 1-10.

Oresanya, M. O., Ebuehi, O. A. T., Aitezemuller, K., & Koleosho, O. A. (2000). Extraction and characterization of Guna melon (*Citrullus cococynthis*) seed oil. *Nigerian Journal of Natural Medicare*, 4:76-78.

Osborne, D. R., & Voogt, P. (1978). *The Analysis of Nutrients in Foods* (pp. 103-134). London Academic Press.

Penuel, B. L., Khan, E. M., & Maitera, M. O. (2013). Properties of proximate composition and Elementary Analysis of *Citrullus Vulgaris* (Guna) seed: *Bill. Env. Pharmacology and Life Science, 2*(2), 39-46.

Shadrach, O. A., & Oyebiodum, G. I. (1999). The physico – Functional characteristics of starches from cowpea, pigeon pea and yam bean. *Food Chemistry, 65*, 460-474

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

The Antioxidant and DNA Repair Activities of Resveratrol, Piceatannol, and Pterostilbene

Justin R. Livingston[1], Jacob J. Peterson[1], Gabriel A. Martinez[1], Connor J. Peck[1], Andrew R. Garrett[1], Rachel A. Uhl[1], Brett H. Thompson[1], Gajendra Shrestha[2], Richard A. Robison[1] & Kim L. O'Neill[1]

[1] Department of Microbiology and Molecular Biology, Brigham Young University, Utah, USA

[2] Department of Biology, Brigham Young University, Utah, USA

Correspondence: Kim L. O'Neill, Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT 84602, USA. Tel: 1-801-422-2449. E-mail: kim_oneill@byu.edu

Received: May 7, 2015 Accepted: June 10, 2015 Online Published: June 12, 2015

doi:10.5539/jfr.v4n5p9 URL: http://dx.doi.org/10.5539/jfr.v4n5p9

Abstract

Lifestyle diseases represent a large burden on developed societies and account for much morbidity worldwide. Research has shown that eating a diet rich in fruit and vegetables helps to ameliorate and prevent some of these diseases. Antioxidants found in fruits and vegetables may provide a substantial benefit in reducing disease incidence. This study examines the antioxidant properties of resveratrol, piceatannol, and pterostilbene, and the ability of Burkitt's Lymphoma (Raji) cells to uptake these three antioxidants. It also studies the effect of the antioxidants in protecting against DNA damage, and their role in DNA repair following oxygen radical exposure in Raji cells. The Oxygen Radical Absorbance Capacity (ORAC) assay was used to measure overall antioxidant contribution as well as the ability of Raji cells to uptake antioxidant following exposure to 2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH). The single cell gel electrophoresis (Comet) assay was used to assess DNA damage and DNA repair rates of cells. Results showed that Raji cells, following oxygen radical exposure, significantly uptake pterostilbene ($p < 0.0001$), but not piceatannol or resveratrol. Piceatannol provided protection against hydrogen peroxide induced DNA damage, but pterostilbene and resveratrol increased DNA damage following hydrogen peroxide treatment. None of the compounds showed any effect on DNA repair. Overall, this study indicates there is merit for further research into the bioactive roles, including antioxidant capacity, of all three compounds. Such research may provide evidence for the more widespread use of these and other food based compounds for preventing lifestyle diseases.

Keywords: antioxidant, comet assay, DNA repair, orac, piceatannol, pterostilbene, resveratrol

1. Introduction

It has been estimated that an unhealthy diet coupled with physical inactivity account for 10% of the global burden of disease (England, Andrews, Jago, & Thompson, 2015). Unhealthy dietary habits include a low consumption of fruits and vegetables. Cardiovascular disease, ischemic stroke, diabetes, and cancer are some diseases that may develop due to poor lifestyle choices such as diets with low consumption of fruits and vegetables. Recent research has shown that a diet with high fruit and vegetable consumption may reduce the risk of these and other diseases (Bussel et al., 2015; Carter, Gray, Troughton, Khunti, & Davies, 2010; Epps, 2013; Joshipura et al., 1999).

Lifestyle diseases are a large area of concern and are the subject of intensive research globally (Edwards et al., 2014). Oxidative stress is believed to be one of the major causes of these diseases (Mahalingaiah & Singh, 2014; Singh, Vrishni, Singh, Rahman, & Kakkar, 2010). Our bodies naturally produce oxygen radicals as part of our cellular metabolism, but our cells also possess mechanisms to deal with those stresses (A. R. Garrett et al., 2014). These natural mechanisms provide considerable protection; however, excess oxygen radicals and reduced antioxidant availability may lead to imbalance and disease (Koppenhofer et al., 2015). Oxidative imbalance due to lifestyle factors can be partially prevented by consuming antioxidants. Antioxidants from diet, such as those found in fruits and vegetables, reduce oxidative stress and prevent oxygen radical DNA damage (Kuate, 2013; Sagrillo et al., 2015). Many fruits and vegetables have been shown to exhibit robust antioxidant activity (Gupta-Elera et al., 2012; Gupta-Elera, Garrett, Martinez, Robison, & O'Neill, 2011). One antioxidant, resveratrol, has previously been reported to have high antioxidant activity and was believed to be a central factor

in what is known as the French Paradox (Aschemann-Witzel & Grunert, 2015; Yamagata, Tagami, & Yamori, 2015). The French Paradox is the observation that even though the French diet includes large amounts of saturated fats, they have a low incidence of coronary heart disease. Their high intake of red wine was believed, among other things, to be responsible for the lower disease incidence rates because it contains the powerful antioxidant resveratrol. Resveratrol can be found naturally in many food sources such as grapes, peanuts, and berries (Calamini et al., 2010; Rimando, Kalt, Magee, Dewey, & Ballington, 2004). There has been evidence suggesting that the *in vivo* effect of resveratrol may be limited due to its low bioavailability (Davidov-Pardo & McClements, 2015; Semba et al., 2014). Bioavailability is the ability of a drug to enter the circulation and cause an effect once it is consumed.

Resveratrol has two relatively lesser studied analogs, pterostilbene and piceatannol, which may provide valuable alternatives to resveratrol for protection against chronic disease. Piceatannol structurally differs from resveratrol by an additional 3' hydroxyl group (Figure 1), which may contribute to better antioxidant and bioavailability properties than resveratrol (A. R. Garrett et al., 2014; Tang & Chan, 2014). Piceatannol can be naturally found in berries, grapes, and passion fruit (Maruki-Uchida et al., 2013; Rimando et al., 2004). Pterostilbene is a dimethyl ether form of resveratrol (Figure 1), and is also found naturally in grapes and blueberries (Sato et al., 2014). Pterostilbene has also been shown to have greater bioavailability than resveratrol and may serve as a more attractive alternative to resveratrol for antioxidant and other functional properties (Kapetanovic, Muzzio, Huang, Thompson, & McCormick, 2011). Overall these three compounds have been reported to exhibit a variety of potential biological effects such as anticancer properties (Xia, Deng, Guo, & Li, 2010), cardiovascular disease prevention (McCormack & McFadden, 2013), and apoptosis induction (Jancinova, Perecko, Nosal, Svitekova, & Drabikova, 2013).

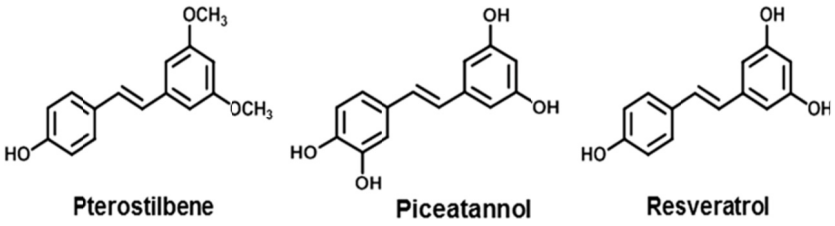


Figure 1. Structures of pterostilbene, piceatannol, and resveratrol

The purpose of this study was: (1) to compare the antioxidant properties of resveratrol, pterostilbene, and piceatannol, (2) to assess the ability of Burkitt's Lymphoma (Raji) cells to uptake these three compounds following challenge with an oxidizing stimulus, (3) to determine the ability of these compounds to protect against DNA damage, and (4) to identify the effect of resveratrol, piceatannol, and pterostilbene on DNA repair rates in Raji cells. We used the Oxygen Radical Absorbance Capacity (ORAC) assay to measure the overall antioxidant properties as well as the ability of cells to uptake antioxidant following exposure to AAPH and the single cell gel electrophoresis (Comet) assay to measure DNA protection and DNA repair. Due to the structural differences discussed earlier, we hypothesized that pterostilbene and piceatannol would exhibit higher utilization and protective effect compared to resveratrol following oxidative challenge. We also hypothesized that these compounds would provide protection against DNA damage and possibly modulate DNA repair when compared to a placebo phosphate buffered saline (PBS) treatment.

2. Materials and Methods

2.1 Chemicals

2,2'-Azobis(2-methyl-propionamide) dihydrochloride (AAPH), Fluorescein sodium salt, Propidium Iodide, Resveratrol, and 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich, Inc. (Milwaukee, WI). Piceatannol and Pterostilbene were purchased from Cayman Chemical (Ann Arbor, MI). Hydrogen Peroxide was purchased from Fisher Scientific (Pittsburg, PA).

2.2 Materials

Costar 3694 96 well plates were obtained from Corning Inc. Cellstar 12 well cell culture plates were obtained from Greiner Bio-One International.

2.3 Equipment

A BMG FLUOstar Optima plate reader (S/N 413-0225) was used to measure fluorescence readings for ORAC assays. A Zeiss Axioscope fluorescence microscope was used to image all Comet experiments. A Misonix Sonicator 3000 was used for cell lysis.

2.4 Cell Culture

Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations at 37 °C and 5% CO_2.

2.5 Compound Preparation

Resveratrol, pterostilbene, and piceatannol were each dissolved initially at 40 mM concentrations in DMSO. These samples were then diluted in PBS to 400 μM aliquots. These aliquots were further diluted, in PBS, as needed to working concentrations for their respective assays. Aliquots were stored in fluorescence protection bags at -20 °C until needed. Aliquots were tested before freezing and after being frozen to ensure effectiveness; no difference in effectiveness was observed.

2.6 Procedure

2.6.1 Oxygen Radical Absorbance Capacity (ORAC) Assay

All samples were diluted to a 24 μM concentration and then analyzed according to the method published in Gupta-Elera et al. (2012) with the following modifications: 20 μL of AAPH was used instead of 25 μL AAPH, and assays were run for 120 minutes instead of 90 minutes. The final concentration of all samples tested was 2 μM. The ORAC assay was used because it is a well-established method of measuring antioxidant activity *in vitro* (Andrew R. Garrett, Murray, Robison, & O'Neill, 2010). The sample to be tested was mixed with a fluorescent molecule (Fluorescein) and an oxygen radical generator (AAPH). The level of fluorescence was measured every 2 minutes over a 120 minute period to create fluorescence decay curves. Experimental samples were run in the same plate as a standard (Trolox) and control samples (Fluorescein alone and Fluorescein plus AAPH). Samples were run in a BMG FLUOstar Optima plate reader with 485 nm emission and 590 nm excitation filters. Samples were run in six different plates with twelve replicates per plate for each compound.

2.6.2 Standard Curve

The area under the curve (AUC) was measured for all experimental and standard samples from their respective fluorescence decay curves. The AUC for each sample and standard was calculated by subtracting the AUC of Fluorescein plus AAPH from the AUC of the sample/standard. The AUC for the standard samples were then plotted to create a standard curve. The AUC of each sample was compared to the standard curve to determine its antioxidant activity in Trolox Equivalents per mg sample (TE/mg).

2.6.3 Cellular Uptake of Antioxidant following Oxygen Radical Generator Exposure

Cellular uptake of antioxidant by Raji cells after oxygen radical generator exposure was measured by following the method published in Gupta-Elera et al. (2011) with some modifications. In short, Raji cells were incubated with AAPH for 10 minutes to simulate oxidative stress. Following incubation, they were washed and then treated with 10 μM antioxidant for 10, 20, 45, or 60 minutes. Following treatment, the samples were washed 2 times and sonicated to lyse. Following sonication, the samples were centrifuged for 30 minutes at 3000 g. The supernatant was then removed as the lysate fraction. The pellet was washed and used as the membrane fraction. Three different plates per compound were run for both membrane and lysate fractions with twenty-four replicates per plate.

2.6.4 Protection Against DNA Damage

Raji cells were incubated for 90 minutes at a 10 μM concentration of the compounds. Following incubation, cells were washed twice and then exposed to 10 μM H_2O_2 for 10 minutes. The cells were then washed and re-suspended in warm RPMI 1640 and prepared for comet assay as described below.

2.6.5 DNA Repair

Raji cells were incubated in 500 μL of 10 μM H_2O_2 for 10 minutes to induce DNA damage. Following incubation, 1 mL of cold RPMI 1640 media was added to the cells. The cells were then centrifuged at 450 g for 5 minutes at 4 °C, washed with cold PBS, and re-suspended in fresh warm media. Cells were then treated with the compounds and allowed to repair for a predetermined amount of time (0, 5, 15, 30, 60, and 90 minutes). Samples were then prepared for comet assay as described below.

2.6.6 Comet Assay

Following both protection and repair protocols, samples were prepared for comet analysis by following the methods described by Xiao et al. (2014) with slight modifications. Briefly, samples were mixed with low melting point agarose and layered on double frosted microscope slides (Xiao et al., 2014). The slides were placed in alkaline lysis buffer for 1 hour, rinsed with ddH_2O and then placed in alkaline electrophoresis buffer for 20 minutes. They were then electrophoresed for 30 minutes. Following electrophoresis, slides were allowed to rest in ddH_2O for 15 minutes, then fixed in -20°C 100% ethanol for 5 minutes and allowed to dry prior to being stained with Propidium Iodide and imaged. All comets were scored using TriTek CometScore Freeware v1.5. Samples were run in three different trials with fifty comets imaged per sample, totaling 150 comets per sample.

Comet assay results are reported in terms of Tail Moment. Tail moment is defined as the product of the tail length and the % of DNA in the tail. These values are given as part of the output by the CometScore software and are widely reported for Comet analysis (Olive, Banath, & Durand, 2012).

2.7 Statistics

All statistics were performed using JMP Pro 11. ANOVA and the Tukey-Kramer HSD tests were used to compare compounds and PBS for all ORAC and cellular uptake data. Welch's T test was used to compare treatment and non-treatment groups for the Comet Repair data. A Dunnett's Procedure was used to compare all treatment groups with the PBS treatment for the DNA protection data. All statistics were calculated at the family-wise $\alpha = 0.05$ level. All error bars represent the 95% confidence interval of the mean.

3. Results

3.1 ORAC Antioxidant Values

The raw antioxidant values of pterostilbene, piceatannol, and resveratrol as measured by ORAC are given in Figure 2. Resveratrol exhibited the highest antioxidant activity with a mean value 311.403 TE/mg (CI $302.63 \leq \mu \leq 320.18$, N=72) followed by piceatannol and pterostilbene with a mean of 283.520 TE/mg (CI $274.75 \leq \mu \leq 292.29$, N=72) and 126.875 TE/mg (CI $118.10 \leq \mu \leq 135.65$, N=72) respectively. There was a significant difference ($p<0.0001$) in the mean TE/mg values among the tested compounds.

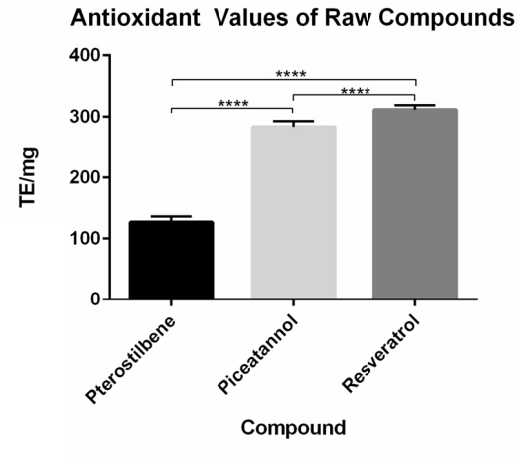


Figure 2. Antioxidant values of raw compounds as measured by ORAC assay reported as mean Trolox Equivalents per milligram (TE/mg). Each mean was significantly different than those of the other two compounds ($p < 0.0001$, $n = 72$ respectively).

3.2 Cellular Uptake of Antioxidant Following Oxygen Radical Generator Exposure

3.2.1 Lysate Fraction

Figure 3 shows the results from our cellular uptake model for the lysate fraction of Raji cells. There was a significant difference in the mean antioxidant capacity between the lysates of cells treated with the different compounds. Treatment of Raji cells with pterostilbene had the highest antioxidant activity with a mean of 22,407 TE/L/10^6 cells followed by piceatannol and resveratrol with mean values of 16,319 and 12,424 TE/L/10^6 cells,

respectively.

Treatment of Raji cells with pterostilbene produced significantly higher antioxidant values ($p<0.0001$), compared to piceatannol, resveratrol and the control. We also found significantly higher antioxidant values in Raji cells treated with piceatannol compared to those treated with resveratrol ($p = 0.0283$).

3.2.2 Membrane Fraction

Contrary to the lysate, the mean antioxidant values for the membranes of cells treated with any of the three compounds were less than raw Trolox (standard), (Figure 4). The treatment with the highest antioxidant value was pterostilbene, (mean = -4614 TE/L/10^6 cells). This treatment was the only treatment that was significantly different from the other groups ($p < 0.0001$). Piceatannol, resveratrol, and PBS had mean antioxidant values of -11024, -11390, and -10612 TE/L/10^6 cells, respectively. Antioxidant values are reported as negative because they are being compared to a standard curve of raw Trolox, as opposed to cells treated with Trolox. Thus, the amount of antioxidant in the membrane fraction is not as high as raw Trolox. There is no precedent for a Trolox standard curve from cells incubated with Trolox so results were compared to a raw Trolox standard curve.

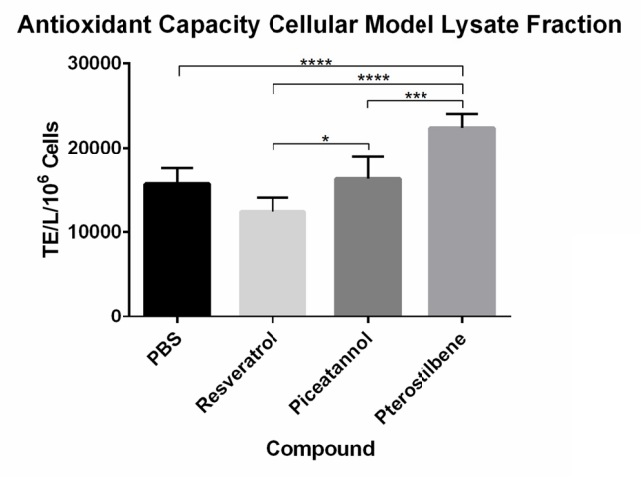


Figure 3. Mean antioxidant values of Raji cell lysates. Antioxidant values of cell lysate following challenge with AAPH and recovery with the specified antioxidant treatments. n = 72 for each treatment

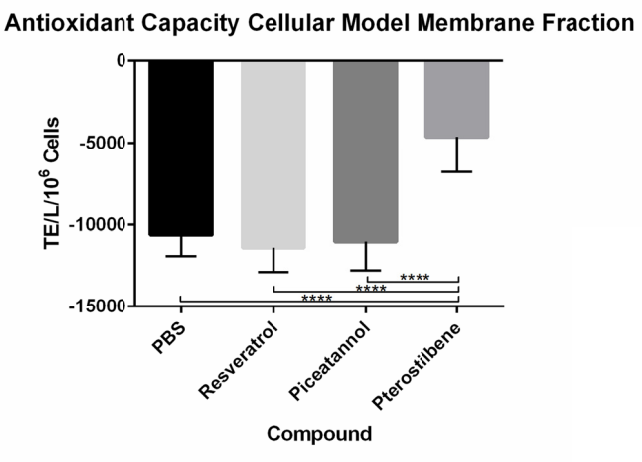


Figure 4. Mean antioxidant values of Raji cell membrane fractions following challenge with AAPH and recovery with the specified antioxidant treatments. n = 72 for each treatment

3.3 DNA Protection

The DNA protection assay was performed by pretreating Raji cells with one of the antioxidant compounds or the control (PBS) prior to treatment with hydrogen peroxide (Figure 5). Surprisingly, samples pretreated with pterostilbene and resveratrol exhibited larger tail moments than samples pretreated with PBS alone, ($p < 0.0001$). Samples pretreated with piceatannol had smaller tail moments than samples pretreated with PBS ($p < 0.0001$). No difference was observed between samples pretreated with PBS and samples receiving no pretreatment.

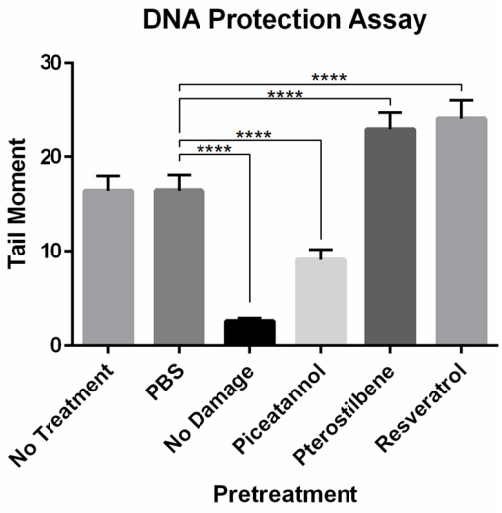


Figure 5. DNA protection. Raji cells were pretreated with antioxidant compounds prior to treating with H_2O_2. Larger tail moments indicate more DNA damage, n = 150 for each treatment

3.4 Comet Repair Assay

The results from our Comet repair assay showed a decrease in the tail moment of cells over time (Figures 6, 7, and 8); however, there was no difference in tail moment for samples treated with or without pterostilbene, piceatannol, or resveratrol after 90 minutes of repair p=0.9488, 0.7750, and 0.6785 respectively.

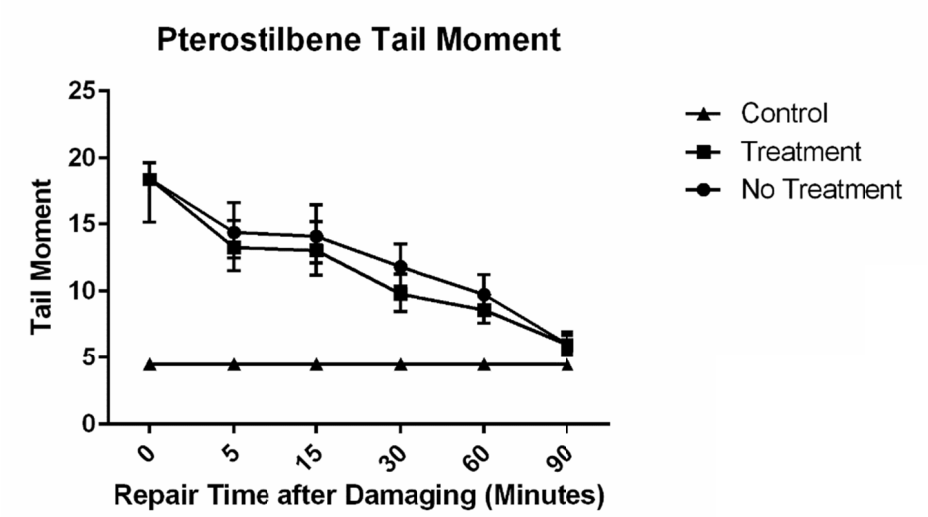


Figure 6. Tail moments for pterostilbene. Tail moments were measured for samples treated either with or without pterostilbene over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.9488$), n = 150 for each treatment at each time point

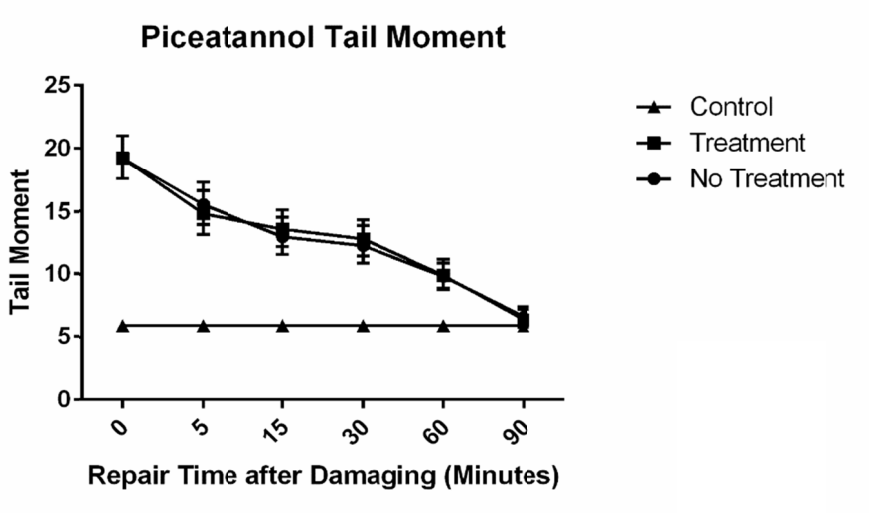


Figure 7. Tail moments for piceatannol. Tail moments were measured for samples treated either with or without piceatannol over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.7750$). $n = 150$ for each treatment at each time point

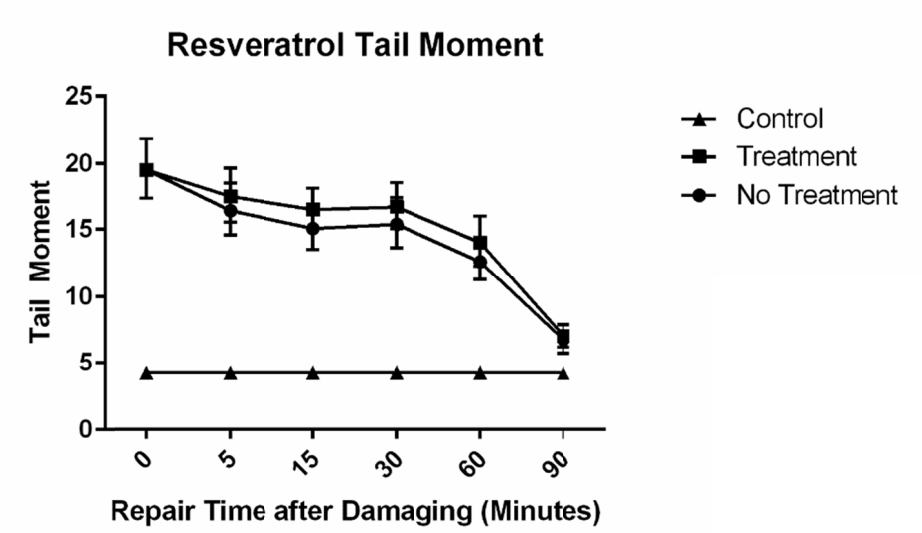


Figure 8. Tail moments for resveratrol. Tail moments were measured for samples treated either with or without resveratrol over 6 different time points. The differences were insignificant after 90 minutes of repair ($p = 0.0612$). $n = 150$ for each treatment at each time point

4. Discussion

The results of this study provide interesting information regarding resveratrol and its analogs: piceatannol and pterostilbene.

4.1 Analysis of Antioxidant Abilities

Even though resveratrol demonstrated significantly higher raw antioxidant activity than either piceatannol or pterostilbene, its ability to be absorbed by Raji cells for future antioxidant protection was weak. As suspected, pterostilbene did show a significantly increased ability to be absorbed compared to piceatannol, resveratrol, and control. Piceatannol was also shown to have more absorption ability than resveratrol in Raji cells. We tested this absorptive capacity at different time points, 10, 20, 45, and 60 minutes, as well as at 1 μM and 10 μM, concentrations. ANOVA revealed no significant differences between the antioxidant capacity between the two concentrations or the four time points for each individual compound, so the data from all time points and

concentrations was combined into 1 group for each compound. These compound groups were then compared to each other for the results reported here. This evidence helps to support our hypothesis that the structures of pterostilbene and piceatannol affect their ability to protect cells from internal oxidizing damage. These results suggest a need for further studies into the biological roles of both pterostilbene and piceatannol.

4.2 DNA Protection

We investigated the ability of these compounds to protect against DNA damage induced by treatment with hydrogen peroxide. We first wanted to ensure that these compounds were not exerting any cytotoxic effects on the Raji cells, so cells were exposed to each of these compounds. We did not see any loss of viability compared to cells incubated with PBS, as measured by Trypan blue exclusion after 24, 48, or 72 hours indicating that the results we obtained were not due to loss of cell viability (data not shown). We also wanted to ensure that no DNA damage was induced by these compounds so we performed comet analyses on Raji cells treated with each compound. We observed no difference in tail moments of Raji cells treated with these compounds when compared to cells treated with PBS, indicating that these compounds themselves had not caused DNA damage directly to the cells (data not shown).

To assess the ability of these compounds to protect Raji cells from DNA damage, we pretreated Raji cells with each compound and then exposed them to hydrogen peroxide. While piceatannol pretreatment demonstrated the ability to protect from DNA damage ($p< 0.0001$), resveratrol and pterostilbene pretreatment resulted in increased DNA damage following hydrogen peroxide exposure, ($p< 0.0001$). Reasons for this are unclear at this point. Increase in tail moment after pretreatment with resveratrol and pterostilbene may be due to anticancer effects or cell death inducing effects of these agents. We did not observe any loss of viability or increase in DNA damage after treatment with these compounds alone, so pretreatment may predispose Raji cells to undergo cell death following oxidative stress. Xia et al. (2010) reported that resveratrol has anticancer effects for prostate, breast and epithelial cancers. These results suggest that this effect may extend to lymphocytic cancers as well. Further work will help to elucidate and characterize these mechanisms.

4.3 DNA Repair Analysis

In an effort to investigate whether or not these compounds could help to repair previously damaged DNA, we tested both a 1 μM (data not shown) and a 10 μM concentration. No positive effects on DNA repair were observed at either concentration. We also looked at several time points to see if there were differences over time. We saw no significant effect on DNA repair rates of Raji cells that received antioxidant treatment versus those that did not. This result was observed for all time points and concentrations tested.

These results indicate that among the many biologically relevant roles of resveratrol, piceatannol, and pterostilbene, DNA repair modulation does not appear to be one of them. This study provided no additional evidence of resveratrol's usefulness as an intracellular antioxidant, but has shown the potential usefulness of pterostilbene in protecting cells from intracellular oxidizing damage. This work also provided information about the possible anticancer activities of resveratrol and pterostilbene. This preliminary study demonstrates the need to further investigate the biologically active roles of all three compounds. Such work may prove to establish mechanisms which may increase longevity and reduce aging. Foods containing antioxidants should be studied further to provide greater insight into the role of a healthy balanced diet in improving health. Such studies may be valuable in helping to alleviate many lifestyle diseases worldwide.

References

Aschemann-Witzel, J., & Grunert, K. G. (2015). Resveratrol food supplements: a survey on the role of individual consumer characteristics in predicting the attitudes and adoption intentions of US American and Danish respondents. *BMC Public Health, 15*, 10. http://dx.doi.org/10.1186/s12889-015-1348-7

Bussel, B. C. T. v., Henry, R. M. A., Ferreira, I., Greevenbroek, M. M. J. v., Kallen, C. J. H. v. d., Twisk, J. W. R., … Stehouwer, C. D. A. (2015). A healthy diet is associated with less endothelial dysfunction and less low-grade inflammation over a 7-year period in adults at risk of cardiovascular disease. *Journal of Nutrition, 145*(3), 532-540. http://dx.doi.org/10.3945/jn.114.201236

Calamini, B., Ratia, K., Malkowski, M. G., Cuendet, M., Pezzuto, J. M., Santarsiero, B. D., & Mesecar, A. D. (2010). Pleiotropic mechanisms facilitated by resveratrol and its metabolites. *Biochemical Journal, 429*(2), 273-282. http://dx.doi.org/10.1042/BJ20091857

Carter, P., Gray, L. J., Troughton, J., Khunti, K., & Davies, M. J. (2010). Fruit and vegetable intake and incidence of type 2 diabetes mellitus: systematic review and meta-analysis. *British Medical Journal, 341*, 8. http://dx.doi.org/10.1136/bmj.c4229

Davidov-Pardo, G., & McClements, D. J. (2015). Nutraceutical delivery systems: Resveratrol encapsulation in grape seed oil nanoemulsions formed by spontaneous emulsification. *Food Chemistry, 167*, 205-212. http://dx.doi.org/10.1016/j.foodchem.2014.06.082

Edwards, B. K., Noone, A. M., Mariotto, A. B., Simard, E. P., Boscoe, F. P., Henley, S. J., … Ward, E. M. (2014). Annual Report to the Nation on the status of cancer, 1975-2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. *Cancer, 120*(9), 1290-1314. http://dx.doi.org/10.1002/cncr.28509

England, C. Y., Andrews, R. C., Jago, R., & Thompson, J. L. (2015). A systematic review of brief dietary questionnaires suitable for clinical use in the prevention and management of obesity, cardiovascular disease and type 2 diabetes. *Eur J Clin Nutr*. http://dx.doi.org/10.1038/ejcn.2015.6

Epps, C. T., Stenquist, B. P., Lowder, K. T., Blacker, B. C., Low, R. M., Eggett, D. L., & Parker, T. L. (2013). Synergistic endo- and exo-Interactions between blueberry phenolic compounds, grape variety fractions, chocolate covered strawberries, and fruit smoothies. *Journal of Food Research, 2*(6), 33-47. http://dx.doi.org/10.5539/jfr.v2n6p33

Garrett, A. R., Murray, B. K., Robison, R. A., & O'Neill, K. L. (2010). Measuring antioxidant capacity using the ORAC and TOSC assays. In D. Armstrong (Ed.), *Advanced Protocols in Oxidative Stress II* (Vol. 594, pp. 251-262)..Totowa, NJ: Humana Press Inc.

Garrett, A. R., Weagel, E. G., Martinez, A. D., Heaton, M., Robison, R. A., & O'Neill, K. L. (2014). A novel method for predicting antioxidant activity based on amino acid structure. *Food Chemistry, 158*, 490-496. http://dx.doi.org/10.1016/j.foodchem.2014.02.102

Gupta-Elera, G., Garrett, A. R., Martinez, A., Kraus, R. D., Robison, R. A., & O'Neill, K. L. (2012). A comparison of antioxidant properties in organic and conventional blueberries. *Journal of Food Research, 1*(3), 1-7. http://dx.doi.org/10.5539/jfr.v1n3p1

Gupta-Elera, G., Garrett, A. R., Martinez, A., Robison, R. A., & O'Neill, K. L. (2011). The antioxidant properties of the cherimoya (Annona cherimola) fruit. *Food Research International, 44*(7), 2205-2209. http://dx.doi.org/10.1016/j.foodres.2010.10.038

Jancinova, V., Perecko, T., Nosal, R., Svitekova, K., & Drabikova, K. (2013). The natural stilbenoid piceatannol decreases activity and accelerates apoptosis of human neutrophils: involvement of protein kinase C. *Oxid Med Cell Longev, 2013*, 136539. http://dx.doi.org/10.1155/2013/136539

Joshipura, K. J., Ascherio, A., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., … Willett, W. C. (1999). Fruit and vegetable intake in relation to risk of ischemic stroke. *Jama-Journal of the American Medical Association, 282*(13), 1233-1239. http://dx.doi.org/10.1001/jama.282.13.1233

Kapetanovic, I. M., Muzzio, M., Huang, Z. H., Thompson, T. N., & McCormick, D. L. (2011). Pharmacokinetics, oral bioavailability, and metabolic profile of resveratrol and its dimethylether analog, pterostilbene, in rats. *Cancer Chemother Pharmacol, 68*(3), 593-601. http://dx.doi.org/10.1007/s00280-010-1525-4

Koppenhofer, D., Kettenbaum, F., Susloparova, A., Law, J. K. Y., Vu, X. T., Schwab, T., … Ingebrandt, S. (2015). Neurodegeneration through oxidative stress: Monitoring hydrogen peroxide induced apoptosis in primary cells from the subventricular zone of BALB/c mice using field-effect transistors. *Biosensors & Bioelectronics, 67*, 490-496. http://dx.doi.org/10.1016/j.bios.2014.09.012

Kuate, D., Kengne, A. P. N., Dakam, W., Etoundi, B. C. O., Paka, G. D.,, Ngondi, J. L., & Oben, J. E.. (2013). Effectiveness of *Dichrostachys glomerata* spice phenolics in reduction of oxidative stress associated with obesity and type 2 diabetes; a randomized, double-blind placebo-controlled clinical trial. *Journal of Food Research, 2*(2), 1-10. http://dx.doi.org/10.5539/jfr.v2n2p1

Mahalingaiah, P. K. S., & Singh, K. P. (2014). Chronic oxidative stress increases growth and tumorigenic potential of MCF-7 breast cancer cells. *PLoS One, 9*(1), 10. http://dx.doi.org/10.1371/journal.pone.0087371

Maruki-Uchida, H., Kurita, I., Sugiyama, K., Sai, M., Maeda, K., & Ito, T. (2013). The protective effects of piceatannol from passion fruit (*Passiflora edulis*) seeds in UVB-irradiated keratinocytes. *Biological & Pharmaceutical Bulletin, 36*(5), 845-849. http://dx.doi.org/10.1248/bpb.b12-00708

McCormack, D., & McFadden, D. (2013). A review of pterostilbene antioxidant activity and disease modification. *Oxid Med Cell Longev, 2013*, 575482. http://dx.doi.org/10.1155/2013/575482

Olive, P. L., Banath, J. P., & Durand, R. E. (2012). Heterogeneity in Radiation-Induced DNA Damage and Repair in Tumor and Normal Cells Measured Using the "Comet" Assay. *Radiation Research, 178*(2), AV35-AV42. http://dx.doi.org/10.1667/RRAV04.1

Rimando, A. M., Kalt, W., Magee, J. B., Dewey, J., & Ballington, J. R. (2004). Resveratrol, pterostilbene, and piceatannol in vaccinium berries. *J Agric Food Chem, 52*(15), 4713-4719. http://dx.doi.org/10.1021/jf040095e

Sagrillo, M. R., Garcia, L. F. M., de Souza, O. C., Duarte, M., Ribeiro, E. E., Cadona, F. C., & da Cruz, I. B. M. (2015). Tucuma fruit extracts (Astrocaryum aculeatum Meyer) decrease cytotoxic effects of hydrogen peroxide on human lymphocytes. *Food Chemistry, 173*, 741-748. http://dx.doi.org/10.1016/j.foodchem.2014.10.067

Sato, D., Shimizu, N., Shimizu, Y., Akagi, M., Eshita, Y., Ozaki, S., … Kubota, N. (2014). Synthesis of glycosides of resveratrol, pterostilbene, and piceatannol, and their anti-oxidant, anti-allergic, and neuroprotective activities. *Bioscience Biotechnology and Biochemistry, 78*(7), 1123-1128. http://dx.doi.org/10.1080/09168451.2014.921551

Semba, R. D., Ferrucci, L., Bartali, B., Urpi-Sarda, M., Zamora-Ros, R., Sun, K., … Andres-Lacueva, C. (2014). Resveratrol levels and all-cause mortality in older community-dwelling adults. *JAMA Intern Med*. http://dx.doi.org/10.1001/jamainternmed.2014.1582

Singh, S., Vrishni, S., Singh, B. K., Rahman, I., & Kakkar, P. (2010). Nrf2-ARE stress response mechanism: A control point in oxidative stress-mediated dysfunctions and chronic inflammatory diseases. *Free Radic Res, 44*(11), 1267-1288. http://dx.doi.org/10.3109/10715762.2010.507670

Tang, Y. L., & Chan, S. W. (2014). A Review of the pharmacological effects of piceatannol on cardiovascular diseases. *Phytotherapy Research, 28*(11), 1581-1588. http://dx.doi.org/10.1002/ptr.5185

Xia, E. Q., Deng, G. F., Guo, Y. J., & Li, H. B. (2010). Biological activities of polyphenols from grapes. *Int J Mol Sci, 11*(2), 622-646. http://dx.doi.org/10.3390/ijms11020622

Xiao, M., Helsing, A. V., Lynch, P. M., El-Naggar, A., Alegre, M. M., Robison, R. A., & O'Neill, K. L. (2014). DNA damage caused by inorganic particulate matter on Raji and HepG2 cell lines exposed to ultraviolet radiation. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis, 771*, 6-14. http://dx.doi.org/10.1016/j.mrgentox.2014.06.004

Yamagata, K., Tagami, M., & Yamori, Y. (2015). Dietary polyphenols regulate endothelial function and prevent cardiovascular disease. *Nutrition, 31*(1), 28-37. http://dx.doi.org/10.1016/j.nut.2014.04.011

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Abnormal Colorations of Mozzarella Cheese Caused by *Phoma glomerata* (Corda) Wollenw & Hochapfel

Francesco Casalinuovo[1], Marinella Rodolfi[2], Paola Rippa[1], Anna Scognamiglio[1] & Rosanna Musarella[1]

[1] Istituto Zooprofilattico Sperimentale del Mezzogiorno, Sezione di Catanzaro, Italy

[2] Dipartimento Scienze della Terra e dell'Ambiente, Lab. Micologia, Università degli Studi di Pavia, Italy

Corrispondence: Francesco Casalinuovo, Istituto Zooprofilattico Sperimentale del Mezzogiorno, Sezione di Catanzaro, viale Crotone snc, 88100 Catanzaro, Italy. Tel: 39-096-173-7763, Fax: 39- 096-173-8088. E-mail: francesco.casalinuovo@cert.izsmportici.it

Received: June 11, 2015 Accepted: July 15, 2015 Online Published: July 22, 2015

doi:10.5539/jfr.v4n5p19 URL: http://dx.doi.org/10.5539/jfr.v4n5p19

Abstract

This paper describes an unusual type of abnormal coloration caused by the fungal species *Phoma glomerata* (*P. glomerata*) detected in samples of commercially available mozzarella cheese produced from cow's milk. The presence of this fungus in dairy and cheese products has already been reported by other authors, along with other fungal contaminants; however, it has never been associated to specific alterations of cheeses. This is the first report of a macroscopic alteration of a soft cheese due to *P. glomerata*. Mozzarella cheese from four packages (two sealed and two already opened, three of which with evident macroscopic alterations) was analyzed by means of ISO methods for the detection of the main bacterial and fungal contaminants of cheese products. Culture tests carried out according to the ISO 21527-1:2008 method revealed presence of *P. glomerata* (from 1,100 CFU/g to 45,000 CFU/g). In addition, in both the previously opened packages, *Acremonium* spp. (100 CFU/g), *Alternaria* spp. (100 CFU/g), *Pseudomonas fluorescens* (25,000 CFU/g) and *Pseudomonas putida* (2,400 CFU/g) were also isolated. In sample N°4, contamination by *P. glomerata* was present, but in the absence of macroscopic changes. These results show that *P. glomerata* is able to contaminate mozzarella cheese, causing macroscopically visible alterations of the product; this may have serious consequences in terms of sales. With regard to the possible effects on human health, further studies are needed in order to assess the toxic effect of the fungus. As a result of the episode described, the Italian health authorities issued a RASFF (Rapid Alert System for Food and Feed) early warning notice, a key E.U. tool to ensure the cross-border flow of information in order to react swiftly when risks to public health are detected in the food chain.

Keywords: adulteration, cheese, molds, *Phoma glomerata*

1. Introduction

Mozzarella is a typical Italian cheese product made by stretching and kneading the drained curd mass. It is mainly made from cow's milk, but over recent years mozzarella made with buffalo's milk has becoming increasingly popular. There has been an increase in national demand and important commercial channel have also opened up both in Europe and toward third party countries, in particular the USA and Asia.

In recent years, color anomalies have been reported in fresh cheeses, and particularly in mozzarella cheeses (so-called "blue mozzarella"), caused by biotypes A and B of the species *Psedomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas gessardii* and *Pseudomonas brassicacearum*, which have been indicated as responsible for yellow/orange color alterations in cottage cheese (Giaccone, 2010) and mozzarella (Cantoni et al., 2006; Cantoni, Stella, Cozzi, Iacumin, & Comi, 2003; Soncini, Marchivio, & Cantoni, 1998). These reports have triggered health alerts in several European and non-European countries and have raised concerns among consumers, who are increasingly attentive to food safety. Indeed, in the case described here, the anomaly of the product was reported to the health authorities by consumers who had purchased the cheese through the regular channels. Cheeses are subject to numerous varieties of microbial and fungal contamination. In certain types of cheese, the growth of mold is desirable, as it enhances the organoleptic characteristics of the product. Some types of mold, however, cause unwanted changes that negatively affect the quality of the product, resulting in commercial and economic losses. In addition, some kinds of molds can produce mycotoxins, representing potential risks for human health. The genera most commonly responsible for such alterations are: *Cladosporium*

(*C. cladosporioides, C. herbarum*), *Penicillium* (*P. commune, P. glabrum*) and *Phoma* (Hocking & Faedo, 1992). *Phoma* is the largest and most widespread genus of the order Pleosporales, which includes more than 2000 cosmopolitan species (*Mold & Bacteria Consulting Laboratories 2005*). Owing to its great ecological versatility, it is widespread in air, sea-water, soil and leaves, and can also be isolated from wood, paper, textiles (wool), leather and aquatic organisms (Dörr et al., 2011). Being a common airborne contaminant and allergen in indoor environments, *Phoma* can cause infections, Type-1 allergies (rhinitis, asthma), pneumonitis, keratitis and skin and subcutaneous lesions in humans. A wide variety of vegetables (Kocić-Tanackov et al., 2010), fresh fruits, meat, dairy and meat-derived products (Sørensen, Jacobsen, Nielsen, Frisvad, & Koch, 2008), and animal and vegetable fat (Samson, Hoekstra, & Frisvad, 2004) have occasionally been reported as contaminated by *Phoma* spp. In particular, the species *Phoma glomerata* (*P. glomerata*) has been found in raw cow's milk (Lavoie, Touchette, St-Gelais, & Labrie, 2012), hard cheeses (Fente-Sampayo et al., 1995; Hocking & Faedo, 1992) and semi-hard cheeses (Hoekstra, Van Der Horst, & Samson, 1998). In a study conducted on cheddar cheese (Basilico, Debasilico, Chiericatti, & Vinderola, 2001), *P. glomerata* accounted for 63% of the fungi isolated. Moreover, the indoor environments of food-production facilities have often proved to be contaminated by various species of environmental fungal genera, including *Phoma*; in such situations, foodstuffs may be contaminated during the phases of processing or wrapping. The present paper is the first to describe adulteration caused by *P. glomerata* in commercially available mozzarella cheeses produced from cow's milk.

2. Materials and Methods

2.1 Samples Analyzed

The matrix tested consisted of four packages of mozzarella cheese immersed in water. Each contained 125 g of cheese, flow-packed in modified-atmosphere packaging, belonging to the same production lot. The cheese had been produced in Italy from bovine milk, with the addition of salt, rennet and lactic ferments, and were purchased by a consumer from a food supermarket. On opening, the consumer noticed strange dark spots on the product and reported the matter to the health authorities, which sent the sample to Department of food microbiology Istituto Zooprofilattico Sperimentale del Mezzogiorno of Catanzaro, Italy, for laboratory investigation. Two packages had already been opened, but the product was whole and unconsumed (indicated as samples N°1 and N°2), while the other two (samples N°3 and N°4) were still intact and sealed.

2.2 Visual Inspection of Samples

The tests were performed on all samples in sterile conditions through the use of biological hood and sterile equipment, and stored in same conditions until the end of analysis. External and internal portions of the samples underwent visual and olfactory examination in order to detect the presence of alterations, abnormal odors and variations in consistency.

2.3 Microbiological Tests

The samples underwent ISO (International Organization for Standardization) testing; the microbiological parameters determined for each sample and the test methods used were as follows: survey of *Salmonella* spp. (UNI EN ISO 6579:2004), enumeration of *E. coli* (ISO 16649-2: 2001), enumeration of coagulase-positive staphylococci (UNI EN ISO 6888-1:2004), enumeration of yeasts and molds by means of the selective medium Dichloran Rose-Bengal Chloramphenicol Agar (DRBCA, Biolife), (ISO 21527-1:2008), enumeration of *Pseudomonas* spp (ISO/TS 11059:2009), and detection and enumeration of *Listeria monocytogenes* (UNI EN ISO 11290-1:2005). The tests were performed on all samples in sterile conditions through the use of biological hood and sterile equipment. From each sample, 25 g of the product was taken for *Salmonella* spp and *Listeria monocytogenes* detection, and 10 g for the other microbiological parameters. Following homogenization and preparation of scalar dilutions on a decimal basis for quantitative testing, this material was seeded in culture media and incubated at the respective temperatures. Subsequently, in addition to colony counting, the various bacterial colonies were identified by means of macroscopic observation of the smears subjected to Gram staining and through biochemical testing with an automated system (VITEK 2TM Compact, bioMérieux France). Similarly, the developed filamentous fungal colonies were counted and separated on the basis of their macro-morphology. For each group detected, representative strains were transferred into appropriate media for identification: PDA (Potato Dextrose Agar, medium composition: 200 g potato, 20 g dextrose, 15 g agar,1000 ml distilled water) and MEA (Malt Extract Agar, medium composition: 30 g malt extract, 15 g agar, 1000 ml distilled water). Identification was carried out both on the basis of the macro-morphology of the colony and on the direct microscopic observation of the reproductive structures and spores (staining with Amman's lactophenol/fuchsin lactophenol). Specific taxonomical keys were finally used for strain characterization (Sutton, 1980; Samson et al., 2004). The incubation times and temperatures used were those recommended for the methods of testing for each specific microbiological

parameter, as were the reading and interpretation of the results.

3. Results

The presence of alterations and the positive results of the microbiological tests are reported in Table 1.

Table 1. Results of visual inspection and microbiological tests for the detection of *Pseudomonas* spp, yeasts and molds carried out on already opened (samples 1 and 2) and sealed (samples 3 and 4) mozzarella packages

	Visual inspection and microbiological tests		
	Alterations	Molds	*Pseudomonas spp*
Sample 1	present	*Phoma glomerata* (45,000 CFU/g) *Acremonium* spp. (100 CFU/g)	*Pseudomonas fluorescens* (2,400 CFU/g)
Sample 2	present	*Phoma glomerata* (4,800 CFU/g) *Alternaria* spp. (100 CFU/g)	*Pseudomonas putida* (25,000 CFU/g)
Sample 3	present	*Phoma glomerata* (9,000 CFU/g)	absent
Sample 4	absent	*Phoma glomerata* (1,100 CFU/g)	absent

Visual examination revealed the presence of superficial alterations of the cheese contained in samples 1 and 2. These were manifested as yellow/dark yellow or dark brown patches covering 2-6 cm^2 of the outer surface (Figures 1 and 2). The interior of the cheese did not present any macroscopic alteration.

Figure1. Macroscopic alteration of sample 1

Figure 2. Macroscopic alteration of sample 2

Alterations similar to those found in samples 1 and 2 were observed in sample 3, while sample 4 appeared macroscopically normal. All samples proved negative for *Staphylococcus aureus*, *E. coli*, *Salmonella* spp. and *Listeria monocytogenes*. In the selective DRBCA medium, three types of filamentous colonies developed. The genera *Alternaria* and *Acremonium* proved to be minor contaminants of the substrate, as shown in Table 1. *Phoma glomerata* emerged as the dominant strain; colonies grew rapidly, reaching 5 cm in diameter, and had already reached maturity after 5 days of incubation. The full development of conidia was observed within 10 days. A grayish to red-brown, sparse aerial mycelium was evident (Figure 3), as was the presence of diffusible reddish-brown pigment. Superficially, on agar, globose, 1-ostiole, reddish-brown picnidia were abundantly produced, coalescing in some sectors of the colonies. Characterization of the species was confirmed after its isolation in pure culture and transfer to the generic media PDA and MEA and by means of the above-mentioned taxonomic keys.

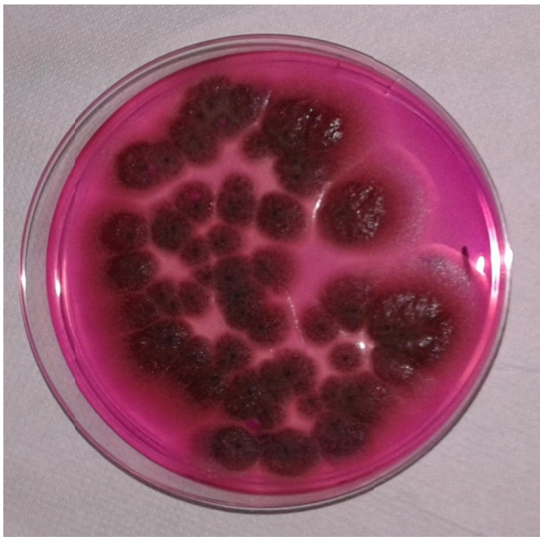


Figure 3. Morphology of *P. glomerata* colonies developed on Dichloran Rose-Bengal Chloramphenicol Agar after 3 days of incubation at 25 °C

4. Discussion

The visible adulteration of the mozzarella cheese samples can be ascribed to the fungal species *P. glomerata*, which was detected in all the samples investigated. Its presence in the sealed packages excludes the possibility that contamination occurred during the marketing phase of the product. On the contrary, it indicates that contamination probably took place during the production of that particular batch. A high fungal presence in the environment or contamination of the water used during the process of production and conservation could have been responsible. The absence of *P. fluorescens* and *P. putida* in the sealed packages (samples 3 and 4) seems to indicate that the presence of these two bacterial species in the opened packs (samples 1 and 2) was due to environmental contamination following the opening, handling and conservation of the cheese for about 2 days. No species of *Pseudomonas* was isolated in the samples from the sealed packages, although the characteristic surface alterations of the cheese were present. The same consideration applies to the presence of *Acremonium* spp. and *Alternaria* spp. By contrast, high levels of *P. glomerata* were detected in all the samples. The observed yellow/brown color alterations on the surface of the mozzarella may have been due to contamination of the product during packaging, of the water used during processing or of the conservation liquid itself. Contamination of the milk used as raw material may also be hypothesized, though this undergoes slow pasteurization (70 °C for 3-5 minutes); moreover, the curd, which is obtained by coagulating the casein of the milk, is usually treated with hot water (80-90°C), thus ensuring good bacterial inactivation. Finally, a particular consideration concerns sample N°4, in which 1100 CFU/g of *P. glomerata* were counted even though no macroscopically evident alterations were present. In such a situation, the potential health risk is far higher, as the product is likely to be eaten by the unsuspecting consumer. Mozzarella is a fresh, soft, stringy, shiny white, rindless cheese. Its qualities are best appreciated if it is eaten soon (a few days) after production. Today, however, industrially produced mozzarella has a shelf-life as long as 30 days. Consequently, the temperature of conservation during the marketing phase becomes extremely important, as microbial counts can rise markedly if the product is not properly conserved. This is probably what happened in the case described here; contamination by *P. glomerata* occurred during the processing phase, and was probably followed by improper conservation during the marketing phase. Thus, the fungus was able to proliferate, producing the pigments responsible for the anomalous color of the surface of the product.

Considering the capability of *P. glomerata* of spreading in the environment, it should pay particular attention to this kind of contamination in the factories production of cheese, in order to avoid the contamination of the products and the considerable economic loss caused by the sales decline, the recall from the market, the destruction of lots and the health risk for the consumers. In Italy, the cheese discoloration, in the last few years, caused the closure of many companies.

5. Conclusions

The clinical significance of *P. glomerata* has not yet been completely defined. To date, there have been no reports of confirmed cases of human or animal mycotoxicosis associated with *Phoma*. Consequently, *P. glomerata* can not

strictly be considered a pathogen; rather, it is an opportunistic fungus that can be involved in mycosis after entering the human body by chance. Nevertheless, the tests conducted in this study highlighted its ability to cause organoleptic alterations in mozzarella cheeses, with inevitable serious commercial consequences. Moreover, these findings should help industrial quality-control systems to identify sources of contamination. As a result of the episode described in this paper, the health authorities issued an RASFF (Rapid Alert System for Food and Feed) early warning notice.

References

Basílico, J. C., Debasílico, M. Z., Chiericatti, C., & Vinderola, C. G. (2001). Characterization and control of thread mould in cheese. *Letters Applied Microbiology, 32*(6), 419-423. http://dx.doi.org/10.1046/j.1472-765X.2001.00934.x

Cantoni, C., Soncini, G., Milesi, S., Cocolin, L., Iacumin, L., & Comi, G. (2006). Colorazioni anomale e rigonfiamento di formaggi fusi e mozzarelle. *Industrie Alimentari, 45*, 276-281.

Cantoni, C., Stella, S., Cozzi, M., Iacumin, L., & Comi, G. (2003). Colorazione blu di mozzarelle. *Industrie Alimentari, 42*, 840- 843.

Fente-Sampayo, C. A., Vazquez-Belda, B., Franco-Abuin, C., Quinto-Fernandez, E., Rodriguez-Otero, J. L., & Cepeda-Saez, A. (1995). Distribution of fungal genera in cheese and dairies. Sensitivity to potassium sorbate and natamycin. *Archiv für Lebensmittelhygiene, 46*(3), 62-65.

Giaccone, V. (2010). *Pseudomonas* e prodotti lattiero-caseari. Medicina Veterinaria Preventiva, 32, supplemento.

Hocking, A. D., & Faedo, M. (1992). Fungi causing thread mould spoilage of vacuum packaged Cheddar cheese during maturation. *International Journal of Food Microbiology, 16*(2), 123-30. http://dx.doi.org/10.1016/0168-1605(92)90005-N

Hoekstra, E. S., Van Der Horst, M. I., & Samson, R. A. (1998). Survey of the fungal flora in Dutch cheese factories and warhouses. *Journal of Food. Mycology, 1*(1), 13-22.

ISO/TS 11059:2009. (IDF/RM 225: 2009). Milk and milk products -- Method for the enumeration of *Pseudomonas* spp.

ISO 21527-1:2008. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of yeasts and moulds -- Part 1: Colony count technique in products with water activity greater than 0, 95.

ISO 16649-2:2001. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta glucuronidase-positive *Escherichia coli* -- Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro- 3-indolyl beta-D-glucuronide.

Kociæ-Tanackov, S. D., Dimiæ, G. R., Leviæ, J. T., Pejin, D. J., Pejin, J. D., & Jajiæ, I. M. (2010). Occurrence of potentially toxigenic mould species in fresh salads of different kinds of ready-for-use vegetables. *Acta periodica technologica, 41*, 33-45. http://dx.doi.org/10.2298/APT1041033K

Lavoie, K., Touchette, M., St-Gelais, D., & Labrie, S. (2012).Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. *Dairy Science & Technology, 11*, 51-4. http://dx.doi.org/10.1007/s13594–011-0051-4

Mold & Bacteria Consulting Laboratories (MBL). (2005). Indoor mould: do not panic! *Indoor Mold and Bacteria, 6*, 30.

Samson, R. A., Hoekstra, E. S., & Frisvad, J. C. (2004). Introduction to food and airborne fungi. (7th, p. 389) Baarn, Centralalbureau voor Schimmellcultures, Institute of the Royal Netherlands Academy of Arts and Sciences.

Soncini, G., Marchivio, G., & Cantoni, C. (1998). Causa delle alterazioni cromatiche di mozzarelle. *Industrie Alimentari, 37*, 850-855.

Sørensen, L. M., Jacobsen, T., Nielsen, P. V., Frisvad, J. C., & Koch, A. G. (2008). Mycobiota in the processing areas of two different meat products. *International Journal of Food Microbiology, 124*(1), 58-64. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.02.019.

Sutton, B. C. (1980). The *Coelomycetes*.. Commonwealth Mycological Institute, Kew, Surrey.

UNI EN ISO 6888-1:2004. Microbiologia di alimenti e mangimi per animali - Metodo orizzontale per la conta di stafilococchi coagulasi- positivi (*Staphylococcus aureus* e altre specie) - Tecnica che utilizza il terreno agar Baird-Parker.

UNI EN ISO 11290-1:2005. Microbiologia di alimenti e mangimi per animali - Metodo orizzontale per la ricerca e la conta di *Listeria monocytogenes* - Parte 1: Metodo per la ricerca.

UNI EN ISO 6579:2008. Microbiologia di alimenti e mangimi per animali - Metodo orizzontale per la ricerca di *Salmonella* spp.

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Physicochemical Properties of *Melipona beecheii* Honey of the Yucatan Peninsula

Victor M. Moo-Huchin[1], Gustavo A. González-Aguilar[2], José D. Lira-Maas[1], Emilio Pérez-Pacheco[1], Raciel Estrada-León[1], Mariela I. Moo-Huchin[3] & Enrique Sauri-Duch[4]

[1] Cuerpo Académico Bioprocesos. Instituto Tecnológico Superior de Calkiní, Av. Ah-Canul, C.P. 24900, Calkiní, Campeche, México

[2] Research Center for Food & Development (CIAD), AC., Carretera a la Victoria Km 0.6, Hermosillo (83000), Sonora, México

[3] Universidad Tecnológica del Poniente, Calle 29 Las Tres Cruces, C. P. 97800, Maxcanú, Mérida, México

[4] Instituto Tecnológico de Mérida, C.P. 97118, km 5 Mérida-Progreso, Mérida, Yucatán, México

Correspondence: Victor M. Moo-Huchin, Instituto Tecnológico Superior de Calkiní, Av. Ah Canul, C.P. 24900, Calkiní, Campeche, Mexico. Fax: 52-996-813-4870 Ext. 2000, Tel: 52-996-813-4870 Ext. 1002. E-mail: vmoo@itescam.edu.mx

Received: April 8, 2015 Accepted: July 12, 2015 Online Published: July 23, 2015

doi:10.5539/jfr./v4n5p25 URL: http://dx.doi.org/10.5539/jfr./v4n5p25

Abstract

The knowledge regarding the physicochemical characteristics of the honey produced by stingless bees is still limited, mainly due to the high diversity of the floral resources and the low production that is inherent to these species. This manuscript describes the physicochemical characterization of 27 honey samples produced by *Melipona beecheii,* from the Yucatan Peninsula, Mexico. The objective of this study was to contribute to the establishment of standards for quality control. The following parameters were evaluated in the honey samples: reducing sugars, moisture content, acidity, pH, hydroxymethylfurfural (HMF), ash, soluble solids, formol index, proline and color. Most of physico-chemical parameters fulfilled the quality criteria established by the International Legislation for Apis honey, with the exception of moisture content, which presented higher values; for that, the results indicate that the international standard procedures are not completing adequate for all the parameters analyzed on *Apis mellifera* honey and therefore is need establish a suitable standard of quality control for honey from *Melipona*.

Keywords: honey, *Melipona beecheii*, physicochemical characteristics, stingless bee

1. Introduction

Honey is the nectar collected from many plants and processed by bees. Honey from different locations possesses a unique combination of components and properties due to the variety of flora, climatic conditions located in its geographical region of production, and finally by the different processing technologies and storage conditions (Turhan, Tetik, Karhan, Gurel, & Tavukcuoglu, 2008). In both ancient and modern civilizations, honey has been seen as a natural product of great importance, with many functional applications. It is a rich source of readily available sugars, organic acids, various amino acids, and a good source of many biologically active compounds (Anupama, Bhat, & Sapna, 2003; Saxena, Gautam, & Sharma, 2010). All these nutritional, therapeutic and social roles have been noted in different cultures even though no distinctions have been observed between *Apis mellifera* and stingless bee honey.

The stingless bees are one of the most diverse, attractive, fascinating, conspicuous, and useful of all the insect groups of the tropical world. The keeping of stingless bees (Apidae, Meliponini), or meliponiculture, is carried out in a rustic and traditional way in tropical America by a variety of ethnic groups and rural populations. Within the stingless bee tribe there are about 500 species, the majority of them present in South America, and some in Australia, Asia and Africa (Vit, Bogdanov, & Kilchenmann, 1994). The distribution of stingless bee honeys in the world market is limited in comparison with honeys from *A. mellifera*. This is consequence of their limited industrial production, shorter shelf life and the lack of international quality standard, which in turn is due to a relatively limited knowledge of the product such as composition of bioactive compounds and some nutritional

M. beecheii is the most important stingless bee species in the Yucatan Peninsula of Mexico due to high honey yield and its reported medicinal properties. In the Yucatan peninsula, the bee *Melipona beecheii* was named "cab" or "kab" in the Mayan language. It was considered of such importance by the Mayan people that, after a long process of appropriation, the bees were deified and named "xunan cab," or "xunan kab" (Ocampo-Rosales, 2013). A wide range of attributes may suggest that *M. beecheii* honey enhances several systems to control digestive disorders, eye diseases, and respiratory infections, wound healing, post-birth recovery, fatigue, casts for fractures and skin ulcers (Vit et al., 2004). These attributes afford stingless bee honey greater value in comparison with *A. mellifera* honey.

Due to the lack of information regarding stingless bee honey, it is not included in the international standards for honey (Codex Alimentarius, 2001) and is not specified by the food control authorities. Vit et al. (2004) have proposed values for the physicochemical parameters for stingless bee honey; however, in the Yucatan Peninsula, Mexico like in other countries, the composition and bioactivity of these honeys is still unknown. Thus, in order to increase the knowledge about shed light quality of honey from stingless bees, the aim of this study was to determine the physicochemical characteristics of *M. beecheii* honey produced in different location of the Yucatan Peninsula, Mexico.

2. Materials and Methods

2.1 Honey Samples

For this study, 27 *Melipona beecheii* honey samples from the Yucatan Peninsula, Mexico were analyzed (Table 1). Samples of honey were collected from traditional hives in tree trunks (Figure 1) at different locations in the states of Yucatan, Quintana Roo and Campeche from March to April, 2010 (Figure 2). The honey from the tree trunk hives was obtained by breaking the pots and decanting it. All samples were filtered and stored in holders and transferred to the laboratory, where they were kept at 4 °C until analysis. The floral origin of *Melipona* honey samples is multi-flora (Chan-Rodríguez et al., 2012).

Table 1. Samples of *M. beecheii* honey, location and date of harvesting from the Yucatan Peninsula, Mexico

Sample Code	Location	Month and year of harvesting
H1	Temozón Norte, Yucatán	March 2010
H2	Yaxcabá, Yucatán	March 2010
H3	Concepción, Calkiní, Campeche	October 2010
H4	Calkiní, Campeche	October 2010
H5	Mérida, Yucatán	January 2010
H6	San Antonio Sahcabchén, Calkiní, Campeche	May 2010
H7	Mérida, Yucatán	December 2010
H8	Celestún, Yucatán	December 2010
H9	Celestún, Yucatán	December 2010
H10	Celestún, Yucatán	December 2010
H11	Quintana Roo	October 2010
H12	Quintana Roo	October 2010
H13	Yaxcabá, Yucatán	March 2010
H14	Santa Cruz Pueblo, Calkiní, Campeche	March 2010
H15	Maní, Yucatán	April 2010
H16	Valladolid, Yucatán	April 2010
H17	Maní, Yucatán	April 2010
H18	Maní, Yucatán	April 2010
H19	Maní, Yucatán	April 2010
H20	Yaxcabá, Yucatán	April 2010
H21	Yaxcabá, Yucatán	April 2010
H22	Yaxcabá, Yucatán	April 2010
H23	Yaxcabá, Yucatán	April 2010
H24	Yaxcabá, Yucatán	April 2010
H25	Yaxcabá, Yucatán	April 2010
H26	Yaxcabá, Yucatán	April 2010
H27	Yaxcabá, Yucatán	May 2010

Figure 1. Harvesting *Melipona beecheii* honey

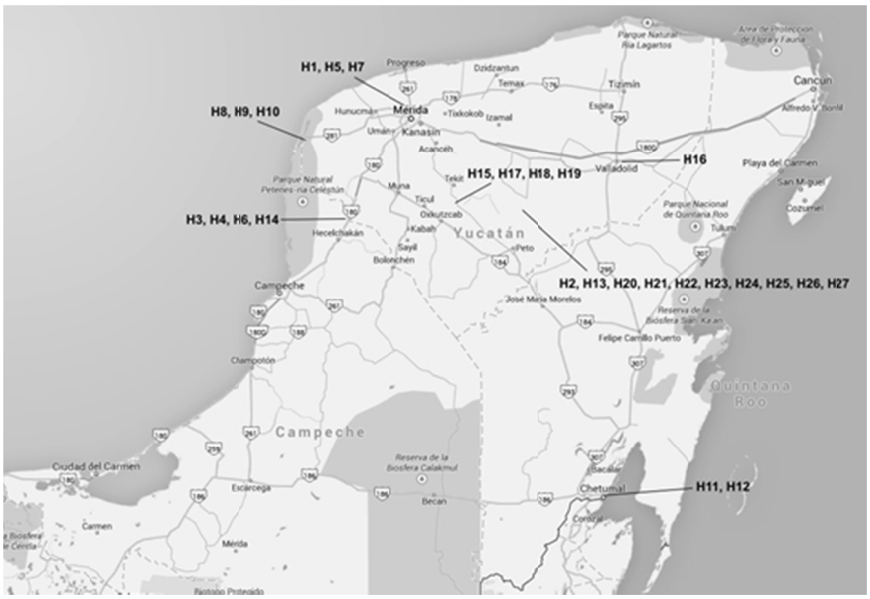


Figure 2. Honey sampling locations in the Peninsula of Yucatan

2.2 Physicochemical Parameters

Physicochemical parameters of *Melipona* honey samples were determined according to the official method of the Association of Official Analytical Chemists (AOAC, 1990) and those reported by Viuda-Martos et al. (2010). The evaluated parameters were: reducing sugars (g/100 g), moisture content (g/100 g), free acidity (meq/kg), pH, hydroxymethylfurfural content (mg/kg), ash (g/100 g), soluble solids (°Brix), formol index (mL/kg), proline (mg/kg). In the determination of the honey's color, it was used a photometer with direct readout in mmPfund. Honey color is measured in millimeters on the Pfund scale compared to an analytical standard scale of reference on the graduation of glycerin (Almeida-Muradian et al., 2013). The use of this meter removes all the guesswork commonly associated with honey color measurement, providing accurate and repeatable results.

2.3 Statistical Analysis

All analyzes were carried out in triplicate and the data were expressed as means±standard deviations (SD), which were calculated using Excel (Microsoft Office, Version 2003).

3. Results and Discussion

Honey is a very complex matrix endowed with very specific physico-chemical properties that make it unique from other viscous solutions. Stingless bee honeys are gaining the attention of researchers given their importance as foodstuffs and traditional remedies in folk medicine of countries where *Melipona* spp. is endemic (Vit et al., 1994). Curiously, although the ethnopharmacobotanical tradition linked to these honeys, there is a lack of specific parameters for quality control stablished by institutional food control authorities comparable to that of *A. mellifera* honeys that allow to marketing in local and external areas.

Table 2 shows the physico-chemical properties of the 27 *Melipona* honey samples from the Yucatan Peninsula in Mexico. Moisture is a physico-chemical parameter that is related to the climatic conditions and degree of maturity of honey (Baroni et al., 2009). Moisture values of samples between 21 and 25.3 g/100 g honey were obtained and excluded in the water range limits (< 20 g/100 g) approved by the Codex Alimentarius Commision standard for honey of *Apis mellifera* (Codex Alimentarius, 2001). However, these moisture values obtained for these honey samples were below the maximum amount of water (30 g/100 g) suggested for *Melipona* honey (Vit et al., 2004). Also, the values of moisture obtained for *Melipona* honey of this study were similar to those reported for stingless bee honeys (Vit, 2009). The moisture content of honey is an important factor contributing to its stability and shelf-life. Higher moisture content could lead to undesirable honey fermentation during storage, commonly caused by the action of osmotolerant yeasts, resulting in the formation of ethyl alcohol and carbon dioxide (Saxena et al., 2010). The ethanol may break down into acetic acid and water, giving the honey a distinctly sour or "off-taste" and a runny texture with small bubbles, surface heaving or foaming. However, from the perspective of traditional medicinal use, it must be stressed that fermented honeys – or honeys particularly rich in water – are considered by natives to be particularly indicated in the treatment of respiratory disorders. This ethnomedical aspect is consistent with other studies conducted on stingless bee honeys from other South American regions (Vit et al., 2004).

The honey samples analyzed showed ash values ranging from 0.01 to 0.6% with a mean of 0.16 ± 0.12%. Similar results were detected by Souza, Marchini, Oda-Souza, Carvalho and Alves (2009) for *Melipona* honey from the northeast area of Brazil. Except for sample H2, all other *Melipona* honeys had ash contents that are in accordance with the Codex Alimentarius Commision standard for honey of *Apis mellifera* (Codex Alimentarius, 2001), which stipulates a maximum of ash value of 0.5%. The variability in the ash content of honeys could be due to the differences in soil, atmospheric conditions, and in the material collected by the bees during foraging on the flora (Finola, Lasagno, & Marioli, 2007). Certain nitrogen compounds, minerals, vitamins, pigments and aromatic substances contribute to the ash content of honey (Mairaj et al., 2008) which is considered a quality criterion indicating the possible botanical origin of honey.

Soluble solids content expressed as °Brix of the *Melipona* honey samples ranged from 72.8 to 77.3, which are lower than those of other honey samples of *Apis mellifera* from other regions (Silva, Videira, Monteiro, Valentão, & Andrade, 2009; Viuda-Martos et al., 2010). Values of soluble solids obtained from the *Melipona* honey coincide with those reported by Lage et al. (2012) for three species of the Brazilian *Melipona*. Moreover, according to Anupama et al. (2003), there was a high negative correlation between °Brix and moisture content in *Apis* honey. Therefore, the lower content of soluble solids from *Melipona* honey may be related to their higher moisture content.

The acidity of the honey contributes to its flavor, improves antioxidant activity and is effective against the action of microorganisms (Cavia, Fernández-Muiño, Alonso-Torre, Huidobro, & Sancho, 2007). Variations in acidity have been attributed to the floral source and harvest season (Ojeda de Rodríguez et al., 2004). Free acidity is due to the presence of organic acids, particularly gluconic acid, which is in equilibrium with the corresponding lactones and some inorganic ions such as phosphate or sulfate. The free acidity of the *Melipona* honey samples varied from 13.0 to 71.3 meq/kg honey, with a mean of 35.0±12.8 meq/kg honey, which is similar to the results published by Souza, Marchini, Oda-Souza, Carvalho and Alves (2009). International regulations specify a free acidity not higher to 50 meq/kg honey of *Apis mellifera* (Codex Alimentarius, 2001; European Union, 2002). Except for samples H4, H21 and H3, all other *Melipona* honeys had free acidity contents in accordance with these specifications.

In this work, the pH values of *Melipona* honey varied from 2.6 to 3.3 with a mean of 3.07 ± 0.2. This mean value of pH was previously reported by Alves, Carvalho, Souza, Sodré and Marchini (2005) for honey samples of the *Melipona* collected in Brazil (pH 3.2).

Table 2. Physicochemical properties of *Melipona beecheii* honey from of the Yucatan Peninsula, Mexico

Sample	Free acidity	Soluble solids (ºBrix)	Moisture	Reducing Sugar	HMF	Ash	Formol index	pH	Proline	Color
	(meq/kg)		(g/100 g)	(g/100 g)	(mg/kg)	(g/100 g)	(mL/kg)		(mg/kg)	(mm Pfund)
H1	28.3±1.1	75.0±1.4	22.8±1.5	74.2±1.8	16.9±1.2	0.01±0.0	2.5±0.1	3.3±0.2	264.5±1.9	34.0±1.2
H2	46.0±3.0	74.3±1.1	23.9±2.0	73.6±1.9	10.7±0.9	0.60±0.02	2.5±0.2	3.1±0.3	959.1±5.0	83.0±1.6
H3	54.3±4.2	73.9±0.8	24.5±1.0	72.1±2.0	23.3±0.7	0.21±0.02	3.1±0.1	3.1±0.1	883.2±9.9	113.0±1.8
H4	71.3±4.1	72.8±0.9	25.3±1.1	65.6±1.1	15.4±0.7	0.19±0.02	2.0±0.1	2.9±0.1	722.2±6.9	53.0±1.0
H5	46.3±4.0	74.7±1.2	23.5±1.1	66.1±2.1	4.0±0.8	0.27±0.01	2.6±0.2	3.2±0.1	1182±10	67.0±1.2
H6	42.3±2.3	73.9±1.1	24.4±1.0	70.1±1.1	22.3±0.9	0.22±0.01	2.0±0.1	3.2±0.1	552±9.8	60.0±1.2
H7	40.3±2.0	75.4±1.1	22.8±1.1	62.6±1.1	45.5±0.4	0.26±0.01	1.7±0.1	3.1±0.2	542.8±9.9	98.5±1.2
H8	43.0±2.1	74.7±1.6	23.6±1.3	68.2±1.3	40.4±1.1	0.01±0.0	2.0±0.1	3.2±0.2	568.1±9.7	81.0±1.3
H9	42.3±2.1	74.6±1.1	23.8±1.3	71.6±1.3	42.7±0.8	0.17±0.01	2.1±0.1	2.9±0.1	568.1±8.9	78.0±1.6
H10	31.3±1.1	73.3±1.5	24.8±1.3	70.2±1.3	10.2±0.3	0.03±0.0	2.0±0.1	3.1±0.1	365.7±8.0	59.0±1.2
H11	30.7±1.2	76.0±1.1	22.4±1.3	67.8±1.3	21.6±0.3	0.17±0.01	2.0±0.2	2.9±0.2	322.0±9.9	50.0±2.0
H12	20.7±1.0	74.8±1.2	23.4±1.1	69.8±1.1	15.7±1.2	0.23±0.02	2.8±0.2	3.1±0.1	308.2±7.9	74.5±1.3
H13	25.0±0.5	73.1±1.2	25.3±1.2	72.8±1.2	16.4±1.1	0.09±0.0	2.5±0.2	3.3±0.1	379.5±5.5	54.0±1.1
H14	20.0±1.1	75.3±1.3	23.1±0.8	74.0±1.2	13.7±0.9	0.15±0.01	2.0±0.2	3.2±0.1	423.2±7.5	121.5±1.1
H15	13.0±1.1	76.0±1.3	22.4±0.9	71.0±1.2	9.5±0.1	0.17±0.01	2.4±0.1	3.3±0.1	784.3±9.6	39.0±1.0
H16	25.0±0.5	75.0±1.0	23.0±0.7	70.6±1.2	29.6±0.5	0.40±0.02	2.0±0.1	3.2±0.2	1193.7±9.0	90.0±1.7
H17	28.0±0.9	77.3±0.8	21.0±0.5	57.1±1.2	11.1±0.6	0.09±0.0	2.0±0.1	3.0±0.2	349.6±7.5	37.5±1.3
H18	30.5±1.1	76.4±1.2	23.3±1.1	59.7±1.3	7.1±0.3	0.07±0.0	1.6±0.1	2.6±0.2	483.0±9.9	19.0±1.5
H19	24.0±1.2	75.1±1.1	21.9±1.2	65.3±2.0	15.6±0.4	0.12±0.01	2.3±0.2	3.0±0.2	342.7±9.8	34.0±1.7
H20	30.5±1.1	76.5±1.1	21.9±1.1	65.3±1.1	17.8±0.7	0.12±0.01	1.9±0.1	3.1±0.2	351.9±9.8	33.0±1.8
H21	60.5±2.1	74.9±1.4	23.5±1.2	66.7±1.5	8.6±0.5	0.10±0.01	3.0±0.3	2.9±0.2	1053.4±9.4	105.0±1.9
H22	41.0±1.1	75.8±1.3	22.7±1.2	67.0±1.5	27±0.6	0.13±0.01	2.0±0.1	2.8±0.2	489.9±9.6	45.0±1.8
H23	32.5±1.2	75.5±1.2	23.0±0.7	60.3±1.4	5.3±0.8	0.09±0.0	1.5±0.1	2.9±0.1	522.1±9.7	37.5±1.7
H24	33.0±0.9	75.6±1.2	22.9±0.7	68.0±1.3	21.3±0.9	0.11±1.1	2.5±0.2	3.1±0.1	653.2±8.7	40.0±1.6
H25	35.5±0.9	76.0±1.3	22.4±0.6	67.0±1.2	4.2±0.9	0.11±0.01	2.5±0.1	3.1±0.2	522.1±9.7	38.0±1.9
H26	25.5±0.8	76.5±0.5	22.0±0.5	72.5±1.6	5.6±0.8	0.10±0.01	2.0±0.1	3.1±0.2	809.6±9.7	39.0±1.6
H27	25.0±0.5	75.7±1.2	22.8±0.5	60.8±1.5	24±0.3	0.12±0.01	2.0±0.1	3.2±0.2	476.1±9.6	60.0±2.0
Average±SD	35.0±12.8	75.1±1.0	23.2±1.0	67.7±4.6	17.9±11.1	0.16±0.12	2.2±0.4	3.07±0.2	595.2±263	60.9±26.8
Minimun value	13	72.8	21	57.1	4	0.01	1.5	2.6	264.5	19
Maximum vaue	71.3	77.3	25.3	74.2	45.5	0.6	3.1	3.3	1193.7	121.5
Coefficient of variation (%)	36.5	1.3	4.3	6.7	62	75	18.1	6.5	44.1	44

On the other hand, the reducing sugars ranged from 57.1 to 74.2 g/100 g *Melipona* honey. All the samples studied attained the minimum amount of reducing sugars (50 g/100 g) established by Vit et al. (2004) as the standard of quality for *Melipona* honey. However, samples H7, H27, H23, H18 and H17 did not reach the minimum amount of reducing sugars, established by the Codex Alimentarius Commision standard for honey of *Apis mellifera* (65 g/100 g) (Codex Alimentarius, 2001). In addition, the values of reducing sugars reported in this study are similar to those found by Vit et al. (2004) for the honeys of stingless bees in Venezuela.

The HMF content of honey is an indicator of freshness, because this compound is not generally present in ~~a~~ fresh honey. The honey samples analyzed in this study showed HMF content values between 4.0 and 45.5 mg/kg *Melipona* honey. Honey samples analyzed in this experiment (Table 1) did not exceed the international regulation and suggested standards for *Melipona* honey, which stablish a maximum HMF of 40 mg/ kg for *Apis Mellifera* honey (Vit et al., 2004; Codex Alimentarius, 2001; European Union, 2002), except three samples (H7, H8 and H9) (40.4-45.5 mg/kg honey) which were not in accordance with the international regulation. This is probably due to overheating, a contributing factor for HMF formation. The heating may have been caused by mishandling during honey harvesting, with the hive exposed to the sun.

Honey commonly have different color spectrum, from straw yellow to amber, and from dark amber to almost black with a hint of red. This property apparently is related to the mineral content, pollen and phenolic compounds present in the honey which varies according to the geographical origin and botanical varieties visited by the bees (Ramalhosa, Gomes, Pereira, Dias, & Estevinho, 2011). The method of production and agricultural practices and extraction procedure also influence the color of honey. Some changes also occur during storage; browning/darkening of honey attributed mainly to Maillard reactions, caramelisation of fructose and

polyphenolic reactions, depending on storage temperature and/or duration influence this negative change (Bertoncelj, Dobersek, Jamnik, & Golob, 2007). In our study, the mean value of color was found at 60.9 mmPfund with a range of 19 to 121.5. According to Color Standards Designations for extracted Honey (USDA, 1985); the color of the *Melipona* honey studied was classified as: white (14.8%), extra light amber (26%), light amber (37%), amber (18.5%) and dark amber (3.7%). However, results obtained for different honeys produced in other areas reveal a significant or similar values to those obtained in this study (Alves et al., 2005; Almeida-Muradian et al., 2013) for *Melipona* honey. The content of proline is an indication of quality of the honey and is also commonly used as an indicator of adulteration, when proline content is below of 183 mg/kg (Bogdanov et al., 1995; Bogdanov, 1999). According to table 2, all the *Melipona* honey samples we studied had good proline levels of up to 183 mg/kg, indicating that all samples obtained in Yucatan, Mexico were free of adulteration. Proline is the most abundant amino acid in honey and is used as a standard to quantify amino acid content.

Formol index is an important parameter and represents a global measurement of amino compounds which facilitate the evaluation of protein and aminoacid content of honey (Alves et al., 2005). The formol index of *Melipona* honey was found to be in the range of 1.5-3.1 mL/kg, which is lower than that reported by Souza et al. (2009) for *Melipona* honey (*M. quadrifasciata anthidioides*) (3.06-9.01 mL/kg). This difference can be attributed to the content of amino acids and proteins of honey from the nectar of flowers of each region.

It should be noted that the values of acidity, HMF, ash, proline and color reported in this work presented a high variability among the samples of *Melipona* honey, as evidenced by the high values of the coefficient of variation (36.5%, 62%, 75%, 44.1% and 44%, respectively). In order to reduce these variations of evaluated parameters, it is necessary to increase the sample number and location which in turn will give us a better idea of quality and compare more properly with other honeys from other locations and production areas with similar environmental conditions.

4. Conclusions

According to our knowledge, this is the first time that is reported that physicochemical parameters of *Melipona* honey produced in the Yucatan Peninsula, Mexico. The results revealed many similarities to other *Melipona* honeys produced in other areas.

A significant number of analyzed honey samples meet the specifications of the International Legislation for physico-chemical characteristics, with the exception of moisture. Therefore, these parameters of honey produced in the Yucatan Peninsula allow to compete with great perspective with similar products in internal and international markets.

The high moisture value of the honey reported in this work could be a limiting factor to consider, due that can affect significantly shelf-life, due to the fermentation processes that are enhanced by the presence of free water.

According to literature and the findings in this paper a maximum moisture content of 35 g /100 g for honey of Melipona is proposed.

The results show that the current legislation regarding *Apis mellifera* honey is not adequate for all the characteristics analyzed, emphasizing the need for a suitable standard for stingless bee honeys in order to prevent adulterations and to allow its commercialization on a formal market.

References

Almeida-Muradian, L., Stramm, K., Horita, A., Barth, O., Freitas, A., & Estevinho, L. (2013). Comparative study of the physicochemical and palynological characteristics of honey from *Melipona subnitida* and *Apis mellifera*. *International Journal of Food Science and Technology, 48*, 1698-1706. http://dx.doi.org/10.1111/ijfs.12140

Alves, R. G., Carvalho, C. A., Souza, B. A., Sodré, G., & Marchini, L. (2005). Características físico-químicas de amostras de mel de *Melipona mandacaia* Smith (Hymenoptera: Apidae). *Ciência e Tecnologia de Alimentos*, *Campinas, 25*, 644-650. http://dx.doi.org/10.1590/S0101-20612005000400004

Anupama, D., Bhat, K. K., & Sapna, V. K. (2003). Sensory and physico-chemical properties of commercial samples of honeys. *Food Research International, 36*, 183-191. http://dx.doi.org/10.1016/S0963-9969(02)00135-7

Association of Official Analytical Chemists [AOAC]. (1990). *Methods of analysis*. Washington, DC: AOAC.

Baroni, M. V., Arrua, C., Nores, M. L., Fayé, P., Diaz, M. P., Chiabrando, G. A., & Wunderlin, D. P. (2009). Composition of honey from Cordoba (Argentina): Assessment of North/South provenance by chemometrics.

Food Chemistry, 114, 727-733. http://dx.doi.org/10.1016/j.foodchem.2008.10.018

Bertoncelj, J., Dobersek, U., Jamnik, M., & Golob, T. (2007). Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. *Food Chemistry, 105*, 822-828. http://dx.doi.org/10.1016/j.foodchem.2007.01.060

Bogdanov, S. (1999). *Harmonised methods of the international honey commission*. Swiss Bee Research Center, FAM, Liebefeld, CH-3003 Bern, Switzerland.

Bogdanov, S., Bieri, K., Figar, M., Figueiredo, V., Iff, D., Känzig, A., Stöckli, H., & Zürcher, K. (1995). *Miel: définition et directives pour l' analyse et l' appréciation*. Centre Suisse de recherche Apicole; Station de recherches laitiéres, Liebefeld, CH-3003 Berne.

Cavia, M. M., Fernández-Muiño, M. A., Alonso-Torre, S. R., Huidobro, J. F., & Sancho, M. T. (2007). Evolution of acidity of honeys from continental climates: Influence of induced granulation. *Food Chemistry, 100*, 1728-1733. http://dx.doi.org/10.1016/j.foodchem.2005.10.019

Chan-Rodríguez, D., Ramón-Sierra, J., Lope-Ayora, J., Sauri-Duch, E., Cuevas-Glory, L., & Ortiz-Vásquez, E. (2012). Antibacterial properties of honey produced by *Melipona beecheii* and *Apis mellifera* against foodborn microorganisms. *Food Science and Biotechnology, 21*, 905-909. http://dx.doi.org/10.1007/s10068-012-0118-x

Codex Alimentarius [CA]. (2001). Commission standards. Standards for honey, CODEX STAN 12–1981 Rev. 2. Retrieved from http://www.codexalimentarius.org/standards/list-of-standards/en/.

European Union [EU]. (2002). European Union Directive, Council Directive 2001/110/EC relating to honey. Retrieved from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri¼OJ:L:2002:010:0047:0052:EN:PDF

Finola, M. S., Lasagno, M. C., & Marioli, J. M. (2007). Microbiological and chemical characterization of honeys from central Argentina. *Food Chemistry, 100*, 1649-1653. http://dx.doi.org/10.1016/j.foodchem.2005.12.046

Lage, L., Coelho, L., Resende, H., Tavares, M., Campos, L., & Fernandes-Salomão, T. (2012). Honey physicochemical properties of three species of the brazilian *Melipona. Anais da Academia Brasileira de Ciências, 84*, 605-608. http://dx.doi.org/10.1590/S0001-37652012005000051

Mairaj, G., Akhtar, S., Khan, A. R., Ullah, Z., Bibi, S., & Ali, S. (2008). Quality evaluation of different honey samples produce in Peshawar valley. *Pakistan Journal of Biological Sciences, 11*, 797-800. http://dx.doi.org/10.3923/pjbs.2008.797.800

Ocampo-Rosales, G. R. (2013). Medicinal Uses of *Melipona beecheii* Honey, by the Ancient Maya. In P. Vit, S. R. M. Pedro & D. W. Roubik (Eds.), *Pot-Honey: A legacy of stingless bees* (pp. 229-240). Springer-Verlag New York, LLC. http://dx.doi.org/10.1007/978-1-4614-4960-7_15

Ojeda de Rodríguez, G., Sulbarán de Ferrer, B., Ferrer, A., & Rodríguez, B. (2004). Characterization of honey produced in Venezuela. *Food Chemistry, 84*, 499-502. http://dx.doi.org/10.1016/S0308-8146(02)00517-4

Ramalhosa, E. E., Gomes, T. T., Pereira, A. P., Dias, T. T., & Estevinho, L. M. (2011). Mead production tradition versus modernity. *Advanced Food Nutritional Research, 63*, 101-118. http://dx.doi.org/10.1016/B978-0-12-384927-4.00004-X

Saxena, S., Gautam, S., & Sharma, A. (2010). Physical, biochemical and antioxidant properties of some Indian honeys. *Food Chemistry*, *118*, 391–397. http://dx.doi.org/10.1016/j.foodchem.2009.05.001

Silva, L. R., Videira, R., Monteiro, A. P., Valentão, P., & Andrade, P. B. (2009). Honey from Luso region (Portugal): physicochemical characteristics and mineral contents. *Microchemical Journal, 93*, 73-77. http://dx.doi.org/10.1016/j.microc.2009.05.005

Souza, B. A., Marchini, L. C., Oda-Souza, M., Carvalho, C. A., & Alves, R. M. (2009). Caracterização do mel produzido por espécies de *Melipona* Illiger, 1806 (apidae: meliponini) da região nordeste do Brasil: 1. Características físico-químicas. *Química Nova, 32*, 303-308. http://dx.doi.org/10.1590/S0100-40422009000200007

Turhan, I., Tetik, N., Karhan, M., Gurel, F., & Tavukcuoglu, H. R. (2008). Quality of honeys influenced by thermal treatment. *LWT-Food Science and Technology, 41*, 1396-1399. http://dx.doi.org/10.1016/j.lwt.2007.09.008

United States Department of Agriculture [USDA]. (1985). *United States Standards for Grades of Extracted Honey*. Processed Products Branch. U.S Department of Agriculture, Washington, D.C.

Vit, P. (2009). Caracterización fisicoquímica de mieles de abejas sin aguijón (Meliponini) de Venezuela. *Revista del Instituto Nacional de Higiene "Rafael Rangel", 40*, 7-12.

Vit, P., Bogdanov, S., & Kilchenmann, V. (1994). Composition of Venezuelan honeys from stingless bees (Apidae: Meliponinae) and *Apis mellifera* L. *Apidologie, 25*, 278-288. http://dx.doi.org/10.1051/apido:19940302

Vit, P., Medina, M., & Enríquez, E. (2004). Quality standards for medicinal uses of Meliponinae honey in Guatemala, Mexico and Venezuela. *Bee World, 85*, 2-5. http://dx.doi.org/10.1080/0005772X.2004.11099603

Viuda-Martos, M., Ruiz-Navajas, Y., Zaldivar-Cruz, J. M., Kuri, V., Fernández-López, J., Carbonell-Barrachina, A., & Pérez-Álvarez, J. (2010). Aroma profile and physico-chemical properties of artisanal honey from Tabasco, Mexico. *International Journal of Food Science and Technology, 45*, 1111-1118. http://dx.doi.org/10.1111/j.1365-2621.2010.02243.x

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Sensory Comparison of Bread Crumb with and without Crust

Katharina Fuckerer[1], Oliver Hensel[2] & Joachim J. Schmitt[1]

[1] Department of Food Technology, University of Applied Science Fulda, Fulda, Germany

[2] Faculty of Organic Agricultural Sciences, Department of Agricultural Engineering/Livestock Systems Engineering, University of Kassel, Kassel, Germany

Correspondence: Katharina Fuckerer, Department of Food Technology, University of Applied Science Fulda, Leipziger Straße 123, 36039 Fulda, Germany. Tel: 49-(0)661-9640-541. E-mail: Katharina.Fuckerer@lt.hs-fulda.de

Received: May 4, 2015 Accepted: June 10, 2015 Online Published: July 31, 2015

doi:10.5539/jfr.v4n5p33 URL: http://dx.doi.org/10.5539/jfr.v4n5p33

Abstract

Rye-bread belongs to traditional food in Europe and is preferred by elderly people in Germany. But those people often have difficulties with chewing the firm crust of those breads which, therefore, gets cut off. Current opinion suggests that flavor substances, produced during baking, diffuse from crust to crumb and therefore, bread baked with weak or without crust does not taste well. In present study, however, it was determined by triangle tests as well as color and firmness measurements, that crumb of crustless bread is nearly similar to crumb of bread with crust.

Keywords: crumb, crustless, rye bread, sensory

1. Introduction

Bread is part of basic food, where the average consumption per person per year is 50 kg in Europe (FoB, 2010). In Germany bread consumption is even above 80 kg per person per year (Heseker, 2008).

Since rye-bread belongs to traditional food, many elderly people prefer eating this bread despite the fact that many very old people often have chewing and swallowing difficulties (Fillion & Kilcast, 2001; Morley, Glick, & Rubenstein, 1995; Rehrmann, 2007; Volkert, Kruse, Oster, & Schlierf, 1991). Therefore those elderly could chew bread without crust more easily and prefer this. The flavor of crumb of crustless bread has to be similar to crumb of bread with crust, because consumer taste is a key attribute of bread (Heiniö, 2006). Therefore it is necessary that crustless bread tastes like bread with crust.

Flavor of bread depends on ingredients (Baker & Mize, 1939; Visser't Hoft & De Leeuw, 1935) and substances produced during fermentation (Baker & Mize 1939; Baker, Parker & Fortmann, 1953; Robinson, Lord, Johnson & Miller, 1958; Salim-ur-Rehman, Paterson, & Pigott, 2006; Visser't Hoft & De Leeuw, 1935; Wiseblatt, 1957). Baker and Mize (1939) and Baker et al. (1953) concluded in their studies, that flavor of bread is influenced by heat reactions in the crust during baking, e.g. maillard reaction. Lindenmeier and Hofmann (2004) also determined that maillard reaction influenced formation of typical bread flavor.

Previous literature assumed that there is a diffusion of flavor substances from crust to crumb. So Baker and Mize (1939) maintained those flavor products from the crust, formed during baking, that reached the crumb. Lorenz and Maga (1972) assumed that aldehydes diffuse from the crust into the crumb via partial diffusion during aging. Thus those authors are convinced that crust influences the flavor of the crumb and that crustless bread offered unacceptable odor and flavor (Wiseblatt, 1961).

These results indicate that there is a sensory difference between crumb of bread with crust and crumb of bread without crust.

Therefore the aim of the following study was to identify if there is a difference between the crumb of bread with crust and the crumb of bread without crust. Both crumbs were compared and an investigation taken based on color and firmness measuring and in addition by sensory triangle test of differences and similarity.

2. Experimental

Bread was baked under laboratory conditions following the recipe shown in Table 1.

Table 1. Recipe for a kilogram of dough

Ingredientes	[g]
Rye flour type 997 [a]	415.2
Wheat flour type 550 [a]	135.1
Breadcrumbs	11.5
Dry Auer (Diamalt) [a]	16.5
Yeast (S. cerevisiae) [b]	7.5
Salt	11.5
Water (pre-warmed 37 °C)	402.7

[a] provided by bakery Storch, Fulda.

[b] stored at 4 °C.

2.1 Dough Preparation

Dough was prepared traditionally. Ingredients were kneaded with the dough kneader (Typ S20G3, DIOSNA, Osnabrück, Germany) initially for five minutes at Level 1 (60 strokes/ min) and a further five minutes at Level 2 (120 strokes/ min). Dough resting followed for 30 minutes at a temperature of 22 °C and was afterwards covered with a damp cloth. The dough was split up into six parts of 800 g. Each part was cylindrically kneaded and added to a fatted iron baking pan. Subsequently, the raw dough was incubated for one hour at 35 °C and 80% humidity in the proofing cabinet (Typ AEG5, MIWE, Arnstein, Germany) for the rising process.

2.2 Baking Procedure for Crustless Bread

Crustless brown bread (shown in Figure 1) was baked in a preserving pan (Typ abc 1410, Westfalia, Hagen, Germany), which was filled with 1 l of water. Before baking, the preserving pan was heated to 100 °C so that water started to steam. Then baking pans, each covered with a second metal pan on top, were placed into the steaming water for baking. Loaves of bread were baked for 70 minutes and ended up with a core temperature of 95 °C.

2.3 Baking Procedure for Bread With Crust

Breads with crust (shown in Figure 1) were baked in conventional oven (Type CE 416/77H, Winkler Wachtel, Hilden, Germany). The Oven was heated to 200 °C and the baking process took place for 50 minutes until the bread with crust had the core temperature of 95 °C. For the first 5 minutes the slide damper was open and steam was added for 5 seconds.

Figure 1. Rye bread with crust (left) and without crust (right)

2.4 Color and Firmness Measurement

The Color of both crumbs were measured with spectro-color-meter (Typ LMG 183, Hach-Lange, Berlin, Germany), after standardization with a white and black calibration plate. The color was recorded using CIE-L*a*b* uniform color space (CIE-Lab), where L* indicates lightness, a* indicates coloring on a green (-) to red (+) axis and b* indicates coloring on a blue (-) to yellow (+) axis. The spectral curves were determined over 400-700 nm range using illuminant D65 and with a 10° standard observer. Color difference between crustless crumb and crumb with crust was calculated, using the following Equation (1):

$$\Delta E = \sqrt{[(L_1^* - L_2^*)^2 + (a_1^* - a_2^*)^2 + (b_1^* - b_2^*)^2]} \quad (1)$$

Note: L_1*, a_1* and b_1*: Values of crustless crumb bread; L_2*, a_2* and b_2*: Values of crumb bread with crust.

Crumb firmness was measured using a TA-XT Plus texture analyzer (Stable-Micro-Systems, Surrey, Surrey, Great Britain), which measured maximal force in Newton. Slices of 25 mm were compressed 9.5 mm with a cylindrical probe (diameter: 35 mm, contact: 962 mm², cylinder aluminum) and with a test speed of 1.00 mm/sec.

For the color and firmness measurement three cooled (3 hours) slices of bread from the center of the loaves were measured and three breads were measured each day. Hence 27 values of each crumb type were determined.

2.5 Storage of Breads for Sensory Test

After baking, both bread types were cooled for three hours and sliced in 1.0 cm thick slices. Sliced loaves of bread were kept in dense plastic bags for 20 hours at room temperature.

2.6 Sensory Analyses

Similarity Tests were performed by using the triangle method (DIN EN ISO 4120: 2007) and to determine if the crumb of bread with crust was similar to the crumb of bread without crust. For the triangle test panelists have to evaluate three samples, two are identical and one is different. Panelists were asked to pick the sample that is different. Triangle test is a forced-choice test, so that panelists have to choose a sample as the different one.

Prior to testing one round piece was cut out from the middle of the crumb of a slice of bread with, respectively, without crust. The samples had a diameter of 3.3 cm and a thickness of 1.0 cm. Sensory analyses were conducted in the sensory laboratory of University of Fulda and were performed by 37 voluntary panelists (20 male, 17 female, mean age 25.6) who had experience with bread (participation at sensory test with bread), but were untrained in regard to the triangle test. All participants were students with normal olfactory and gustatory function and from University of Fulda. Untrained panelists were chosen to determine whether consumers detect the difference between both crumbs.

Bread crumbs were tested in three different ways over a two week period. During one session a panelist analyzed four different triangles. Hence 148 triangles were tested. Bread samples were served in small glass bowls at room temperature (23 °C) and were coded with a three-digit random number. Each triangle had a different and random order of the samples, so that one triangle was built of two samples of crumb of crustless bread and one samples of crumb with crust. Another triangle was built of two samples of crumb of bread with crust and one crumb sample of crustless bread. Each panelist received four random, different orders of the triangles. Therefore all serving orders were analyzed (AAB, ABA, BAA, BBA, BAB, ABB). Four of six possible combinations were researched, because more than four triangles could overburden panelists. The four combinations were selected randomly. Pure water was served for neutralization. The panelists were informed that one of the samples was different from the others and were asked to identify this sample and to explain their decision. If a panelist were unable to detect a difference between all three samples, they were instructed to make a guess and to note that it was so.

2.7 Statistical Analysis

Means and standard deviations of color and firmness measurement were calculated with SPSS statistical software (Version 20.0, SPSS, Chicago, USA). Normal distribution was determined with Shapiro-Wilk-Test ($p = 0.05$) and test of homogeneity of variance was passed by Levene Test ($p = 0.05$). Significant differences ($p < 0.05$) among crustless bread and bread with crust were calculated with single-factor ANOVA.

Sensory test results were analyzed by a statistical significance template for triangle tests of similarity (Meilgaard, Civille, & Carr, 1999; Quadt, Schwarz, & Schönberger, 2009) according on a binomial distribution. Parameters for triangle method of similarity were defined with α-risk-level of 0.01 (probability of concluding that a perceptible difference exists when 1 does not) and β-risk-level of 0.01 (probability of concluding that no perceptible difference exists when 1 does) and p_d-level of 0.3 (true proportion of population able to detect a difference between samples). A p_d-level of 0.3 was chosen because it equates a medium range of population, which are able to detect difference between samples (Busch-Stockfisch 2003).

3. Results

3.1 Color Measurement

L*a*b*-values of both crumb types are shown in Table 2. Lightness values (L*) of crumb with crust and crustless crumb were 55.96 ± 1.39 and 55.79 ± 0.88, respectively. Between both L*-values there is no significant

difference. Crumb of crustless bread showed higher a*-values (4.41 ± 0.21) then crumb of bread with crust (4.27 ± 0.21). The yellow hue (+b*) was also higher in crumb of crustless bread (16.61 ± 0.46) then in crumb of bread with crust (16.20 ± 0.40). a*- and b*-values of both crumbs differed significantly ($p < 0.05$).

Crumb color difference of both bread types was evaluated in terms of Δ E. The calculated difference between crumb of crustless bread and bread with curst was $\Delta E = 0.47$.

3.2 Firmness Measurement

Crumb firmness values of bread with and without crust are shown in Table 2. The firmness of crumb of bread with crust (22.124 ± 2.257 N) is higher than crumb of crustless bread (19.100 ± 1.637 N). Evaluation of ANOVA shows, that both crumbs differed significantly ($p < 0.05$).

Table 2. L*a*b*-values and crumb firmness of bread with crust and crustless bread

	Crumb of bread with crust	Crumb of crustless bread
Color		
L*	55.96 ± 1.39	55.79 ± 0.88
a*	4.27 ± 0.21	4.41 ± 0.21
b*	16.20 ± 0.40	16.61 ± 0.46
Firmness in N	22.124 ± 2.257	19.100 ± 1.637

3.3 Sensory

The results of triangle test of the two types of bread crumbs are demonstrated in Table 3. The sensory test shows that 85 triangles out of 148 triangles were detected incorrectly which corresponds to 57.4% and 63 triangles out of 148 triangles were identified correctly, which corresponds to 42.6%. These results of the triangle test of similarity revealed a similarity between crumb of bread with crust and crumb of bread without crust.

Table 3. Results of triangle test

	Number of judgments	Expressed as a percentage
Correct judgments	63	42.6 %
Wrong judgments	85	57.4 %

Figure 2 shows, reasons for correct judgments which panelists noted. Thereby it is noted, that 57% of panelists could differentiate the samples because of differences in texture and 13% of panelists guessed correct judgment. Just 30% of panelists identified correct judgments because of differences in flavor.

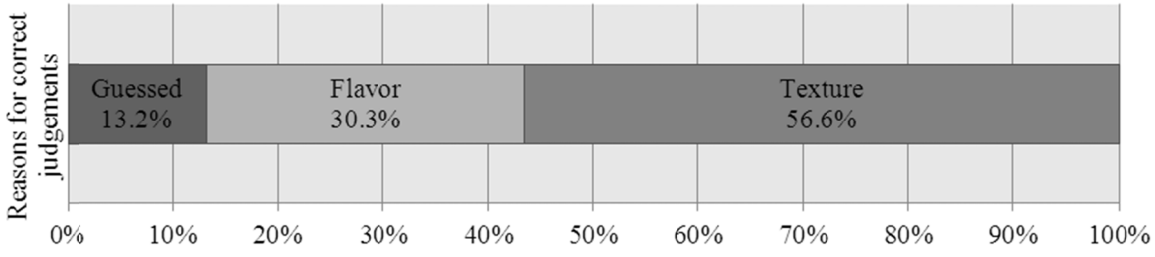


Figure 2. Reasons for correct judgments, which were noted by panelists

4. Discussion

4.1 Color and Firmness

Color measuring demonstrated that there was no significant difference between both L*-values. However a*- and b*-values differed significantly. Although color distance (Δ E) of both crumbs was 0.47 which means that

the observer cannot see any differences between both colors (Dreher, 2009). Therefore, it is assumed that panelists cannot determine a color difference between crumb of crustless bread and crumb of bread with crust during sensory tests.

Measurement of firmness showed that there was a significant difference of texture between crustless bread and bread with crust, although crumb of crustless bread was softer then crumb of bread with crust. Differences in firmness is the result of a different baking procedure. Crustless bread was baked in stream of water and therefore the texture was softer, like Baik and Chinachoti (2003), Curti, Carini, Bonacini, Tribuzio and Vittadini (2013) and Piazza and Masi (1995) also reported.

Comparing the results of firmness measurement and sensory test, it was recognized that panelists who identified the correct sample, detected correct sample very often (57%) due to the difference texture. This relation indicated that a different texture was the major difference between crustless crumb and crumb of bread with crust and not the difference in flavor.

4.2 Sensory

The test showed that crumbs of bread baked with and without crust were similar. Thus the results suggest that there was no diffusion of flavor substances from crust to crumb, which was sensory perceptible by panelists.

In contrast to this data, many authors determined slightly milder flavor in crustless bread and stronger flavor in bread baked with crust. Hence they supposed that flavor substances produced in crust during baking diffuse to the crumb (Baker et al., 1939, Baker & Mize, 1953, Lorenz & Maga, 1972, Wiseblatt, 1961).

In case of the experiments conducted by Baker and Mize (1939), this difference could be explained by their different way of baking crustless breads. The scientists succeeded in getting crustless breads by heating the dough between electrodes in a field of alternating current whereas in the constant study those breads were baked in steam of water. Providing alternating current heating may possibly produce some flavor active substances in the crumb of those breads. Another difference may be caused by the kind of bread baked. In the present study, 80 % rye bread with 20 % wheat flour enriched with sour dough was made which might be more enriched in flavor than pure wheat flour breads. Nevertheless, this study proves that the widely accepted assumption that the flavor of bread crumb is influenced by the crust is not true in general and is not detectable by sensory tests; at least for rye-breads.

5. Conclusion

Low color distance (ΔE) between both crumbs showed that observers normally cannot see differences between the crumbs. Significant softer texture of crustless crumb indicates that major differences between both bread types are different in texture and not in different flavor.

Sensory comparison of the crumb of crustless breads and breads with crust indicated in this study a similarity of both crumbs. Because many panelists guessed differing sample or recognized differing sample due to different texture, it is assumed that there was no sensory noticeable diffusion of flavor substances from crust to crumb in bread with crust. This data indicates that the generally accepted and taughed assumption that the flavor of crust diffuses into the crumb of bread is, at least for rye-breads, not detectable by sensory tests.

Acknowledgements

This project (HA project no. 359/12-49) was funded in the framework of Hessen ModellProjekte financed with funds of LOEWE-Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz, Förderlinie 3: KMU-Verbundvorhaben.

References

Baik, M. Y., & Chinachoti, P. (2003). Water self-diffusion coefficient and staling of white bread as affected by glycerol. *Cereal Chem, 80*, 740-744. http://dx.doi.org/10.1094/CCHEM.2003.80.6.740

Baker, J. C., & Mize, M. D. (1939). Some observations regarding the flavor of bread. *Cereal Chem, 16*, 295-297.

Baker, J. C., Parker, H., & Fortmann, K. (1953). Flavor of bread. *Cereal Chem, 30*, 22-30.

Busch-Stockfisch, M. (2003). *Dreiecksprüfungen – Triangeltest*. In S. Praxishandbuch, M. Busch-Stockfisch, (4th ed., pp. 1-12). Behr's Verlag.

Curti, E., Carini, E., Bonacini, G., Tribuzio, G., & Vittadini, E. (2013). Effect of the addition of bran fractions on bread properties. *J. Cereal Sci, 57*, 325-332. http://dx.doi.org/10.1016/j.jcs.2012.12.003

Dreher, A. (2009). *Proseminar Farbmanagement - Thema 2 - Grundlagen II.* Universität Koblenz-Landau,

Institut für Computervisualistik.

FoB. (2010). *European Bread Market.* Retrieved 7. August 2014, from http://www.bakersfederation.org.uk/the-bread-industry/industry-facts/european-bread-market.html

Fillion, L., & Kilcast, K. (2001). Towards a measurement of oral tactile sensitivity and matication performance: development of texture tests. *Leatherhead Food RA Research Report, 781. Surrey (UK): Leatherhead Food.*

Heiniö, R-L. (2006). *Sensory attributes of bakery products*. In Y. H. Hui (Ed.), *Bakery products: science and technology* (pp. 285-298), Blackwell Publishing. http://dx.doi.org/10.1002/9780470277553.ch16

Heseker, H. (2008). Ernährungssituation in Deutschland. In E. V. (Ed.), *Deutsche Gesellschaft für Ernährung Ernährungsbericht 2008*. (pp. 26-36). Bonn.

Lindenmeier, M., & Hofmann, T. (2004). Influence of baking conditions and precursors supplementation on the amounts of the antioxidant pronyl-L-lysine in bakery products. *J. Agric. and Food Chem, 52*, 350-354. http://dx.doi.org/10.1021/jf0346657

Lorenz, K., & Maga, J. (1972). Staling of White Bread: and Glc Headspace Profiles. *J. Agric. Food Chem, 20*(2), 211-213. http://dx.doi.org/10.1021/jf60180a035

Meilgaard, M., Civille, G. V., & Carr, B. T. (1999). *Sensory evaluation techniques* (3rd ed.), Boca Raton, CRC Press. http://dx.doi.org/10.1201/9781439832271

Morley, J. E., Glick, Z., & Rubenstein, L. Z. (1995): *Geriatric nutrition: A comprehensive review*. (2nd ed.), New York: Raven Press.

Piazza, L., & Masi, P. (1995). Moisture redistribution throughout the bread loaf during staling and its effect in mechanical properties. *Cereal Chem, 72*, 320-325.

Quadt, A., Schwarz, M., & Schönberger, S. (2009). *Statistische Auswertungen in der Sensorik. Vol. I,* Hamburg, Behr's Verlag: CD-Rom.

Rehrmann, N. (2007). Gut ernährt im Alter. *UGB-Forum, 2*, 61-65.

Robinson, R. J., Lord, T. H., Johnson, J. A., & Miller, B. S. (1958). The aerobic microbiological population of pre-ferments and the use of selected bacteria for flavor production. *Cereal Chem. 35,* 295-305.

Salim-ur-Rehman, Paterson, A. & Pigott J. R. (2006). Flavour in sourdough breads: a review. *Trends Food Sci. Tech., 17*, 557-566. http://dx.doi.org/10.1016/j.tifs.2006.03.006

Visser't Hooft, F., & De Leeuw, F. J. G. (1935). The occurrence of acetylmethylcarbinol in bread and its relation to bread flavor. *Cereal Chem, 12*, 213-229.

Volkert, D., Kruse, W., Oster, P., & Schlierf, G. (1991). Malnutrition in geriatric patients: diagnostic and prognostic significance of nutritional parameters. *Ann. Nutr. Metab, 36*, 97-112. http://dx.doi.org/10.1159/000177704

Wiseblatt, L. (1957). The flavor of bread. *Milling Production, 22*(1-2), 18-19.

Wiseblatt, L. (1961). *Baker's Dig, 35, 5,* 60.

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Morphological and Molecular Characterization of Ochratoxin A Producing Black Aspergilli from Grape Pomace

Bernice Karlton-Senaye[1], Jianmei Yu[2] & Leonard Williams[1]

[1] Center for Excellence in Post Harvest Technologies, North Carolina Agricultural and Technical State University, Kannapolis, U.S.A

[2] Department of Family and Consumer Sciences, North Carolina Agricultural and Technical State University, Greensboro, U.S.A

Correspondence: Bernice Karlton-Senaye, Center for Excellence in Post Harvest Technologies, North Carolina Agricultural and Technical State University, Kannapolis, NC 28081, U.S.A. Tel: 1-704-250-5702. E-mail: bekarlton@gmail.com

Received: June 12, 2015 Accepted: July 16, 2015 Online Published: August 4, 2015

doi:10.5539/jfr.v4n5p39 URL: http://dx.doi.org/10.5539/jfr.v4n5p39

Abstract

Grape pomace (GP), a winery by-product is increasingly being explored as food ingredients. Ochratoxin A (OTA), a natural toxigenic metabolite frequently found in wine and its by-products. Black Aspergilli are mainly responsible for OTA build-up and contamination of grapes and winery by-products. The fungal population in GP of five grape cultivars were enumerated and characterized. Fungal population ranged from 4.27±0.05 to 5.35±0.04 Log CFU/mL with GP from Chardonnay being the most contaminated. *Aspergillus niger* (81.1%) was found to be the major source of contamination and most frequently isolated fungal species. Other fungal isolates were *A. carbonarius* (13.51%) and *A fumigatus* (5.39%). Fungal contamination of GP correlated with the type of grape cultivars used for the pomace. Fourteen identified mold isolates were confirmed by PCR using primer pairs ITS1/NIG, ITS1/CAR and ITS1/FUM. Random amplified polymorphic DNA (RAPD) analysis with eight primers OPC-04, OPC-06, OPC-08, OPC-10, OPC-11, OPC-12, OPC-13 and OPC-14 revealed similarity in band patterns between the isolates and the control. Clustering of banding patterns generated from amplification with primer OPC-12 using Pearson's coefficient detected similarity at 99.10%, 97.60%, 86.30% and 99.40%, 99.10%, 87.60%, 78.50% among *Aspergillus niger* and *Aspergillus carbonarious* strains, respectively, confirming the identification of potential ochratoxigenic black *Aspergillus* strains in the GP. The findings from this study suggest that GP obtained from some grape cultivars could be unsafe as food ingredients due to contamination by ochratoxigenic-producing molds, which is an indicative factor for the presence of ochratoxin A and other mycotoxins.

Keywords: grape pomace (GP), ochratoxin A (OTA), fungal contamination, *Aspergillus carbonarius*, *Aspergillus niger*, internal transcription spacer (ITS), RAPD-PCR

1. Introduction

GP is a by-product of grapes derives from wine processing. In recent times, grape pomace (GP) is gaining use in food products. Many studies have estimated pomace to be 20-30% of the original weight of grape processed into wine (Chand et al., 2009; Yu & Ahmedna, 2013). About 75% of harvested grape fruit is used for wine production. The remaining 25 %, which consist of skin, seed and pulp referred to as GP, is typically composted as fertilizer, or used in animal feed (Dwyer et al., 2014). Numerous studies have also shown that GP could serve as a functional food ingredient for the prevention of diet related diseases such as cardiovascular diseases, obesity, and certain cancers (Bousetta et al., 2009). Due to its high chemical and rich nutrients content, GP also has a potential to support the growth of microorganisms (Borrero et al., 2004, 2005). Over a decade, GP has increasingly been used as food ingredient in products such as sourdough, cookies, minced fish, extruded snacks, frankfurters, and muffins (Canett Romero et al., 2004; Altan et al., 2008; Sánchez-Alonso et al., 2008; Mildner-Szkudlarz et al., 2011a, 2015a).

Ochratoxin A (OTA), a naturally occurring toxin, with nephrotoxic, carcinogenic, immunotoxic, genotoxic, teratogenic effects (Pfohl-Leszkowicz & Manderville, 2007; Reddy & Bhoola 2010; Matsuda et al., 2013), and

Balkan Endemic Nephropathy tendencies (Abozied et al., 2002; Stefanović et al., 2009) found in a variety of different agricultural products and their by-products, including GP are creating food safety concerns. Members of *Aspergillus* section *Nigri* (black aspergilli) are mainly responsible for OTA accumulation in GP. Numerous studies showed that grapes could be contaminated from different fungal species during harvesting or processing by pressing for wine making, or sun drying for raisings (Somma et al., 2012). Fungal contamination of grape is mainly by *Aspergillus*, *Botrytis*, and *Penicillium* species (Guzev et al., 2008, Terra et al., 2012). However, *Aspergillus* section *Nigri* is considered to be principal source of ochratoxin A contamination in grapes and wine in various regions around the world. *A. carbonarius* and *A. niger* aggregate are the major OTA producer isolated and observed more frequently from grapes (Bau et al., 2006; Perrone et al., 2011). Although *A. niger* aggregate is more commonly found in grapes, the ability to produce OTA is lower than that of *A. carbonarius*. In addition, *A. fumigatus*, which belong to *A. niger* aggregate, isolated from grapes has also been found to be pathogenic. *A. fumigatus* has been report to be the cause of most invasive aspergillosis (IA) cases (Serrano et al., 2011). Worldwide IA cases was estimated to be only a few thousand per year, with an overall fatality of >50% (Latge, 1999). *Aspergillus* has the ability to penetrate the lung causing pulmonary conditions such allergic brochopulmonary aspergillosis, sinus infections, aspergilloma and invasive aspergillosis, which may spread to other organs such as brain, heart and kidney (Lasker, 2002; Vargas, 2006).

Determination of the mycoflora and OTA-producing mold in GP is vital in determining the potential OTA contamination of the GP and subsequently, leading to the development of appropriate storage and treatments of GP to ensure safety of consumers of such products. Although many work were reported on the world- wide occurrence of ochratoxin-producing *Aspergillus* species in grape and many other agricultural products no work has been published on contamination of GP with OTA producing fungi. Therefore, the aims of this study were to determine fungal population of the selected GP cultivars, to isolate and characterize ochratoxigenic black *Aspergilli* from GP.

2. Materials and Methods

2.1 Samples Collection

GP from five grape cultivars namely, Cabernet Franc, Cabernet Sauvignon, Merlot, Chardonnay and Sangiovesse were collected from two grape farms/wineries in North Carolina (USA). Samples weighing between 2-4 kg each were collected in sterile plastic bags, transported in cool boxes and frozen immediately at -20 ^{o}C for analyses.

2.2 Mycological Analyses

Thirty (30 g) of GP were mixed with 0.1% peptone water and blended with a stomach blender for 2 minutes, serially diluted and 100 µl of appropriate dilutions extract were plated in triplicates on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Oxoid, USA), Dichloran Glycerol 18% (DG18, Oxoid, USA) and Potato Dextrose Agar (PDA, Oxoid, USA) plates. Three grams (3 g) of each GP samples was also directly plated on DRBC to compare the fungal population. Both sets of samples were then incubated at 25 ^{o}C for 7 days in the dark. The total colonies of yeast and mold were enumerated and results were reported in colony forming units CFU/ml from average fungal counts (Pitt & Hocking, 1997; Dachoupakan et al., 2009; US Food and Drug Bacteriological Analytical Methods, FDA-BAM).

2.3 Isolation and Maintenance of Culture

Colonies of mold that appeared to look different were selected and coded. Selected molds were isolated by three point inoculation onto PDA (potato dextrose agar), MEA (malt extract agar) and CYA (Czapek yeast extract), 5 g $NaNO_3$; 1.0 g K_2HPO_4; 0.5 g KCl; 0.5 g $MgSO_4 \cdot 7H_2O$; 5.0 g yeast extract; 30.0 g sucrose; and 20.0 g agar in 1 liter water at pH 6.5 incubated for 7 days at 25 ^{o}C in the dark. Mold isolates were then maintained on Dichloran Glycerol 18% (DG18, Oxoid, USA) plates for further analysis (Pitt & Hocking, 1999; Dachoupakan et al., 2009; US Food and Drug Bacteriological Analytical Methods, FDA-BAM).

2.4. Identification of Mold

Molds isolates were identified morphologically according to Pitt and Hocking (1997) based on colony appearance, color, exudates and colony diameter on CYA, PDA, and MEA.

2.5 DNA Extraction

Samples taken from three point inoculated CYA plates were aseptically transferred into the labeled 1.5-ml microcentrifuge tubes. Approximately 100 mg of lyophilized mycelium extract was mixed in 1 ml of ChargeSwitch lysis buffer (Invitrogen, Carlsbad, CA, USA) lysis buffer containing reagent A. Four microliters

(4 µl) RNase A was added to each sample homogenized and centrifuged at 13,000 rpm for 5 min. One hundred microliters (100 µl) of 10% SDS was added to lysate and incubated at room temperature for 5 mins. Four hundred (400 ul) microliters of precipitation buffer (N5) (ChargeSwitch Plant DNA Kit) was added and centrifuged at 13,000 rpm for 3 min. Batches of 1200 ml of cleared supernatant were carefully transferred to a 1.5-ml centrifuge tubes for washing and purification followed by eluting according to the manufacturer's instruction to complete DNA extraction (Dachoupakan et al., 2009; Peronne et al., 2010).

2.6 Molecular Detection of OTA Producing Aspergillus Strains

2.6.1 PCR Amplification

PCR analysis was performed according to the method by Dachoupakan et al. (2009) with a slight modification using GoTaq PCR Core Systems I (Madison, WI, USA). ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') was used in combination with each of the following primers: CAR (5'-AGACAGGGGGACGGC-3'), NIG (5'-AGACAGGGGGACGGC-3'), FUM (5'-CGG CCC TTA AAT AGC CCG GTC-3') ELL (5'-CCCGGGATGGGGGACGG-3'), HET (5'-GCAAAATGGTTGGAGAGGTGC-3'), and JAP (5'-GAGAAGATTGGGGGTCGAGG-3'). A total PCR mixture of 25 µL containing: 14.75 µL deionized water, 2.5 µl of 5x buffer green, 2.5 µL of $MgCl_2$ (25mM), 1 µL dNTP (10 pmol/µl), 1 µl of primer ITS1 (10pmol/ µL), 1 µl of primer NIG for *A. niger* or 1 µl of primer CAR (10 pmol/ µl) for *A. carbonarius* or 1 µl of primer FUM (10 pmol/ µl) for *A. fumigatus*, 0.25 µL of Taq DNA polymerase and 2 µL of DNA template. PCR amplification was performed in a Eppendorf Master Pro (Applied Biosystem, Califonia, USA) Cycler set at 1 cycle of 4 min 30 s at 95 ^{o}C, 25 cycles of 30 s at 95 ^{o}C (denaturation) 25 s at 66 (annealing), 40 s at 72 ^{o}C (extension) and finally 1 cycle of 5 min at 72 ^{o}C (Dachoupakan et al., 2009; Gonzalez-Salgado et al., 2005), and 4 ^{o}C 10 mins (hold). The PCR products were separated on 2.5 % (w/v) agarose (Bio-Rad, California, USA) stained with 0.5µg/ml ethidium bromide and visualize with a UV trans-illuminator. A 1 Kb plus DNA ladder Generuler (Thermo Scientific, California, USA) was used as a standard marker to compare molecular masses of amplified DNA.

2.6.2 RAPD-PCR Analysis

Amplification of fungal DNA was performed according to the method described by Dachoupakan et al., (2009) with slight modifications using each of the following random primers (Applied Biosystems, California, USA): OPC-04 (5'-CCGCATCTAC-3'), OPC-06 (5'-GAACGGACTC-3'), OPC-08 (5'-TGGACCGGTG-3'), OPC-10 (5'-TGTCTGGGTG-3'), OPC-11 (5'-TGTCTGGGTG-3'), OPC-12 (5'-TGTCATCCCC-3'), OPC-13 (5'-AAGCCTCGTC 3'), and OPC-14 (5'-TGCGTGCTTG-3'). PCR was performed using GoTaq PCR Core Systems I (Madison, WI, USA) in a total volume of 25 µL containing: 12.5 µL deionized water, 2.5uL 5x buffer green, 2.5 µL25mM MgCl, 1 µL nucleotide mixture, 2 µL primer (10 pmol/ µL), 4 µL template DNA. Parameters for PCR were as follow: 1 cycle of 2 min at 92 ^{o}C for *A. carbonarius* and at 94 ^{o}C for *A. niger* isolates, 45 cycles for *A. carbonarius* and 35 cycles for *A. niger* aggregate of 1 min at 92 ^{o}C (denaturation), 1 min at 35 ^{o}C annealing, 2 min at 72 ^{o}C (extension) and finally 1 cycle of 5 min at 72 ^{o}C (Dachoupakan et al., 2009). The amplified DNA were separated by electrophoresis in 1 % (w/v) agarose (Bio-Rad, California, USA) in 10X TBE buffer and stained with ethidium bromide solution (10mg/mL) (Promega, Madison, WI, USA) and visualized under UV light. The molecular masses of amplified DNA were estimated by comparing with a 1 Kb plus DNA ladder (Thermo Scientific, California, USA).

2.7 Data Analysis

Statistical analysis of the random amplified polymorphic DNA (RAPD) analysis was carried out using GelCompare software (Applied Maths BVBA, Belgium). Similarity in band patterns was computed by cluster analysis using tree clustering with unweighted pair-group averaging (UPGMA) algorithm and corresponding tools in Biometrics.

3. Results and Discussion

3.1 Determination of Fungal Population

The yeast and mold counts ranged from 4.27±0.05 to 5.35±0.04 log CFU/mL (Table 1) with *Aspergillus* spp., being the predominant fungi detected. The highest population of mold (5.35±0.04 log CFUml-1) was detected in pomace derived from Chardonnay grape cultivar, which also showed lowest water activity (0.979±0.01) and highest pH (3.93±0.01). According to Diaz et al. (2009), ochratoxigenic species grow between water activity of 0.900 and 0.996. This indicates the role of water activity and pH in the growth of ochratoxigenic mold. It was observed that pomace from Chardonnay cultivar was the most contaminated with all the three strains of *Aspergillus* detected namely, *A niger, A. carbonarius and A. fumigatus.* The Chardonnay grape cultivar from

which this pomace was produced is used mainly for white wine production. The average total soluble sugar content (TSSC), which is a measure of the soluble sugar content for white and red grape cultivar are 78.15% and 26.03% respectively (Jiang et al., 2010). In white wine processing, grape peels and seeds are not fermented together with juice leading to relatively higher sugar content of GP derived from Chardonnay grape cultivar used in white wine processing. High sugar content could increase carbon source that promote fungal growth. No mold was detected in pomace from Cabernet Franc grape cultivars. The absence of mold in pomace from Cabernet Franc grapes cultivars could be due to its relative high acidity and low water activity (Figures not shown). Slightly higher fungal population was found on PDA compared to DRBC and DG18 agar (Table 1).

Table 1. Fungal population and percentage contamination of grape pomace cultivars on DRBC, PDA and DG18 for 7 days at 25 ^{o}C

GP	Yeast and mold counts (Log CFU mL^{-1})			Contamination by mold (%)		
	DRBC	PDA	DG18	*A. niger*	*A. carbonarius*	*A. fumigatus*
Cabernet Franc	NG	NG	NG	ND	ND	ND
Cabernet Sauvignon	<10	<10	<10	100	-	-
Merlot	<10	<10	<10	66.67	16.67	16.67
Chardonnay	4.47±0.56	5.35±0.04	4.27±0.05	81.10	13.51	5.39
Sangiovesse	NG	NG	NG	ND	ND	ND

NG-no growth; ND-not determined; <10 = less than 10 colonies/mL.

3.2 Determination of Contamination and Comparing the Morphological Characteristics of Different Molds

3.2.1 Percentage Contamination

Percentage contamination of the GP was determined. Pomace derived from Chardonnay was found to have the most contamination from mold. Merlot and Cabernet Sauvignon were also contaminated but to a lesser extent. Predominant fungal population belonging to black *Aspergillus* species made up 94.61 % of the total fungal population. *Aspergillus niger* (81.10%) was found to be the major source of contamination and most frequently isolated fungal species. Other fungal isolates were *A. carbonarius* (13.51%) and *A fumigatus* (5.39%). Several studies reported black *Aspergillus* as primary sources of contamination and major OTA producers in grape and related products (Bau et al., 2005; Medina et al., 2005; Lucchetta et al., 2010; Somma et al., 2012) from a span of studies on grapes and related products. Fungal contamination of the pomace correlated with the type of grape cultivar from which the pomace was derived.

3.2.2 Identification of Mold Isolates

A total of 7 out of the 14 previously selected isolates (Figure 1) including *A. carbonarius, A. niger and A. fumigatus* obtained from the GP samples collected from two winery in North Carolina (Figure 1) were further studied morphologically (Table 2, 3, 4) using three different culture media and molecularly by RAPD analysis.

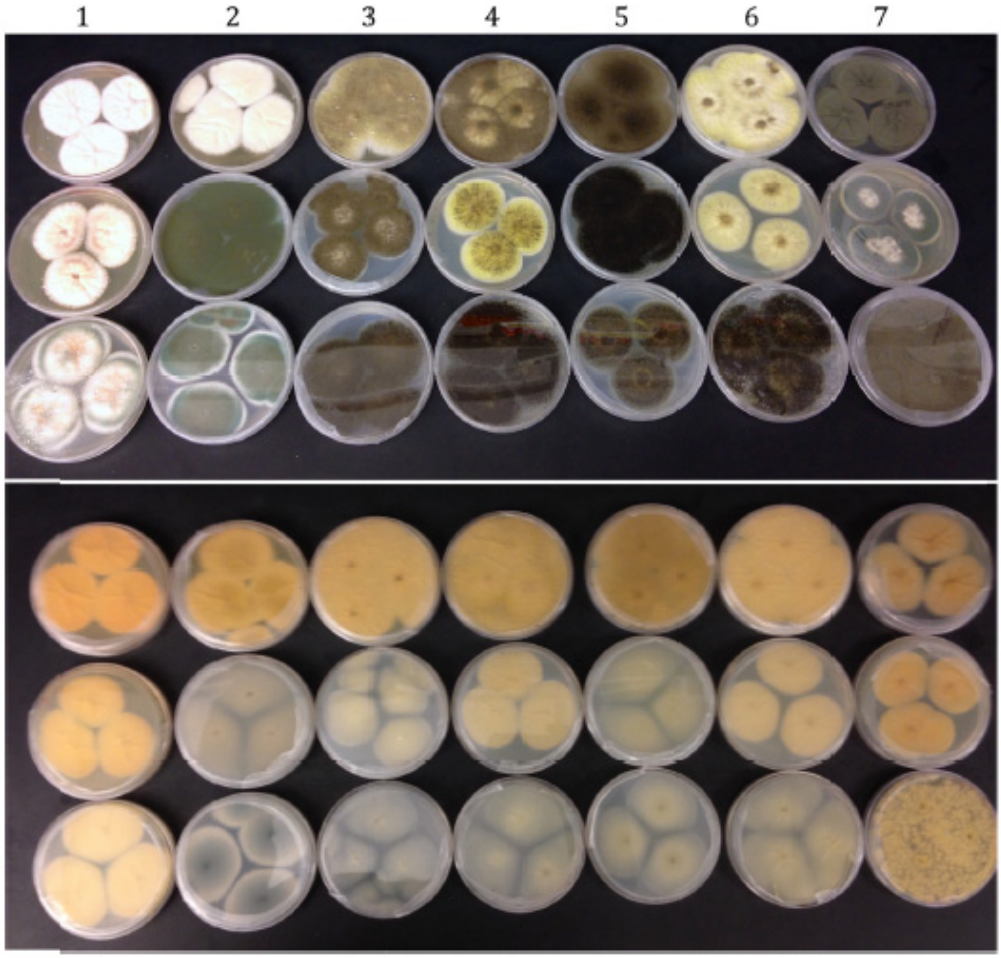


Figure 1. Front and reverse views of different mold (from left to right) isolated on CYA (lane 1), PDA (lane 2) and MEA (lane 3). *Aspergillus niger* (4,6); *Aspergillus carbonarius* (3, 5); *Aspegillus fumigatus* (1, 2, 7)

Table 2. Morphological identification of *A. carbonarius* isolates on CYA for 7 days at 25 ^{o}C

Morphology characteristics		Average colony diameter (mm) on		
Colony Appearance	Light brown and dark brown	PDA	CYA	MEA
Wrinkle colony	Not wrinkled	65	78	71
Reverse coloration	Cream to yellow			
Exudate production	Nil			

Table 3. Morphological identification of *A. niger* isolates on CYA for 7 days at 25 ^{o}C

Morphology characteristics		Average colony diameter (mm) on:		
Colony Appearance	Yellow and black	PDA	CYA	MEA
Wrinkle colony	Wrinkled	58	78	72
Reverse coloration	Florescent Yellow			
Exudate production	Nil			

Table 4. Morphological identification of *A. fumigatus* isolates on CYA for 7 days at 25 ^{o}C

Morphology		Average colony diameter (mm) on		
Colony Appearance	Light brown, dark brown, and dark green	PDA	CYA	MEA
Wrinkle colony	Wrinkled	97	64	57
Reverse coloration	Uncolored to dull yellow			
Exudate production	Nil			

3.3 Molecular Characterization of Fungal Isolates

Specific primers were used to amplify specific regions to confirm morphological identification of the isolates. In all, a total of four *A. niger*, seven *A. carbonarius* and three *A. fumigatus* isolates were tested for amplification using each primer pairs ITS1/NIG and ITS1/CAR and ITS1/FUM respectively. Single fragments estimated to be 400-500 bp amplified was detected only for suspected *A. carbonarius* when ITS1/CAR was tested (Figure 2).

Isolates that did not amplify previously were suspected to be either *A. niger* isolates or *A. fumigatus*, which were further amplified using ITS1/NIG also showed single bands of between 400-500 bp (Figure 3) and were identified as *A. niger*. Isolates that failed to amplify using ITS1/CAR and ITS1/NIG were confirmed as *A. fumigatus* using ITS1/FUM primer set. The remaining sets: ITS1/JAP, ITS1/HET, and ITS1/ELL showed no amplification of the genomic DNA for *A. carbonarius, A. niger*, and *A. fumigatus* (Table 3).

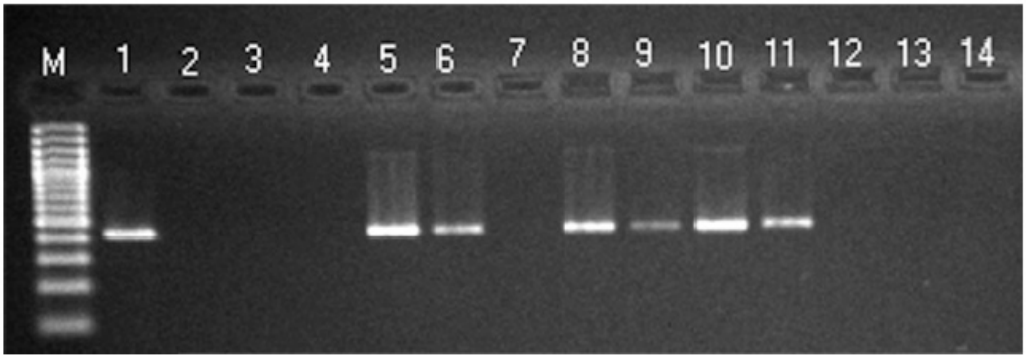


Figure 2. PCR amplification using ITS1/CAR and DNA from *A. carbonarius* isolates; Lane 1: *A. carbonarius* control (ATCC MYA-4641); Lanes 2, 3, 4 & 7: *A. niger* isolates; Lanes 5, 6, 8, 9, 10 & 11: *A carbonarius isolates*; Lanes 12, 13, 14: *A. fumigatus* isolates Lanes; M: DNA marker

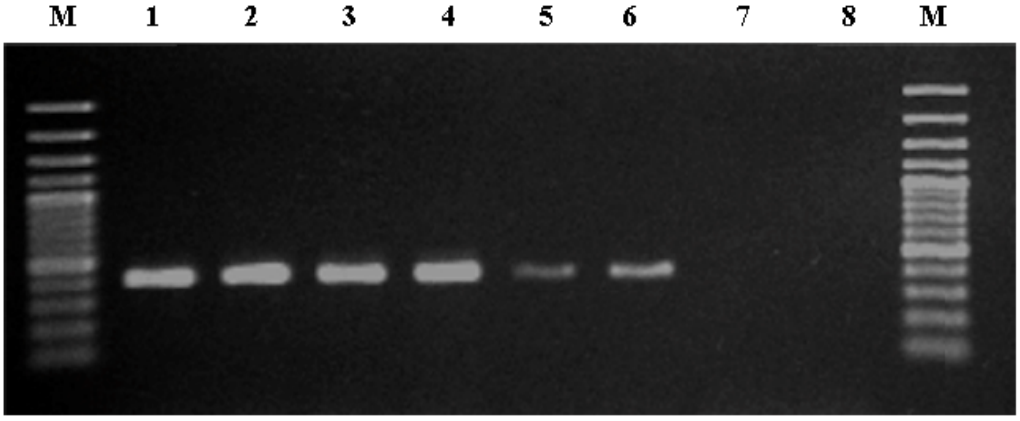


Figure 3. PCR amplification using ITS1/NIG and DNA from *Aspergillus niger* isolates; Lanes 1: *A. niger* control (ATCC 1015D-2); Lanes 2-6: *A. niger* isolates; Lanes 7, 8: *A. fumigatus*; Lanes M: DNA marker

Table 5. Fungal strains analyzed indicating, origin, species, host and the occurrence of PCR amplification product with primers: ITS1-NIG, ITS1-CAR, ITS1-FUM, ITS1-HET, ITS1-JAP and ITS1-ELL

Mold isolate	GP (Cultivar/source)	ITS1-NIG	ITS1-CAR	ITS1-FUM	ITS1-HET	ITS1-ELL	ITS1-JAP
101	Cabernet Franc	-	-	-	-	-	-
102	Cabernet Sauvignon	+	+	+	-	-	-
103	Chardonnay	+	+	+	-	-	-
104	Merlot	+	+	+	-	-	-
105	Sangiovesse	-	-	-	-	-	-

Aspergillus spp. have been isolated from many agricultural products include grape and its associated products such as wine (Somma et al., 2012; Guzev et al., 2008, Terra et al., 2012). Our findings corroborate with results obtained by other studies (Dachoupakan et al., 2009; Diaz et al., 2009; Gonzalez-Salgado et al., 2005).

3.4 Genotypic Studies

Series of RAPD analyses were conducted using 8 random primers (OPC-04, OPC-06, OPC-08, OPC-10, OPC-11, OPC-12, OPC-13, and OPC-14) for detecting similarity through amplified polymorphism between the genomic DNA of isolated *A. niger* and *A. carbonarius* strains and their respective control.

For *A. niger* isolates, similar banding patterns between bands 3 to 9 denoting random amplification of polymorphic DNA were detectable with primers OPC-06, OPC-08, OPC-10, OPC-11, OPC-12, and OPC-14 (Figure 4.) being the most interpretable. Primers OPC-04 and OPC-13 produced amplification that resulted into faint polymorphic bands. All isolates identified as *A. niger* showed identical sequence pattern that are also identical to banding in *A. niger* control samples. Primer OPC-11 displayed the faintest yet the highest discriminatory power (6) and OPC- 04 the least (3). Similar polymorphic banding was observed between isolates and *A. niger* control when primer OPC-12 was amplified with genomic DNA from *A. niger* isolates (Figure 7).

Similarly, *A. carbonarius* isolates also demonstrated similar banding with OPC primers with the highest (8) being with primer OPC 04 and the least (3) with OPC 06. Relatively more distinct bands were observed when primers OPC were used in amplification with genomic DNA of *A. carbonarius* isolates compare to *A. niger*. Except for primer OPC-11, all primers used produced distinct and interpretable polymorphic bands (Figure 5).

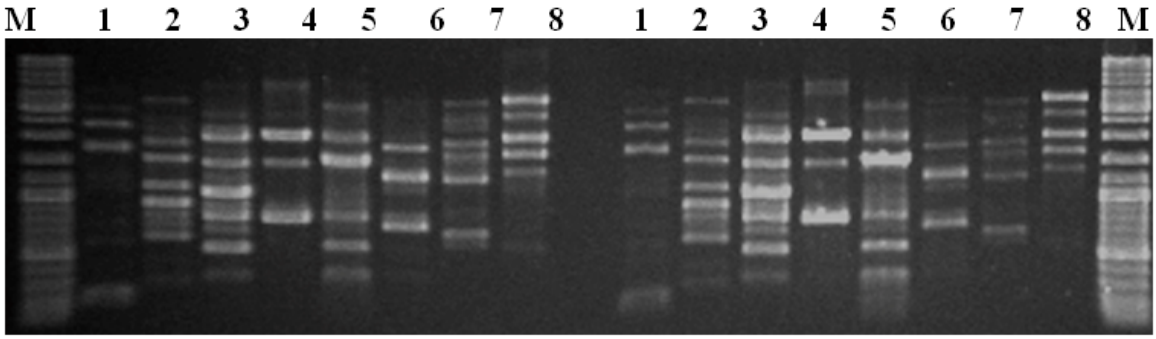


Figure 4. RAPD patterns of *A. niger* isolate, generated with OPC primers: Lane 1: OPC-04, Lane 2: OPC-06, Lane 3: OPC-08; Lane 4: OPC-10; Lane 5: OPC-11; Lane 6: OPC-12; Lane 7: OPC-13; Lane 8: OPC-14; M: DNA marker

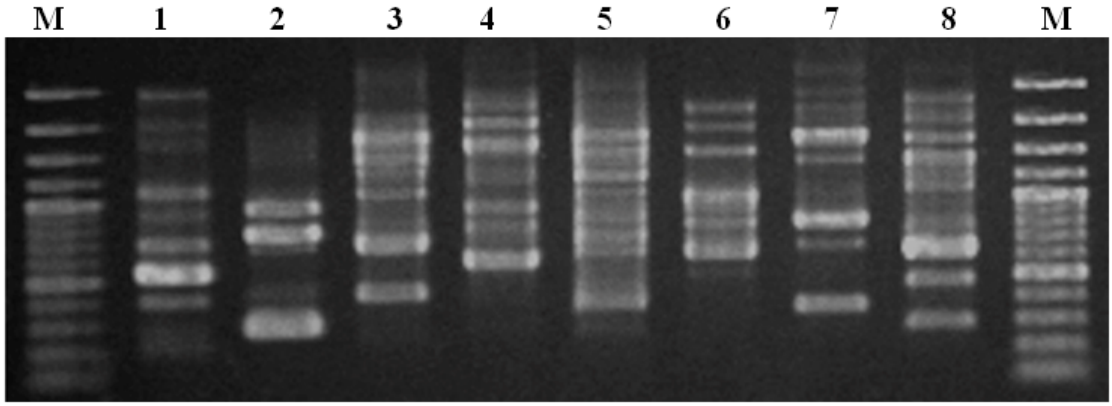


Figure 5. RAPD patterns of *A. carbonarius* isolate, generated with OPC primers: Lane 1: OPC-04; Lane 2: OPC-06; Lane 3: OPC-08; Lane 4: OPC-10; Lane 5: OPC-11; Lane6: OPC-12; Lane 7: OPC-13; Lane 8: OPC-14; M: DNA marker

Except for primers OPC 06 and OPC13 that showed distinct bands when used in amplification of genomic DNA of *A. fumigatus* isolates, all other primers used displayed faint bands (Figure 6). However, banding patterns observed in all isolates were similar to those found in respective controls (Figure 7).

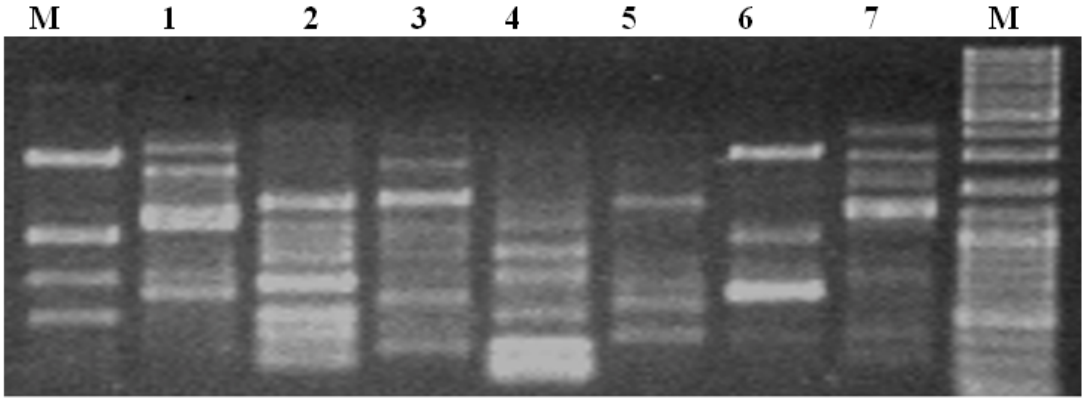


Figure 6. RAPD patterns of *A. fumigatus* isolate, generated with OPC primers: Lane 1: OPC-04; Lane 2: OPC-06; Lane 3: OPC-08; Lane 4: OPC-10; Lane 5: OPC-11; Lane 6: OPC-12; Lane 7: OPC-13; Lane 8: OPC-14; M: DNA marker

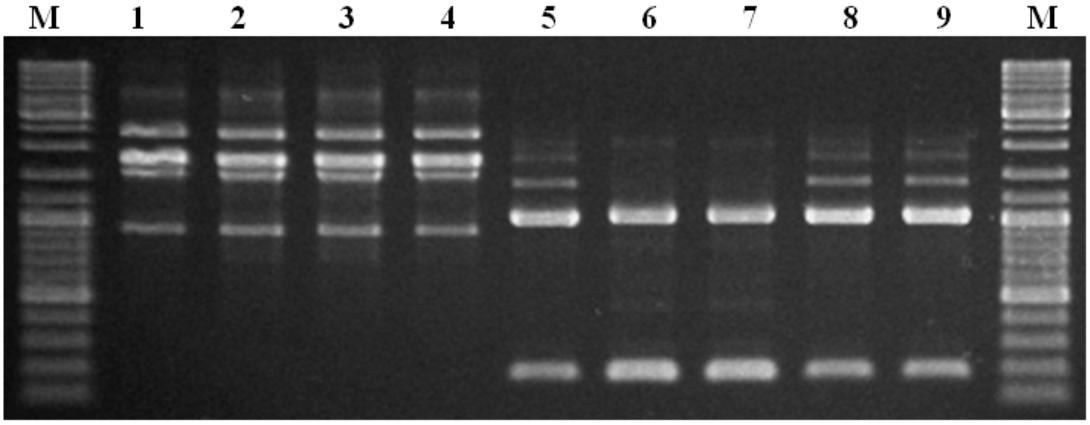


Figure 7. RAPD patterns of *A. niger* and *A. carbonarius* isolates and their respective controls, generated with primer OPC-12: Lane 1: *A. niger* control (ATCC 1015D-2); Lanes 2-4: *A. niger* isolates; Lane 5: *A. carbonarius* control (ATCC MYA-4641); Lanes 6-9: *A. carbonarius* isolates; M: DNA marker

To further confirm the fungal species isolated from the pomace primer OPC-12 was used for RAPD analysis (Figure 7.) for both *A. niger* (N1, N2, N3, *A. niger* control: **N4**) and *A. carbonarius* (isolates: C1, C2, C3, C4; *A. carbonarius* control: **C5)** strains. Figure 8 shows the dendogram, which was generated from the RAPD banding patterns using unweighted pair group average linkage clustering revealed three main clusters within *A. carbonarius* and *A. niger* strains (Figure 8). *A. carbonarius* and *A. niger* isolates and their respective controls obtained by cluster analysis showed close degree of similarity among the strains. The similarity percentages ranged from 78.5-97.6% among *A. carbonarius* isolates, N1 and N2 clustered together showing similarity at 99.1% and with N3 at 97.6%. Similarly, all *A. niger* isolates showed similarity at 86.3% to the *A. niger* control **(N4**). Similarly, *A. carbonarius* isolates C1 and C2 clustered together at 99.1% similarity. Isolate C4 is similar to the *A. carbonarius* control (**C5**) at 99.4% similarity. All strains belonging to *A. carbonarius* and *A. niger* showed clustering at 2.2% similarity, indicating a wide genotypic variation between *A. carbonarius* and *A. niger*.

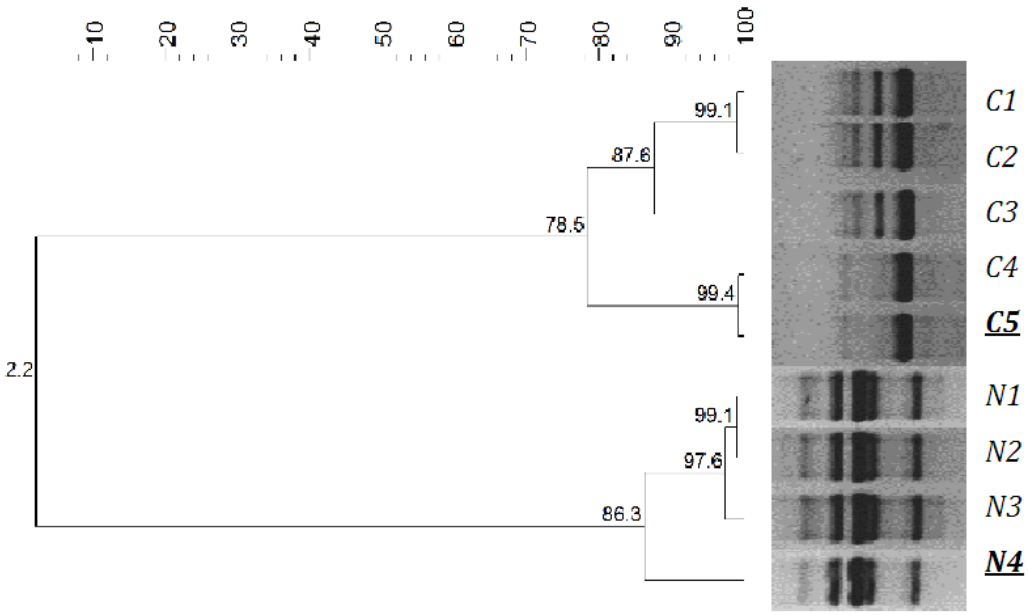


Figure 8. Dendogram based on UPGMA cluster analysis of *A. carbonarius* and *A. niger* isolates assessed from the comparison of RAPD fingerprints generated with primer OPC-12 using cluster analysis with percent similarity

Morphological group is indicated in letters: C1-C4 (*A. carbonarius* isolates); **C5** (*A. carbonarius* control); and N1-N3 (*A. niger* isolates); **N4** (*A. niger* control).

Black Aspergilli *are* considered primary producers and contaminant of OTA, a mycotoxin with lethal effects including immunosuppressive, teratogenic and carcinogenic consequences, in grapes and it's associated products (Varga & Kozakiewicz). *A. carbonarius* and *A. niger* have been the course of OTA production in grapes and its by-products (Cabanes et al., 2002; Varga et al., 2007; Peronne et al., 2007).

Our results agree with the study of Martinez et al. (2009) using ap-PCR and phylogenic analysis based on ITS and IGS sequences. The data obtained are also consistent with several other studies (Lasker, 2002; Varga et al., 2007; Peronne et al., 2008; Moslem et al., 2010). Therefore, a close relationship appeared to exist between the isolates and the control, confirming the identification of potential ochratoxin A-producing *A. niger,* and *A. carbonarius* in tested GP.

4. Conclusion

Our study found high levels of fungal population and contamination (94.61%) by potential ochratoxigenic *A. niger* and *A. carbonarious* in certain GP cultivars. Additionally, clear evidence of the presence of potential OTA producing *A. niger* and *A. carbonarius* isolates was established through PCR amplification of ITS region and close RAPD-PCR similarity matrix found between the isolates and their respective references. Our findings suggest that GP could be contaminated with high levels pathogenic black Aspergillus, which is an indicative factor for the presence of ochratoxin A and other mycotoxins rendering certain GP unsafe as food ingredients.

The contamination of grapes by mold is closely related to agricultural practices such as the use of fungicide and removal of grapes contaminated with mold before harvest, and handling after harvest. There is a pressing need to enhance awareness among wine processors to develop more stringent and appropriate protocols for proper handling of GP from vineyard to winery sites and to table to ensure they are safe as food products for both humans and animals.

Acknowledgement

This study was financially supported by a USAD-NIFA Capacity Building grant. Award number: 2013-38821-21147.

References

Abouzied, M. M., Horvath, A. D., Podlesny, P. M., Regina, N. P., Metodiev, V. D., Kamenova-Tozeva, R. M., … Ganev, V. S. (2002). Ochratoxin concentrations in food and feed from a region with Balkan Endemic Nephropathy. *Food Additives and Contaminants, 19*, 755-764. http://dx.doi.org/10.1080/02652030210145036

Altan, A., McCathy, K. L., & Maskan, M. (2008). Twin-screw extrusion of barley-grape pomace blends: extrudate characteristics and determination of optimum processing conditions. *Journal of Food Engineering, 89*, 24-32.

Bau, M., Castella, G., Bragulat, M. R., & Cabañes, F. J. (2006). RFLP characterization of *Aspergillus niger* aggregate species from grapes from Europe and Israel. *International Journal of Food Microbiology, 111*, S18-S21. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.03.008

Borrero, C., Trillas, M. I., Ordovás, Tello, J.C, & Aviles, M. (2004). Predictive factors for the suppression of Fusarium wilt of tomato in plant growth media. *Phytopathology, 94*, 1094-1101. http://dx.doi.org/10.1094/PHYTO.2004.94.10.1094

Bousetta, N., Lebovka, N., Vorobiev, Adenier, H., Bedel-Cloutour, C., & Lanoiselle, J. L. (2009). Electrically assisted extraction of soluble matter from chardonnay grape skins for polyphenol recovery. *Journal of Agricultural Food Chemistry, 57*, 1491-1497. http://dx.doi.org/10.1021/jf802579x

Cabañes, F.J, Accensi, F, Bragulat, Abarca, M.L., Castella, G., Minguez, S., & Pons, A. (2002). What is the source of ochratoxin A in wine? *International Journal of Food Microbiology, 79*, 213-215.

Canett Romero, R., Ledesma Osuna, A. I., Maribel, R., Sanchez, R., Morales Castro, R., Leon Martinez, L., & Leon-Galvez, R. (2004). Characterization of cookies made with deseeded grape pomace. *ALAN, 54*, 93-99.

Chand, R., Narimura, K., Kawakita, H., Ohto, K., Watari, T., & Inoue, K. (2009). Grape waste as a biosorbent for removing Cr(VI) from aqueous solution. *Journal of Harzardous Materials, 163*, 245-250. http://dx.doi.org/10.1016/j.jhazmat.2008.06.084

Dachoupakan, C., Ratomahenina, Martinez, R., Guiraud, J. P., Baccou, J. C., & Schorr Galindo, S. (2009). Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black aspergilli isolated from grapes. *International Journal of Food Microbiology, 132*, 14-23. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.03.011

Dayani, O., Ghiasi, A., & Tahmasbi, R. (2014). Aranian Journal of applied Animal Science. Chemical Composition, Physical Characteristics, Digestibility and Degradability of Grape Pomace Processed with Neurospora sitophila. *Iranian Journal of Applied Animal Science, 4*, 733-739.

Díaz, G.A., Torres, R., Vega, M., & Latorre, B. A. (2009). Ochratoxigenic Aspergillus species on grapes from Chilean vineyards and Aspergillus threshold levels on grapes. *International Journal of Food Microbiology, 133*, 195-199. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.04.018

Dwyer, K., Hosseinian, F., & Rod, M. (2014). The Market Potential of Grape Waste Alternatives. *Journal of Food Research*, *3*, 91-106. http://dx.doi.org/10.5539/jfr.v3n2p91

Gonzalez-Salgado, A., Patno, B., Vazquez, C., & Gonzalez-Jaen, M.T. (2006). Discrimination of Aspergillus niger and other Aspergillus species belonging to section Nigri by PCR assays. *FEMS Microbiology. Letters, 245*, 353-361. http://dx.doi.org/10.1007/s13213-014-0803-5

Jiang, Y., Simonsen, J., & Yanyun, Z. (2010). Compression-molded biocomposite boards from red and white wines. *Journal of Applied Polymer Science, 119*, 2834-2846. http://dx.doi.org/10.1002/app.32961

Khoury, E. I., & Atoui, A. (2010). Ochratoxin A: general overview and actual molecular status. *Toxins, 4*, 461-493. http://dx.doi.org/10.3390/toxins2040461

Latgé, J. (1999). Aspergillus fumigatus Aspergillosis. *Clinical Microbiology Review, 12*, 310-350.

Lasker, B. A. (2002). Evaluation of Performance of Four Genotypic Methods for Studying the Genetic Epidemiology of Aspergillus fumigatus Isolates. *Journal of Clinical Microbiology, 40*, 2886-2892. http://dx.doi.org/10.1128/JCM.41.4.1798-1800.2003

Lucchetta, G., Bazzo, I., Cortivo, G.D., Stringher, L., Bellotto, D., Michele Borgo, M., & Angelini, E. (2010). Occurrence of Black Aspergilli and Ochratoxin A on Grapes in Italy. *Toxins (Basel), 2*, 840–855. http://dx.doi.org/10.3390/toxins2040840

Matsuda, Y., Wakai, T., Kubota, M., Osawa, M., Sanpei, A., & Fujimaki, S. (2013). Mycotoxins are conventional and novel risk biomarkers for hepatocellular carcinoma. *World Journal of Gastroenterology, 19*, 2587-2590. http://dx.doi.org/10.3748/wjg.v19.i17.2587

Medina A., Mateo, R., Lopez-Ocana, L., Valle-Algarra, F. M. & Jimenez, M. (2005). Study of Spanish grape mycobiota and ochratoxin A production by isolates of Aspergillus tubingensis and other members of Aspergillus section Nigri. *Applied and Environmental Microbiology, 71*, 4696-4702. http://dx.doi.org/10.1128/AEM.71.8.4696-4702.2005

Moslem, M. A., Bahkali, A. H., Abd-Elsalam, K. A., & Wit, P. J. G. M. (2010). An efficient method for DNA extraction from Cladosporioid fungi. *Genetic* and *Molecular Research, 9*, 2283-2291. http://dx.doi.org/10.4238/vol9-4gmr936

Mildner-Szkudlarz, S., Zawirska-Wojtasiak, R., Szwengiel, A., & Pacynski, M. (2011). Use of grape by-product as a source of dietary fibre and phenolic compounds in sourdough mixed rye bread. *International Journal of Food Science & Technology, 46*,1485–1493. http://dx.doi.org/10.1111/j.1365-2621.2011.02643.x

Mildner-Szkudlarz, S., Siger, A., Szwengiel, A., & Bajerska, J. (2015). Natural compounds from grape by-products enhance nutritive value and reduce formation of CML in model muffins. *Food Chemistry, 172*, 78-85. http://dx.doi.org/10.1016/j.foodchem.2014.09.036

Perrone, G., Varga, J., Susca, A., Frisvad, J. C., Stea, G., Kocsube, S., … Samson, R. A. (2008). Aspergillus uvarum sp nov., an uniseriate black Aspergillus species isolated from grapes in Europe. *International Journal of Systematic and Evolutionary Microbiology, 58*, 1032-1039. http://dx.doi.org/10.1099/ijs.0.65463-0

Perrone G., Stea G., Epifani, F., Varga, J., Frisvad, J.C. & Samson, R.A. (2011). *Aspergillus niger* contains the cryptic phylogenetic species A. awamori. *Fungal Biology, 115*, 1138-1150. http://dx.doi.org/10.1016/j.funbio.2011.07.008

Varga, J. (2006). Molecular typing of aspergilli: Recent developments and outcomes. *Medical Mycology, 44*, 149-161. http://dx.doi.org/10.1080/13693780600823266

Pfohl-Leszkowicz, A., & Manderville, R. A. (2007). Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutrition Food Research, 51*, 61-99. http://dx.doi.org/10.1002/mnfr.200600137

Pitt, J., & Hocking, A. (1997). *Fungi and Spoilage* (2nd ed.). London, N.Y. Blackie Academy and Professional.

Pitt, J. I., & Hocking, A. D. (1999). *Fungi and Food Spoilage* (2nd ed.). The United Kingdom: Aspen Publishers, Inc.

Reddy, L., Bhoola, K., 2010. Ochratoxins-food contaminants: impact on human health. *Toxins (Basel), 4*, 1-9. http://dx.doi.org/10.3390/toxins2040771

Sánchez-Alonso, I., Jiménez-Escrig, A., Saura-Calixto, F., Sánchez-Alonso, I., Jiménez-Escrig, A., Saura-Calixto, F., & Borderías, A. J. (2008). Antioxidant protection of white grape pomace on restructured fish products during frozen storage. *LWT- Food Science & Technology, 41*, 42-50. http://qap2.onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.2012.03151

Serrano, R., Gusmão, L., Amorim, A., & Araujo, R. (2011). Rapid identification of Aspergillus fumigatus within the section Fumigati. *BMC Microbiology, 11*, 82. http://dx.doi.org/10.1094/PHYTO.2004.94.10.1094

Stefanović, V., & Polenaković, M. (2009). Fifty years of research in Balkan endemic nephropathy: where are we now? *Nephron Clinical Practice, 112*, 51- 56. http://dx.doi.org/10.1159/000213081

Varga, J., & Kozakiewicz, Z. (2006). Ochratoxin A in grapes and grape derived products. *Food Science & Technology, 17*, 72-81. http://dx.doi.org/10.1016/j.tifs.2005.10.007

Varga, J., Kocsube, S., Toth, B., Frisvad, J. C., Perrone, G., Susca, A., … Samson, R. A. (2007). Aspergillus brasiliensis sp. nov., a biseriate black Aspergillus species with world-wide distribution. *International Journal of Systematic Evolutionary Microbiology, 57*, 1925-1932. http://dx.doi.org/10.1099/ijs.0.65021-0

Yu, J., & Ahmedna, M. (2013). Functional components of grape pomace: their composition, biological properties and potential applications. *International Journal of Food Science & Technology, 48*, 221-237. http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2621.2012.03197

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Transcriptomic Response of *Salmonella* Typhimurium Heat Shock Gene Expression Under Thermal Stress at 48 °C

Sujata A. Sirsat[1,2], Christopher A. Baker[2], Si Hong Park[2], Arunachalam Muthaiyan[2,3], Scot E. Dowd[4] & Steven C. Ricke[2]

[1] Present address: Conrad N. Hilton College, University of Houston, Houston, TX 7720, USA

[2] Center for Food Safety, Dept. of Food Science, University of Arkansas, Fayetteville, AR 72704, USA

[3] Present address: Dept. of Biological Sciences, Chicago State University, Chicago, IL 60628, USA

[4] MR DNA (Molecular Research) 503 Clovis Road, Shallowater, TX 79363, USA

Correspondence: Steven C. Ricke, University of Arkansas, Center for Food Safety and Department of Food Science, 2650 N. Young Ave. Fayetteville, AR 72704-5690, USA. Tel: 1-479-575-4678. E-mail: sricke@uark.edu

Received: May 5, 2015 Accepted: June 28, 2015 Online Published: August 13, 2015

doi:10.5539/jfr.v4n5p51 URL: http://dx.doi.org/10.5539/jfr.v4n5p51

Abstract

Salmonella enterica has been associated with a variety of food products, and thermal treatments are commonly used to reduce or eliminate pathogens from these foods. While the physiological response of *Salmonella* to a wide range of lethal and sublethal heating temperatures has been examined, only limited information is available at the transcriptional level. The objective of this study was to investigate the transcriptional profile of *Salmonella* Typhimurium when subjected to thermal shock at 48 °C (10 min). Transcriptomic analysis was performed using partial microarrays (1152 genes) consisting of quorum sensing, virulence, membrane, and stress related genes. Apparent *Salmonella* viability based on the optical density decreased in response to thermal exposure. Transcriptomic analysis revealed induction of several heat shock and stress related genes due to thermal exposure at 48 °C. This research reveals that there is an impact of exposure to a sublethal temperature (48°C) on the subsequent transcriptional responses of *S.* Typhimurium.

Keywords: *Salmonella* Typhimurium, heat response, microarray, RT-qPCR, gene expression

1. Introduction

Salmonella enterica has been estimated to cause over 1 million foodborne illnesses annually (Scallan et al., 2011), each of which has been estimated to cost $3,221 (based on 2009 currency) (Hoffman, Batz, Morris Jr., 2012; Batz, Hoffman, & Morris Jr., 2014). Additionally, these illnesses often lead to a reduced quality of life due to post-infection complications (Batz et al., 2014). *Salmonella enterica* continues to be linked to foodborne infections, and has previously been associated with a wide variety of food products that are heated at some point during processing, retail, or in the home (Finn, Condell, McClure, Amézquita, & Fanning, 2013; Jackson, Griffin, Cole, Walsh, & Chai, 2013).

In particular, thermal treatment is a common preventative measure taken by food processing facilities, and the appropriate temperature and exposure duration for inactivation is pathogen-dependent (FDA, 2011). It has been suggested that *Salmonella* may become more heat resistant when cells are heated during incremental temperature increases (Mackey & Derrick, 1987), and sublethal heat-shock treatment could potentially cause *Salmonella* to become more virulent and result in more severe illnesses (Mackey & Derrick, 1987a; Humphrey, Richardson, Statton, Rowbury, 1993). For instance, Mackey and Derrick, 1987b found that exposure to 60 °C for 24 minutes resulted in a 10^7-log reduction of *Salmonella* Thompson in reconstituted dried milk, but when cells were exposed to 48 °C for 30 minutes prior to 60 °C treatment, only a 2.4-log reduction was observed. Additionally, Humphrey et al. 1993 reported that when *Salmonella* Enteritidis was exposed to 46 to 48 °C, these cells became more tolerant to heat treatment at 56 °C in comparison to lower pre-exposure temperatures.

Less is known about *Salmonella* transcriptomic responses to thermal exposure (Ricke, Khatiwara, & Kwon, 2013). Milillo et al. (2011) investigated the effects of organic acid and heat exposure at 55 °C on *S.* Typhimurium and observed several differentially expressed genes associated with heat shock following treatment. Additionally,

previous research has focused on *S*. Typhimurium growth response and gene expression responses to heat stress at 42 °C, and transcriptomic analysis of the response to these conditions indicated that several key virulence and fimbrial genes were upregulated (Sirsat, Burkholder, Muthaiyan, Dowd, Bhunia, & Ricke, 2011). While 42 °C was examined as being representative of conditions associated with live poultry body temperature, less is known about higher intermediate non-lethal temperatures that *Salmonella* may become exposed to during post-harvest processing. Further investigation into the growth and transcriptional analysis of *S.* Typhimurium at these temperatures will provide additional insight into how cells respond and tolerate sublethal temperatures. The objective of this study is to use microarray transcriptomic analysis to investigate the effects of heat treatment after exposure at 48 °C for 10 minutes on the expression of *S.* Typhimurium genes that are associated with virulence and heat shock in response to this intermediate sublethal temperature.

2. Materials and Methods

2.1 Growth Conditions and Thermal Treatment

One colony of *S.* Typhimurium ATCC 14028 was inoculated in 5 mL of Luria-Bertani (LB) broth for 18 h incubation at 37 °C. The *S.* Typhimurium ATCC 14028 culture was inoculated in 100 mL LB broth (1%) and incubated at 30 °C to mid-log phase as previously described (Sirsat et al., 2011), and growth measurements were assessed on a spectrophotometer (Spectronic 20D, Milton Roy Company, Rochester, NY, USA) at OD_{600} over 24 h. A 1 mL aliquot of a mid-log (approximately 0.4 OD_{600}) S. Typhimurium culture was centrifuged (6,000 × g for 10 min) and prepared for transcriptional microarray by resuspension in 2 mL phosphate buffered saline (pH 7.4 PBS). Thermal stress was applied to bacterial cells for 10 min in a 48°C water bath.

2.2 Microarray Design

A microarray chip embedded with 43 to 45-mers oligonucleotides was designed for transcriptomic analysis of *S.* Typhimurium LT2 (McClelland et al., 2001). A total of 1152 genes out of nearly 6000 currently available were chosen for microarray analysis. These genes were chosen due to their association with virulence, membrane, stress, quorum sensing and transcriptional regulation for evaluation of gene expression under thermal stress at 48 °C. Oligos were synthesized, normalized, resuspended, and printed as previously described (Sirsat et al., 2011). Information for the array format had previously been submitted (accession number GPL9181) (Sirsat et al., 2011) in the NCBI Gene Expression Omnibus (GEO) (Edgar, Domrachev, & Lash, 2002).

2.3 RNA Extraction and Probe Synthesis

Three biological replicates of RNA extraction and probe synthesis were performed as previously described (Sirsat et al., 2011). Briefly, cells were exposed to 48 °C for 10 min, resuspended in RNAprotect Bacteria Reagent (Qiagen, Valencia, CA, USA), and the RNeasy Bacteria Mini Kit (Qiagen Inc.) was used to extract total RNA. After treating with RNase-free DNase (Qiagen) to remove residual DNA, RNA quality and quantity was assessed with a sprectrophotometer. Following reverse transcription, the cDNA was subsequently labeled and hybridized to the microarray as previously described (Sirsat et al., 2011).

2.4 Microarray Analysis

A Genepix 4000B (Molecular Devices Corporation, Union City, CA, USA) was used to scan microarray images followed by analysis with GenePix 6.0 software (Molecular Devices Coporation). Matrix Laboratory (MATLAB) 2009 software and Microsoft Excel were used for statistical analyses. Logically weighted scatterplot smoothing (LOWESS) normalization was used to remove any dye and/or systematic biases (Sirsat et al., 2011; Quackenbush, 2002), and additional data analysis was conducted using LOWESS normalized log ratio values. A student's t-test was used to generate P-values from normalized log ratio values after correcting for a false discover rate (FDR less than 0.05) (Benjamini & Hochberg, 1995).

Genes that exhibited at least a 2.0 fold up or down regulation after the FDR correction were included in the final data set. The functional categories used to classify genes were derived from previous research data sets generated by Sirsat et al., (2011) using the comprehensive microbial resource of TIGR (http://cmr.tigr.org/tigr-scripts/CMR/shared/Genomes.cgi), as well as the KEGG GENOME resource (http://www.genome.jp/kegg-bin/show_organism?org=T00065). This data is accessible via series accession number GSE18089 after having been deposited in NCBI's GEO (Edgar et al., 2002), and can be accessed through series accession number GSE18089 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE18089) (Sirsat et al., 2011).

2.5 Real-time Quantitative RT-PCR (qRT-PCR)

For the real time quantitative RT-PCR (qRT-PCR) assay, specific primer pairs were constructed for *dnaK*, *ibpA*, *uspA*, *fliL*, and *flgF* genes to validate the microarray data. The RNA harvested from control and thermally stressed (48 °C) microbial populations were analyzed using ABI 7500 Sequence Detection system (PE Applied Biosystems, Foster City, CA, USA) and QuantiTect SYBER Green RT-PCR. The relative induction or repression of genes was reported via the $2^{-\Delta\Delta Ct}$ method using the 16S rRNA as a reference gene to normalize cycle threshold values (Livak & Schmittgen, 2001; Dowd, Killinger-Mann, Blanton, San Francisco, & Brashears, 2007).

3. Results and Discussion

To investigate the effects of thermal stress at 48 °C on the growth response of *S.* Typhimurium, populations of mid-log phase *S.* Typhimurium were measured (OD_{600}) after transferring to a 48 °C water bath. The optical density (OD_{600}) measurements indicate that thermal stress at 48°C caused a decrease in *Salmonella* viability similar to previous work (Sirsat et al., 2011) with the organism (Figure 1). The average OD_{600} values (average ± standard deviation) of *S.* Typhimurium grown at 48 °C were slightly lower after 6, 11, and 24 h of growth (0.33 ± 0, 0.03 ± 0, and 0.30 ± 0.01), respectively) when compared to previous work by Sirsat et al. (2011). In the previous research, *S.* Typhimurium was grown at 30 °C, and after 6, 11, and 24 h of growth (0.38 ± 0.01, 0.43 ± 0, and 0.45 ± 0.01, respectively), these cultures exhibited OD_{600} values similar to the *S.* Typhimurium cells at 42 °C.

The effects of thermal stress (48 °C) on gene expression in *S.* Typhimurium was determined by subjecting a mid-log cell population to 48 °C for 10 min, and performing transcriptomic analysis using a microarray. The ten-minute treatment provided a means of comparison with previous work by Sirsat et al. (2011). A microarray chip used in previous research (Sirsat et al., 2011) that consisted of 1152 genes from virulence, membrane, stress, quorum sensing, and transcriptional regulation functional groups was evaluated. The induced and repressed *Salmonella* genes in response to thermal stress at 48°C are available in supplemental files 2 and 3, respectively.

Comparison among microarray and qRT-PCR revealed some differences between these two methods with regard to gene expression for 48 °C. For instance, *dnaK* was determined to be upregulated 23-fold, while qRT-PCR indicated that *dnaK* was upregulated 44-fold (Table 1). Additionally, *fliL* and *flgF* genes were repressed roughly 20-fold as indicated by microarray, but minimal changes in gene expression were observed with qRT-PCR (Table 1). Sirsat et al. (2011) also observed similar inconsistencies between these two methods. The particular system being investigated factors into how well qRT-PCR data supports microarray analysis, and it has been suggested qRT-PCR data is supportive of microarray results roughly 70% of the time (Ding et al., 2007). Smaller differences in gene expression are typically observed with qRT-PCR in comparison to microarray data, and it is often difficult to obtain similar results with different technology platforms (Ding et al., 2007; Sinicropi et al., 2007; Wang et al., 2006).

Table 1. Comparison of microarray and qRT-PCR data

Locus ID	Gene	Gene-Protein name	Microarray [a]	qRT-PCR [a]
AAL18976.1	*dnaK*	Chaperone Hsp70	23.38	44.04
AAL22668.1	*ibpA*	Small heat shock protein	16.47	16.56
AAL22451.1	*uspA*	Universal stress protein A	3.21	1.92
AAL20887.1	*fliL*	Flagellar biosynthesis	-22.48	1.29
AAL20108.1	*flgF*	Flagella biosynthesis protein	-18.07	-0.12

[a]All values represent fold-change.

The microarray transcriptomic analysis revealed induction of several heat shock related genes (Table 2). Both *dnaK* and *dnaJ* encode chaperones that are involved in several cellular processes including binding to misfolded proteins, which allows sigma H (encoded by *rpoH*) to direct the transcription of heat shock proteins (Spector & Kenyon, 2012). Additionally, the DnaK-DnaJ complex has been suggested to enhance survival of *Salmonella* in macrophages (Takaya, Tomoyasu, Matsui, & Yamamoto, 2014). Given that heat shock response is transient, it follows that several stress factors including genes in the Rpo regulon *(rpoS, rpoE*, and *rpoH*) would be upregulated after being exposed to 48 °C.

Table 2. Expression fold value of virulence-related genes in thermal-stressed *Salmonella*

Locus ID	Gene symbol	Gene/Protein name	48°C for 10 min
AAL22428.1	*rpoH*	Sigma H factor of RNA polymerase	11.16
AAL20321.1	*sseA*	Secretion system effector	7.25
AAL19173.1	*htrA*	Periplasmic serine protease	4.11
AAL20322.1	*sseB*	Secretion system effector	3.46
AAL21534.1	*rpoE*	Sigma E (sigma 24) factor of RNA polymerase	3.11
AAL21804.1	*rpoS*	Sigma S (sigma 38) factor of RNA polymerase	2.75
AAL20341.1	*ssaP*	Secretion system apparatus protein	2.32
AAL21778.1	*invG*	Outer membrane invasion protein	-3.01
AAL21754.1	*prgH*	Cell invasion protein	-3.74
AAL21774.1	*invC*	Surface presentation of antigens	-4.89
AAL21751.1	*prgK*	Cell invasion protein	-5.48

As expected, these genes were induced slightly more at 48 °C (*rpoS* (2.75), *rpoE* (3.11), and *rpoH* (11.16)) when compared to the gene expression of cells exposed to heat stress at 42°C (*rpoS* (2.58), *rpoE* (2.2), and *rpoH* (7.61)) in our previous research (Sirsat et al., 2011). However, it should be noted that the transcription levels of *rpoH* are considered to be mostly independent of temperature fluctuations, and that the concentration of sigma H is regulated at the translation level (Spector & Kenyon, 2012). RpoS is associated with RNA polymerase and controls the expression of up to 50 *Salmonella* proteins (Humphrey, 2004). RpoS is produced in response to starvation, as well as pH and temperature fluctuations (Humphrey, 2004; Lianou & Koutsoumanis, 2013).

Food products that are temperature abused may possibly cause *Salmonella* cells to be more virulent. The changes in gene expression observed in this particular study could be used as a preliminary finding to track these changes over time while a food product is being thermally processed. Interestingly, Millilo et al. (2011) observed repression of several heat shock-related genes following exposure to both sodium acetate and sodium propionate at 55 °C. However, the transcriptional response to these conditions likely differ from an independent analysis of gene responses to 55 °C, which would be necessary to determine these responses to various heat treatments. Previous studies indicate that when *Salmonella* had been exposed to 48 °C, cells were still heat resistance for up to 10 hours in temperatures ranging from 50 to 59 °C (Mackey & Derrick, 1986). *Salmonella* may respond to sublethal heat differently depending on the absolute temperature(s) in which they are exposed. To address this question, *Salmonella* could be transitionally exposed to a range of specific sublethal temperatures over time followed by an assessment of transcriptional responses. Additionally, future research may need to focus on the expression of *Salmonella* genes over an extended period of time after the exposure and return to a more optimal temperature. A time-course gene expression study would be useful to indicate the potential risk heat shocked *Salmonella* cells could pose once the food they harbor is being consumed. As suggested by Millilo et al. (2011), additional studies on the transcriptional profile of *Salmonella* would help guide effective multiple hurdle approaches for *Salmonella* at lower thermal temperatures. Additionally, future research can implement RNA sequencing procedures as a means to validate quantitative PCR data as well as avoid the use of microarray techniques that can pose questionable results.

References

Batz, M., Hoffman, S., & Morris Jr., J. G. (2014). Disease-outcome trees, EQ-5D scores, and estimated annual losses of quality-adjusted life years (QALYs) for 14 foodborne pathogens in the United States. *Foodborne Pathogens and Disease, 11*, 395-402. http://dx.doi.org/10.1089/fpd.2013.1658

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistial Society: Series B (Statistical Methodological), 57*, 289-300.

Ding, Y., Xu, Li., Jovanovic, B. D., Helenowski, I. B., Kelly, D. L., Catalona, W. J., … Bergaan, R. C. (2007). The methodology used to measure differential gene expression affects the outcome. *Journal of Molecular Techniques, 18*, 321-330.

Dowd, S. E., Killinger-Mann, K., Blanton, J., San Francisco, M., & Brashears, M. (2007). Positive adaptive state: microarray evaluation of gene expression in *Salmonella enterica* Typhimurium exposed to nalidixic acid. *Foodborne Pathogens and Diseases, 4*, 187-200. http://dx.doi.org/10.1089/fpd.2006.0075

Edgar, R., Domrachev, M., & Lash, A. E. (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Research*, *30*, 207-210. http://dx.doi.org/10.1093/nar/30.1.207

FDA. (2011). *Appendix 4: Bacterial Pathogen Growth and Inactivation. In: Fish and Fishery Products Hazards and Controls Guidance. Fourth Edition – April 2011*. Retrieved from http://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/seafood/ucm251970.pdf

Finn, S., Condell, O., McClure, P., Amézquita, A., & Fanning, S. (2013). Mechanisms of survival, responses, and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiol*ogy, *4*(331), 1-15. http://dx.doi.org/10.3389/fmicb.2013.00331

Hoffman S., Batz, M. B., & Morris Jr., J. G. (2012). Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. *Journal of Food Protection, 75*(7), 1292-1302. http://dx.doi.org/10.4315/0362-028X.JFP-11-417

Humphrey, T. J., Richardson, N. P., Statton, K. M., & Rowbury, R. J. (1993). Effects of temperature shift on acid and heat tolerance in *Salmonella enteritidis* phage type 4. *Applied Environmental Microbiology, 59*(9), 3120-3122.

Humphrey, T. (2004). *Salmonella*, stress responses and food safety. *Nature Reviews in Microbiology, 2*, 504-509. http://dx.doi.org/10.1038/nrmicro907

Jackson, B. R., Griffin, P. M., Cole, D., Walsh, K. A., & Chai, S. J. (2013). Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998-2008. *Emerging Infectious Diseases, 19*(8), 1239-1244. http://dx.doi.org/10.3201/eid1908.121511

Lianou, A., & Koutsoumanis, K. P. (2013). Evaluation of the strain variability of *Salmonella enterica* acid and heat resistance. *Food Microbiology, 34*, 259-267. http://dx.doi.org/10.1016/j.fm.2012.10.009

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$CT method. *Methods, 25*, 402-408. http://dx.doi.org/10.1006/meth.2001.1262

Mackey, B. M., & Derrick, C. M. (1986). Elevation of the heat resistance of *Salmonella typhimurium* by sublethal heat shock. *Journal of Applied Bacteriology, 61*(5), 389-393. http://dx.doi.org/10.1111/j.1365-2672.1986.tb04301.x

Mackey, B. M., & Derrick, C. M. (1987a). Changes in the heat resistance of *Salmonella typhimurium* during heating at rising temperatures. *Letters in Applied Microbiology, 4*(1), 13-16. http://dx.doi.org/10.1111/j.1472-765X.1987.tb01571.x

Mackey, B. M., & Derrick, C. M. (1987b). The effect of prior heat shock on the thermoresistance of *Salmonella thompson* in foods. *Letters in Applied Microbiology, 5*(6), 115-118. http://dx.doi.org/10.1111/j.1472-765X.1987.tb01627.x

McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P. Courtney, L., … Wilson, R. K. (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature, 413*, 852-856. http://dx.doi.org/10.1038/35101614

Milillo, S. R., Martin, E., Muthaiyan, A., & Ricke, S. C. (2011). Immediate reduction of *Salmonella enterica* serotype Typhimurium viability via membrane destabilization following exposure to multiple-hurdle treatments with heated, acidified organic acid salt solutions. *Applied and Environmental Microbiology, 77*, 3765-3772. http://dx.doi.org/10.1128/AEM.02839-10

Quackenbush, J. (2002). Microarray data normalization and transformation. *Nature Genetics, 32*, 496-501. http://dx.doi.org/10.1038/ng1032

Ricke, S. C., Khatiwara, A., & Kwon, Y. M. (2013). Application of microarray analysis of foodborne *Salmonella* in poultry production: A review. *Poultry Science, 92*, 243-2250. http://dx.doi.org/10.3382/ps.2012-02740

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., … Griffin, P. M. (2011). Foodborne illnesses acquired in the United States—Major pathogens. *Emerging Infectious Diseases, 17*(1), 7-15. http://dx.doi.org/10.3201/eid1701.P21101

Sinicropi, D., Cronin, M., & Liu, M. L. (2007). Gene expression profiling utilizing microarray technology and RT-PCR. In *BioMEMS and Biomedical Nanotechnology* (pp. 23-46).

Sirsat, S. A., Burkholder, K. M., Muthaiyan, A., Dowd, S. E., Bhunia, A. K., & Ricke, S. C. (2011). Effect of sublethal heat stress on *Salmonella* Typhimurium virulence. *Journal of Applied Microbiology, 110*(3), 813-822. http://dx.doi.org/10.1111/j.1365-2672.2011.04941.x

Spector, M. P., & Kenyon, W. J. (2012). Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Research International, 45*, 455-481. http://dx.doi.org/10.1016/j.foodres.2011.06.056

Takaya, A., Tomoyasu, T., Matsui, H., & Yamamoto, T. (2004). The DnaK/DnaJ chaperone machinery of *Salmonella enterica* serovar Typhimurium is essential for invasion of epithelial cells and survival within macrophages, leading to systemic infection. *Infections and Immunity, 72*(3), 1364-1373. http://dx.doi.org/10.1128/IAI.72.3.1364-1373.2004

Wang, Y., Barbacioru, C., Hyland, F., Xiao, W., Hunkapiller, K. L., Blake, J., … Samaha, R. R. (2006). Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. *BMC Genomics, 7*, 59. http://dx.doi.org/10.1186/1471-2164-7-100

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Whole Grain Gluten-Free Vegetable Spicy Snacks

Talwinder S. Kahlon[1], Roberto J. Avena-Bustillos[2], Mei-Chen M. Chiu[1] & Marlene B. Hidalgo[1]

[1] Western Regional Research Center, USDA-ARS, 800 Buchanan St, Albany, CA 94710, USA

[2] University of California, Davis, CA, USA

Correspondence: Talwinder S. Kahlon, Western Regional Research Center, USDA-ARS, 800 Buchanan St, Albany, CA 94710, USA. Tel: 1-(510)-559-5665. E-mail: Talwinder.Kahlon@ars.usda.gov

Received: June 24, 2015 Accepted: July 25, 2015 Online Published: August 17, 2015

doi:10.5539/jfr.v4n5p57 URL: http://dx.doi.org/10.5539/jfr.v4n5p57

Abstract

Four kinds of spicy snacks (gluten-free, whole grains with fresh vegetables, low in fat, sugar and salt) were evaluated. Acceptance of spicy snacks tested was Carrot-Garlic 77%, Broccoli-Garlic 68%, Spinach-Garlic 61% and Red Onion 53%. This is the first report of spicy gluten-free, 50% vegetable snacks, which offer a nutritional snack choice for all and an option for gluten sensitive individuals. In addition, it offers spicy snacks that would encourage low fat, sugar and salt foods more palatable and increased health promoting vegetable consumption. The snacks reported herein can be prepared in any house kitchen and/or commercial production. These crispy snacks would have desirable microbial stability resulting in longer shelf life. Each 30 g serving of these healthy chips would provide 1.2-1.3 g of dietary fiber. Currently available chips in the market contain up to twice as much salt as the chips reported herein. These gluten-free vegetable spicy chips could be considered good source of dietary fiber and potassium.

Keywords: carrot, broccoli, gluten-free, snacks, spinach, red onion, whole grain

1. Introduction

Snacks play a significant role in family as well as public events. In children increases is snacking have been in salty and sugary products (Piernas & Popkin, 2010). In the efforts to promote healthfulness of our diets, whole grain foods and savory snacks have been reported (Smith et al., 2013, Kahlon et al., 2014). Low dietary fat, sugar and salt intake is highly recommended to reduce obesity related lifestyle diseases. Since food flavors are fat soluble, low fat foods are not as tasty as high fat flavorful foods. Spicy healthy snacks would make low fat, sugar and salt foods more palatable. Serving healthy snacks to children at home and in school programs would develop eating habits that would prevent obesity and premature degenerative diseases later in life. It is recommended that at least one/half of all the grains eaten should be whole grains (Nutrition Policy and Promotion; Dietary Guidelines for Americans, USDA, 2010). Healthy products should incorporate whole grains instead of refined grains. Eating whole grains lowers the risk many life style chronic diseases (Whole Grain Council, 2009). Consuming 3 servings of whole grain have been reported to results in significant health benefits. Foods containing more than one/half whole grains and providing one/third daily requirement of dietary fiber are allowed label health claims (US Food and Drug Administration, 2003), http://www.fda.gov/Food/LabelingNutrition/LabelClaims/FDAModernization ActFDAMAClaims/ucm073634.htm). Gluten-free snacks are needed as hypersensitivity to gluten has been increasing in the world. In order to meet desired amount of vegetables in daily diets snacks containing vegetables would be desirable. Gluten-free products are considered health promoting with a clean label. In a family even if one member is gluten sensitive (with celiac disease) the whole family needs to remove gluten products from the house. However, food and snacks available in US contain high salt, fat and simple sugar and would be considered unhealthy (Mother-Jones, 2012). Home cooked and commercially offered foods and snacks need to be health promoting gluten-free, vegetable spicy snacks. Acceptance of gluten-free savory snacks containing 50% vegetables have been reported to be Carrot 88%, Broccoli 77%, Spinach 68% and Red Onion 65% (Kahlon et al., 2014). Currently there are many spicy snacks available in the market high in salt, sugar and fat. There is a need for gluten-free healthy spicy snacks to meet changing demographics and ethnic character of the world population. Spicy healthy whole grain gluten-free snacks would make lower salt, sugar and fat foods more palatable. In the study reported herein, sixty two in house volunteers evaluated four kinds of low fat, sugar and salt spicy snacks containing gluten-free whole grains and fresh vegetables.

2. Materials and Methods

2.1 Preparation

All the ingredients were purchased from local markets. Brown rice flour was obtained by pin-milling whole grains rice (160Z, Alpine Pin Mill). The composition of the ingredients is listed in Table 1. Brown rice and sorghum samples were analyzed for nitrogen, using AOAC method 990.03 (2000) by Leco FP628 analyzer (Leco Corporation, St Joseph, MI); total dietary fiber, using AOAC method 985.29 (2000), crude fat with petroleum ether and an accelerated solvent extractor (ASE 350, Dionex Corp.); ash, using AOAC method 942.05 (1990); and moisture, using AOAC method 935.29 (1990).

Table 1. Ingredient composition of the gluten-free whole grain vegetable snacks dry matter (DM) basis, %

Ingredients	Protein	Fat	Minerals	Carbohydrate	TDF	DM
Brown Rice	9.04	7.24	1.1	82.62	3.08	88.94
Sorghum	8.63	3.44	1.45	86.48	6.63	90.67
Tapioca flour	- - -	- - -	- - -	100	- - -	96.15
Potato Flakes	8.34	0.41	1.45	81.17	6.6	93.42
Carrots, fresh	7.94	2.05	4.01	86	23.91	11.71
Broccoli, fresh	26.36	3.46	4.52	65.66	24.3	10.7
Spinach, fresh	33.26	4.53	10.08	52.13	25.58	8.6
Red Onion, fresh	10.1	0.92	3.12	85.86	15.61	10.89
Garlic, fresh	15.35	1.21	1.88	81.56	5.07	41.42
Cayenne Pepper	13.06	18.78	2.88	65.28	29.58	91.95

Brown Rice and Sorghum were analyzed by AOAC (1990, 2000). Nitrogen to protein factors used were brown rice 5.95 and sorghum 6.25 (4). Total Dietary Fiber, TDF; Dry matter, DM. Carbohydrate = [100 – (Protein + fat + Ash)].

Tapioca flour data from product label.

Vegetables values from USDA Nutrient data base (http://ndb.nal.usda.gov/ndb/search/list).

2.2 Formulation

Spicy snack formulation contained gluten-free whole grains (base formulation) and fresh vegetable (1:1) as is basis. Table 2 shows the base formulation composition. The vegetable used were fresh carrots, broccoli, spinach, red onion and garlic. Table 3 shows the composition of the whole grain gluten-free vegetable snacks. Snack dough was prepared by mixing ingredients in a KitchenAid table top mixer (KitchenAid Proline, St. Joseph, MI). Water was added slowly, it took about 4 minutes to thoroughly mix the ingredients. Snack dough was covered and held at room temperature for 30 min. Two equal portions of snack dough (about 10 g each) were cooked for two minutes in 750 watts double 5 inch Krumkake Baker (CucinaPro.com) Figure 1. Cooked snacks were vacuum heat sealed in plastic bags by Food Saver 2200 Series (Foodsaver.com).

Table 2. Base mix of spicy gluten free snacks (As Is Basis)

Ingredients	Brown Rice flour	Sorghum flour	Tapioca flour	Mashed Potato	Canola Oil	Guar gum	Baking powder	Salt	Cayenne Pepper	Total
%	45	20	16.75	8	6	2	1.5	0.5	0.25	100

Table 3. Composition of spicy gluten-free vegetable snacks (As Is Basis)

Snack	Base Mix, g	Vegetable, g	Garlic, g	Water, mL
Carrot-Garlic	100	100	10	200
Broccoli-Garlic	100	100	10	200
Spinach-Garlic	100	100	10	200
Red Onion	100	100	---	200

All the vegetables and garlic were fresh, chopped.

Figure 1. CucinaPro Krumkake Baker

2.3 Sensory Evaluation of Snacks

Sixty two in-house volunteers judged gluten free whole grain vegetable snacks for Color/Appearance, Odor/Aroma, Taste/Flavor, Texture/Mouth feel on a scale of 1-5 (like very much = 5, like slightly = 4, neutral = 3, dislike slightly = 2, dislike very much = 1). The overall acceptable preference was on a scale of 1-2 (acceptable = 2, not acceptable = 1).

2.4 Water Activity

Water activity (Aw) was measured at 25.01 ± 0.02 °C by triplicate using an AquaLab dew point water activity meter 4TE (Decagon Devices, Inc., Pullman, WA).

2.5 Texture

Compression tests were done on 55 mm diameter flat snacks mounted between two 3.5 x 3.5 inch square, ¼ in thick, iron plates with 1 in diameter centered holes and puncturing over smooth and sound snack surfaces with 1.14 ± 0.17 mm thickness with a flat 3 mm diameter cylindrical probe attached to a TA-XT2i Texture Analyzer (Texture Technologies Corp., Scarsdale, NY) set at 1 mm/s test speed and 2 mm rupture test distance. Temperature during testing was 18.7 ± 0.3 °C and samples were tested right after opening vacuum sealed plastic bags used for packaging the snacks. Number of cracks, force needed for first crack, first crack area, maximum force and maximum force area were obtained by 10 replicates for each snack.

2.6 Density

The bulk density (ρ_b) was obtained by triplicate from perfectly round flat snacks by weighing to the nearest 0.0001 g using an analytical balance and measuring the diameter and maximum and minimum thickness in five random places with a digital micrometer to the nearest 0.001 mm and averaged. True density (ρ_t) was determined using an AccPyc II 1340 gas pycnometer (Micromeritics Instrument Co., Norcross, GA) at 21.4 ± 0.4°C. Samples of each snack were cut into small pieces and dried at room temperature for 15 h at 0% relative humidity in a vacuum desiccator with anhydrous calcium sulfate (W. A. Hammond Drierite, Xenia, OH). After drying, samples were compressed into a measuring cylinder for five true density measurements.

Where: ρ_t = true density; ρ_b = bulk density.

2.7 Statistical Analysis

Data were analyzed with Minitab statistical software (version 14.12.0, Minitab Inc., State College, PA) by one-way analysis of variance and Tukey's multiple comparison tests with ($P \leq 0.05$) as criteria of significance.

3. Results and Discussion

Four kinds of whole grain gluten-free vegetable spicy snacks are shown in Figure 2. Fresh baked whole grain gluten-free low salt, low sugar and low fat vegetable spicy snacks were cooled to room temperature, before sealing in vacuum food saver packages. Color/Appearance of Broccoli-Garlic snacks was determined to be significantly ($P \leq 0.05$) better than Spinach-Garlic snacks (Table 4). Roasted brown appearance of Broccoli-Garlic snacks was preferred over greenish color of cooked Spinach-Garlic snacks. Red Onion snacks Odor/Aroma was significantly preferred compared with Spinach-Garlic snacks. Carrot-Garlic snacks Taste/Flavor and Acceptance was significantly higher than that of Red Onion snacks. Red Onion snack Texture/Mouth feel was significantly lower than other snacks tested. Acceptance of spicy snacks tested is shown as bar graph (Figure 3). Acceptance values for whole grain gluten-free vegetable spicy snacks were Carrot-Garlic, 77%; Broccoli-Garlic, 68%; Spinach-Garlic, 61% and Red Onion, 53%. These acceptance values were 7-12% lower than those reported for similar savory snacks (Kahlon et al., 2014). With the view that spicy snacks would make low sugar, salt and fat meals more palatable, acceptance reported herein for spicy healthy snacks is desirable and could be further enhanced with fortifications and/or processing. Any house kitchen or commercial production could make these health promoting naval gluten-free vegetable spicy snacks. These spicy snacks have the potential of increasing palatability of low sugar, salt and fat health promoting diets.

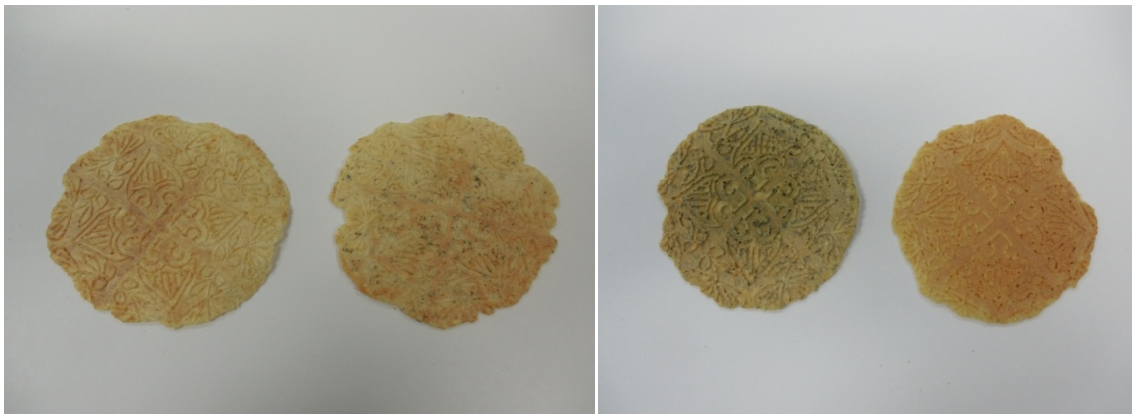
Table 4. Results of taste panel of brown rice – sorghum whole grain (gluten-free), vegetable, low salt spicy snacks[abc]

Gluten free Vegetable Snacks	Color/ Appearance	Odor/ Aroma	Taste/ Flavor	Texture/ Mouth feel	Acceptance
Carrot-Garlic	3.76±0.13^{ab}	3.42±0.08^{ab}	3.44±0.13^{a}	4.00±0.10^{a}	1.77±0.05^{a}
Broccoli-Garlic	4.05±0.12^{a}	3.34±0.10^{ab}	3.23±0.14^{ab}	3.77±0.12^{a}	1.68±0.06^{ab}
Spinach-Garlic	3.48±0.15^{b}	3.24±0.09^{b}	3.19±0.16^{ab}	3.97±0.12^{a}	1.61±0.06^{ab}
Red Onion	3.81±0.12^{ab}	3.53±0.10^{a}	2.97±0.14^{b}	3.26±0.12^{b}	1.53±0.06^{b}

[a]Values are mean±SEM; n = 62.

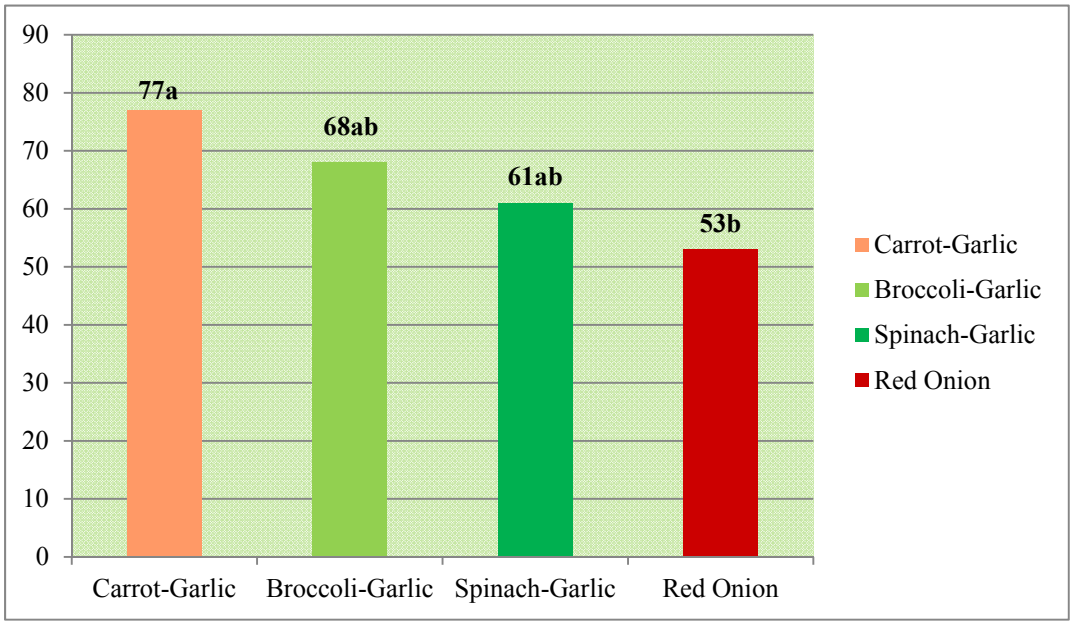
[b]Color/Appearance, Odor/Aroma, Taste/Flavor and Texture/ Mouth feel were on a scale of 1-5 (Like very much = 5, like slightly = 4, neutral = 3, dislike slightly = 2 and dislike very much = 1); Acceptance was on scale of 1-2 (Acceptable = 2 and unacceptable = 1).

[c]Values within columns with different superscript letters differ significantly ($P \leq 0.05$).



Carrot-Garlic Broccoli-Garlic Spinach-Garlic Red Onion

Figure 2. Spicy snacks are shown (left to rights), Carrot-Garlic, Broccoli-Garlic, Spinach-Garlic and Red Onion



Bars with different letters differ significantly ($P \leq 0.05$).

Figure 3. Panelist acceptance % for whole grain, gluten-free vegetable, carrot-garlic, broccoli-garlic, spinach-garlic and red onion spicy snacks

3.1 Water Activity

Water activity (Aw) of four kinds of snacks tested was significantly different from each other Broccoli-Garlic > Red Onion > Carrot-Garlic > Spinach-Garlic (Table 5). Water activity values ranged from 0.20-0.27. These Aw values are typical of very crispy snacks (Katz and Labuza 1981). Carrot snacks with Aw 0.44 have been reported as crisp and with desired microbial stability (Adams and Moss 1997; Dueik et al., 2013).

Table 5. Water Activity, Texture and Density Brown Rice – Sorghum Whole Grain (Gluten-Free), Vegetable, Low Salt Spicy Snacks[ab]

Gluten free Vegetable Snacks	Water Activity	Texture (Newtons)	Density g/ml	Bulk density g/ml	Porosity
Carrot-Garlic	0.240±0.002[c]	3.527±0.174[b]	1.406±0.001[a]	0.295±0.010[d]	0.790±0.001[a]
Broccoli-Garlic	0.273±0.008[a]	2.564±0.217[c]	1.371±0.001[d]	0.328±0.004[b]	0.761±0.001[c]
Spinach-Garlic	0.201±0.006[d]	3.525±0.309[b]	1.396±0.001[c]	0.341±0.006[a]	0.756±0.001[d]
Red Onion	0.258±0.002[b]	4.331±0.140[a]	1.399±0.001[b]	0.308±0.003[c]	0.780±0.001[b]

[a]Values are mean±SEM; Values are mean of triplicate analysis for water activity (n=3), texture, (n=10) and density (n=5).

[b]Values within columns with different superscript letters differ significantly ($P \leq 0.05$).

Porosity $=1-(\rho_{bulk}/\rho_{true})$

3.2 Texture

Red Onion snack texture was significantly higher than Carrot-Garlic and Spinach-Garlic snacks (Table 5). Texture values for Carrot-Garlic and Spinach-Garlic were similar and significantly higher than those for Broccoli-Garlic snacks. Data suggest that crispiness for the snacks tested was Red Onion > Carrot-Garlic = Spinach-Garlic > Broccoli-Garlic. Previously similar texture of savory snacks of Red Onion, Carrot and Spinach and significantly higher than that of Broccoli have been reported (Kahlon et al., 2014). Data suggest that spicy formulation lowered

crispiness of Carrot-Garlic and Spinach-Garlic snacks but did not change relative crispiness of Red Onion or Broccoli-Garlic snacks.

3.3 Density (ρ_{true})

There were significant differences in the true density of the gluten-free spicy vegetable snacks tested (Table 5). Values were Carrot-Garlic > Red Onion > Spinach-Garlic > Broccoli-Garlic. True density of Carrot-Garlic and Red Onion snacks was consistent with higher dry matter content of these two vegetable (Table 1). However, Spinach-Garlic snacks had significantly higher density than Broccoli-Garlic snacks even with 20% lower dry matter in spinach than broccoli.

3.4 Bulk Density (ρ_{bulk})

Bulk density values were significantly different among the gluten-free spicy vegetable snacks tested (Table 5). Bulk density values were Spinach-Garlic > Broccoli-Garlic > Red Onion > Carrot-Garlic.

3.5 Porosity

Porosity was calculated using the equation [Porosity = 1 – (ρ_{bulk} / ρ_{true})]. Porosity values were significantly different among the four gluten-free spicy vegetable snacks tested (Table 5). Values were Carrot-Garlic > Red Onion > Broccoli-Garlic > Spinach-Garlic. Porosity values were inversely related to the bulk density values of these snacks.

3.6 Expansion Values

Expansion values can be calculated [Expansion Value = 1 / (1 – Porosity) = ρ_{true} / ρ_{bulk}). Expansion values (fluffiness) of the four gluten-free spicy vegetable snacks tested were Carrot-Garlic, 4.8; Broccoli-Garlic, 4.2; Spinach-Garlic, 4.1 and Red Onion, 4.5. The four snacks tested had desirable expansion of 4-5, suggesting that these snacks would encourage lower caloric consumption and would give desired presentation in commercial packaging.

4. Conclusions

Any house kitchen or commercial production can prepare these naval health promoting gluten-free, vegetable low salt, low sugar and low fat spicy snacks. Spicy snack would make low fat, low sugar and low salt foods more palatable. Acceptability of spicy snacks evaluated by in house volunteers (n=62) was Carrot-Garlic 77%, Broccoli-Garlic 68%, Spinach-Garlic 61% and Red Onion 53%. These crispy snacks had desirable microbial stability that would result in longer shelf life. Each 30 g serving of these healthy snacks would provide 1.2-1.3 g of dietary fiber. These snacks contain 33-50% less salt and have no added sugar than most chips currently available in the market. One serving of these spicy chips is a good source of dietary fiber and potassium.

References

Adams, M. R., & Moss, M. O. (1997). *Food microbiology*. Cambridge: The Royal Society of Chemistry.

Agricultural Research Service, United States Department of Agriculture, National Nutrient Database for Standard Reference Release 26 (http://ndb.nal.usda.gov/ndb/search/list).

AOAC. (1990). *Official Methods of Analysis of the Association of Official Analytical Chemists* (15th ed., pp. 70, 725, 1st Suppl. p. 83). The Association: Arlington VA.

AOAC. (2000). *Official Methods of Analysis of the Association of Official Analytical Chemists* (17th ed., Chap. 45, pp. 78E-80). The Association: Arlington, VA.

Dueik, V., Marzullo, C., & Bouchon, P. (2013). Effect of vacuum inclusion on the quality and the sensory attributes of carrot snacks. *Food Science and Technology, 50*, 361-365. http://dx.doi.org/10.1016/j.lwt.2012.05.011

Food and Drug Administration. (2003). Retrieved from http://www.fda.gov/Food/LabelingNutrition/LabelClaims/FDAModernizationActFDAMAClaims/ucm073634.htm

Kahlon, T. S., Avena-Bustillos, R. J., Chiu, M. M., & Hidalgo, M. B. (2014). Whole Grain Gluten-Free Vegetable Savory Snacks. *Journal of Food Research, 3*, 1-7. http://dx.doi.org/10.5539/jfr.v3n5p1

Katz, E. E., & Labuza, T. P. (1981). Effect of water activity on the sensory crispness and mechanical deformation of snack food products. *Journal of Food Science, 46*, 403-409. http://dx.doi.org/10.1111/j.1365-2621.1981.tb04871.x

Mother Joes. (2012). Retrieved from http://www.motherjones.com/blue-marble/2012/01/america-food-spending-less

New Product Trends. (2015). Sports Drinks, Nutrition Bars Reshape Market. Retrieved from PreparedFoods.com

Piernas, C., & Popkin, B. M. (2010). Trends in snacking among U. S. children. *Health Affairs, 29*, 398-404. http://dx.doi.org/10.1377/hlthaff.2009.0666

Smith, L. P., Ng, S. W., & Popkin, B. M. (2013). Trends in US home food preparation and consumption: Analysis of national nutrition surveys and time use studies from 1965-1966 to 2007-2008. *Nutrition Journal, 12*, 45-53. http://dx.doi.org/10.1186/1475-2891-12-45

UN.A.F.P.A. Statistics. (2012). Retrieved from http://www.pasta-unafpa.org/ingstatistics5.htm

US Food and Drug Administration. (2003). Retrieved from http://www.fda.gov/Food/LabelingNutrition/LabelClaims/FDAModernizationActFDAMAClaims/ucm073634.htm.

USDA. (2010). *Center for Nutrition Policy and Promotion; Dietary Guidelines for Americans*. Retrieved from http://www.choosemyplate.gov/

Whole Grain Council. (2009). Retrieved from http://www.wholegrainscouncil.org/

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Branding, Ingredients and Nutrition Information: Consumer Liking of a Healthier Snack

Mary R. Yan[1,2], Dave Brown[1], Andrew Parsons[1], Gillian A. Whalley[2], Nazimah Hamid[1], Kevin Kantono[1], Bruce Donaldson[1], & Elaine Rush[1]

[1] AUT Food Network, Auckland University of Technology, Auckland, New Zealand

[2] Faculty of Social and Health Sciences, Unitec Institute of Technology, Auckland, New Zealand

Correspondence: Elaine Rush, AUT Food Network, Auckland University of Technology, PO Box 92006, Wellesley St, Auckland, New Zealand. Tel: 0064-9-9219758. E-mail: elaine.rush@aut.ac.nz

Received: July 12, 2015 Accepted: August 9, 2015 Online Published: August 19, 2015

doi:10.5539/jfr.v4n5p64 URL: http://dx.doi.org/10.5539/jfr.v4n5p64

Abstract

Taste appeal, sustainable ingredients and valid health claims are challenges for successful marketing of healthier food products. This study was designed to compare the effects of branding, ingredients and nutrition information on consumer liking towards a prototype of the Nothing Else healthier snack bar with the top three brands of New Zealand snack bars, and another product with a good nutrient profiling score. Sixty-four consumers were recruited to evaluate the five snack bars. Participants initially blind-rated on visual analogue scales their liking scores in relation to colour, taste, flavour, texture and overall liking. Packaging for the products was then presented alongside each of the five products and participants rated their liking scores for a second time. Participants also ranked the five products from 1 to 5 for healthiness, taste, naturalness, and purchase intent if prices were the same. In both blind and informed tests, the Nothing Else bar was the least liked snack bar among all the tested samples. However, after the packaging for the products was presented, overall liking of the Nothing Else bar increased by 14% (p = 0.023), while overall liking for the four commercial products were unchanged. While the most popular commercial bar was ranked the highest for taste and purchase intent, the Nothing Else bar was ranked the highest for the healthiness and naturalness. Our findings confirmed that the branding and health related nutrition information could improve consumer liking and brand perception particularly if backed by marketing.

Keywords: brand perception, healthier snacking, Nothing Else, sensory properties

1. Introduction

In response to the growing consumer demands for healthier foods (Siró, Kápolna, Kápolna, & Lugasi, 2008), many food manufacturers have reformulated established brand products. However, taste appeal, sustainable ingredients and valid health claims are challenges for successful marketing of healthier food products. While taste is the most important factor for consumer perception in general (Fernqvist & Ekelund, 2014), healthiness is suggested to be another factor which influences consumers' choice (Roininen, Lähteenmäki & Tourila, 1999; Provencher, Polivy, & Herman, 2009; Carillo, Varela, Salvador, & Fiszman, 2011). Extrinsic product factors, such as region of origin of a wine can influence consumer perception (Mueller & Szolnoki, 2010). Mueller and Szolnoki (2010) reported that labelling and branding have a strong impact on product liking. In recent years, nutrition information, and front-of-pack labelling particularly, have been introduced as ways to encourage healthier food choices (Steenhuis et al., 2010; van Herpen & Trijp, 2011). However, consumers pay little attention to this information which limits its effectiveness (Bialkova & van Trijp, 2010).

Snacking between main meals has become increasingly important in daily life (Savige, MacFarlane, Ball, Worsley, & Crawford, 2007). However snacking behaviour has not been well researched or understood (Bilman, van Trijp & Renes, 2010). Snack foods usually are categorized around the eating situations, rather than their health properties (Bilman et al., 2010). Although 'muesli-style' snack bars are regarded as being healthy, in a survey undertaken by the primary researcher of this study (data not published), most of the snack bars in New Zealand supermarket are high in sugar and fat, low in protein, fruits, nuts and dietary fibre, with many E numbered additives. They do not have nutrient profiling scores as defined by Food Standards Australia New Zealand (FSANZ) (2012) that allow them to make a health claim. In New Zealand, the score should be 4 or less

but in the survey we found that scores ranged from 6 to 22.

The Nothing Else, a brand created at Auckland University of Technology, New Zealand (Brown et al., 2015), was introduced in 2010 with the intent to promote sustainable consumption. Since 2013, the Nothing Else healthier snack bar has been developed, and the resultant almonds and dates bar was the third product of the Nothing Else brand. The Nothing Else bar uses eight perceived natural ingredients, is high in fibre and low in sodium according to FSANZ (2012), and has a low glycaemic index of 52 (data not published). An informal sensory trial showed that potential consumers found the taste, healthiness and natural ingredients as favourable features of the bar (Brown et al., 2015).

This study aimed to investigate the effects of branding, ingredients and nutrition information on sensory acceptability/liking of the Nothing Else snack bar, compared to top snack bars brands in New Zealand. The secondary aim was to investigate the relative importance of healthiness, taste, naturalness of foods to consumers. The main hypothesis was that branding, ingredients and nutrition information would influence consumer liking.

2. Method

2.1 Experimental approach

Sensory evaluation (Lawless & Heymann, 2010) describes procedures in food manufacturing and technology to determine the acceptability and consumer responses towards the quality of new food products. Direct consumer testing is where sensory information is collected directly from individuals without formal training in sensory evaluation. The test enables manufacturers to predict likely market behaviour (Giacalone, Bredie, & Frøst, 2012). In addition, consumer opinions can be incorporated as part of the refinement and optimal design of food products (Lawless & Heymann, 2010). Consumers rate their liking for various attributes of a given food product on a defined scale (Schutz, 1999; Lawless & Heymann, 2010). A scaling method with low inter-individual variability will allow more sensitive tests and thus a lower risk of missing a true difference (Lawless & Heymann, 2010). Unstructured horizontal line scaling with only the endpoints marked with short line segments was adopted for the current study because the visual analogue scale allows consumers choice to be more continuous and therefore less limited.

2.2 Participants

Sixty-four participants (Table 1) were recruited for the present study. Participants were comprised of staff members and students from two tertiary education institutes in Auckland, New Zealand. The demographic of this population meant that about half the participants were of Asian origin (Table 1). Eligibility criteria required that participants were 18 years or older, consumed snack bars at least once a week, and had no known allergies to food ingredients including nuts, gluten, egg, and milk. The sample size to measure the degree of liking of consumers by the use of visual analogue scales (VAS) was determined from the usual requirement for fifty or more untrained persons (Lawless & Heymann, 2010; Sensory analysis, 2014). The effective sample size, nevertheless, was based on what is seen during the course of the study (Cohen, 1988). Ethics approval for this study was provided by Auckland University of Technology Ethics Committee (Reference no. 13/184).

Table 1. Demographic characteristics of the 64 participants

	Total	Age 20 to 29 yrs	Age ≥ 30 yrs
Gender			
Men	23	13	10
Women	41	28	13
Ethnicity			
Asian	38	31	7
Non-Asian	26	10	16

2.3 Sample Preparation for Sensory Analysis

The Nothing Else prototype bar was prepared in the food laboratory in School of Applied Science, AUT University. Four commercial products included the three top New Zealand brands of snack bars (45% of the market share, Euromonitor International, 2012), and another product that was similar in nutrition profiling score to the Nothing Else bar (Table 2). All commercial products were purchased from local supermarkets in Auckland, New Zealand.

Snack bars were cut into a 2 x 2 cm^2 pieces and were presented to the consumers at room temperature (20 °C) on white, covered food grade plastic containers under white light. The samples were identified by individual three digit codes, which were randomized, and counter balanced to order of presentation using a Latin square design (Macfie, Bratchell, Greenhoff, & Vallis, 1989) to reduce participant and researcher bias.

Table 2. Samples for consumer liking testing

Code	Brand	Market share[1], MAT[2] to (%)	Nutrient profiling score[3]
B1	Brand 1	15.9	11
B2	Brand 2	15.9	12
B3	Brand 3	14.7	15
B4	Brand 4		1
NE	Nothing Else		-1

[1]Ranked in order of sales value in NZ in 2012 (Euromonitor International, 2012)
[2]MAT: moving annual total
[3]Derived from nutrient profiling model (FSANZ, 2012). A food with a score > 4 is unable to make health claims.

2.4 Experimental Protocol

Participants were asked to attend test sessions at least two hours after breakfast in the morning. Each participant was seated at a table separated by partitions to ensure they did not interact or influence other participants. After reading the study information and asking any questions, participants were asked to sign the consent form before the testing started.

A three stage evaluation procedure was applied: a) sensory evaluation in a blind condition; b) sensory evaluation with the knowledge of branding, ingredients and nutrition information; c) ranking of the importance of healthiness, taste, and naturalness to consumers.

In the first stage blind condition, the Nothing Else prototype was presented twice to measure the reliability of participants. Each participant tasted six samples in a blind condition and evaluated his or her liking in relation to colour, taste, flavour, texture, and overall liking on five 100 mm unstructured line scales anchored at dislike extremely (left end) and like extremely (right end) (Figure 1). Participants were asked to rinse their mouth with water between tasting each sample.

In the informed condition, five samples were presented to participants together with the actual front and back pack labels showing the brand, the name of the bar, all the ingredients and nutrition information. For all products, the labels and nutrition panels were colour printed and presented in the same format. Each participant tasted the five samples and evaluated his or her liking in relation to colour, taste, flavour, texture, and overall liking again on five unstructured line scales. When participants completed this, they were additionally asked to rank the perceived healthiness, taste, and naturalness and purchase intent each from 1 to 5, with 1 being the lowest and 5 being the highest ranking if prices were the same for the snack bars. Participants were also asked to state the importance of healthiness, taste and naturalness from 1 to 5, with 1 being not important and 5 being very important.

Figure 1. Line scaling for measuring consumer liking/disliking in relation to sensory attributes on a 100 mm visual analogue scale (VAS)

2.4 Data Analysis

Distance data, to the nearest mm, were measured with a digital calliper (Warrior, Canada) from the left hand anchor point. To compare the repeat measures of the Nothing Else bar, two tailed paired *t*-test was used. The mean value of the replicate tests for each attribute of the Nothing Else bar was used in the comparison with the four commercial bars. Separately for blind and informed tests means of hedonic liking on 5 sensory attributes (overall liking, colour, taste, flavour, and texture) were calculated and statistically tested using one-way analysis of variance (ANOVA) to determine if a statistical difference existed at $p < 0.05$. Tukey's post-hoc test was then used to identify which specific means were significantly different. Friedman test was carried out for rank sum total (sum total of ranking each of all participants) data on perceived healthiness, taste, naturalness, and purchase intent. Multiple pairwise comparisons with the Bonferroni correction were carried out in order to identify statistical separation among the sum ranking total. The effects of age and ethnicity on liking were analysed using two-way ANOVA. The univariate analysis in this study was carried out using SPSS version 14.0.1, 2005 (SPSS Inc., Chicago, Ill.). Multivariate analysis in this study was carried out using XLSTAT version 2013.4.08 (Addinsoft, USA).

3. Results

When the Nothing Else bar was presented to the 64 participants as two of six randomised samples in a blind test, there was no meaningful difference between the mean values of liking for the 5 sensory attributes. For instance, there was only, on average, a 0.5 mm difference for a mean score of 34 mm out of 100 mm for overall liking (Table 3). However, across all 5 sensory attributes, the standard deviations of the paired differences were large (mean difference < 1.0, SD 15 to 21).

The Nothing Else bar had meaningfully lower scores compared with all four commercial snack bars across all 5 sensory attributes for both blind and informed tests with the 64 subjects (Table 3). The four commercial bars were rated by the participants in the same order of liking as the value of commercial sales (Table 2). The rating scores of Brand 1 were similar to that of Brand 2, but were meaningfully different to Brand 3 and Brand 4, across all 5 sensory attributes for both blind and informed tests (Table 3).

In the blind test, the Nothing Else bar was consistently and significantly ($p < 0.0001$) the least overall liked of all other bars. Brand 1 was overall liked more than all the other bars. Brand 2 was liked more than both of Brand 3 and Brand 4. There was however no difference in overall liking between Brand 3 and Brand 4. The Nothing Else bar was rated lower for taste than all other brands except for Brand 4.

Table 3. Sensory attribute ratings obtained from blind and informed consumer tests (n = 64), comparing the Nothing Else bar with four commercial products

Product	NE*	B1	B2	B3	B4	
Attribute (mm)	Mean (SE)					‡*p* value
Blind						
Overall liking	34.3 (2.52)	73.0[a] (2.07)	62.1[a,b] (2.56)	52.2[a,b,,c] (2.93)	49.6[a,c] (2.77)	< 0.0001
Colour	38.2 (2.36)	73.6[a] (1.86)	63.1[a,b] (2.47)	46.3[a,b,c] (2.60)	47.8[a,c] (2.59)	< 0.0001
Taste	32.2 (2.49)	72.6[a] (2.09)	61.7[a,b] (2.80)	52.8[a,b,c] (3.22)	49.0[a,c] (3.12)	< 0.0001
Flavour	33.6 (2.78)	73.3[a] (2.06)	64.1[a,b] (2.70)	54.3[a,b,c] (3.10)	49.8[a,c] (3.13)	< 0.0001
Texture	33.8 (2.62)	70.8[a] (2.06)	64.1[a,b] (2.79)	51.3[a,b,c] (2.99)	50.0[a,c] (3.32)	< 0.0001
Informed						
Overall liking	39.0[†] (2.89)	71.0[a] (2.25)	60.0[a,b] (2.62)	52.5[a,b,c] (2.79)	52.0[a,c] (2.97)	< 0.0001
Colour	38.9 (3.01)	71.1[a] (2.23)	60.1[a,b] (2.62)	52.5[a,b,c] (2.79)	48.5[a,c] (2.66)	< 0.0001
Taste	36.8[†] (2.87)	70.9[a] (2.25)	59.6[a,b] (2.80)	52.8[a,b,c] (2.88)	49.5[a,c] (3.12)	< 0.0001
Flavour	37.0 (2.93)	72.5[a] (2.00)	60.2[a,b] (2.74)	53.4[a,b,c] (2.83)	50.3[a,c] (3.17)	< 0.0001
Texture	35.3 (2.85)	71.2[a] (2.18)	61.1[a,b] (2.73)	50.4[a,b,c] (3.01)	48.3[a,c] (3.36)	< 0.0001

NE, Nothing Else bar; B1 to B4, four commercial products; *In blind test, the NE values are the mean values of the replicate tests; ‡ ANOVA.

Within the same row, [a]Mean value was significantly different to NE ($p < 0.05$); [b]Mean value was significantly different to B1 ($p < 0.05$); [c]Mean value was significantly different to B2 ($p < 0.05$).

[†]Different to blind test ($p = 0.023$, 0.042 for overall liking, taste, respectively).

After the packaging and branding for the bars were presented alongside the samples for testing in the informed test, the overall liking of the Nothing Else bar remained the lowest but increased by 5 percentage points from 34 to 39, showing a 14% improvement ($p = 0.023$) compared to the blind test. The taste score of the Nothing Else bar increased by 4.6 percentage points from 32.2 to 36.8% ($p = 0.042$). However, overall liking and taste scores for all the other bars did not change with packaging and brand knowledge.

When participants were asked to rank the five bars on healthiness, taste, naturalness, and purchase intent provided that prices were the same for all brands, Brand 1 was ranked the highest for both taste and purchase intent, while the Nothing Else bar was ranked the lowest (Table 4). Conversely the Nothing Else bar was ranked the highest for both naturalness and healthiness while Brand 1 was rated the lowest. The healthiness and naturalness rank sum total of the Nothing Else and Brand 4 were similar, and were significantly higher than those of the top three brands (B1, B2 and B3). When taste and purchase intent scores were compared, the rank sum total for Brand 1 was much higher than that for all other bars which were not different to each other.

Of the perceptions of importance of healthiness, taste and naturalness to consumers, taste was considered the most important, followed by healthiness, and then naturalness. However, there was no meaningful difference in importance of these three perceptions when the mean rankings were compared (3.9, 3.6 and 3.3, respectively).

Table 4. Rank sum total of healthiness, taste, naturalness, and purchase intent for five products from informed test (n = 64)

Product Perception	NE	B1	B2	B3	B4
Taste	161	251[a]	197	188	168
Healthiness	242	136[a]	142[a]	191[a]	234
Naturalness	253	137[a]	146[a]	185[a]	208
Purchase intent	166	222[a]	181	182	178

NE, Nothing Else; B1 to B4, four commercial products. Each perception was ranked from 1 (lowest) to 5 (highest). Within the same row, [a]Rank sum total was significantly different to others at $p < 0.05$ (Friedman test post hoc).

There were no meaningful differences based on age, gender and ethnicity of participants on either the blind or the informed test. However, when the tests of between-subjects effects were performed, differences were found (Table 5). The informed overall liking scores increased significantly with the older age group ($p = 0.019$), the male participants ($p = 0.014$), and non-Asian ethnic group ($p = 0.011$), in comparison with blind overall liking scores.

Of the perceptions of importance of healthiness, taste and naturalness to consumers, taste was considered more important than healthiness and naturalness for all age, gender, and ethnic groups.

Table 5. The effects of impact factors (age, gender and ethnicity) with 64 participants on overall liking scores of the Nothing Else bar in blind and informed tests

Characteristics	Overall liking (mm) Mean (SE)		*p* value
	Blind	Informed	
20 to 29 yrs	35.1 (3.18)	38.6 (3.64)	0.524
≥ 30 yrs	33.7 (4.25)	39.7 (4.87)	0.019
Men	30.3 (4.11)	37.3 (4.82)	0.014
Women	37.0 (3.14)	39.9 (3.64)	0.305
Asian	31.9 (3.26)	33.9 (3.65)	0.127
Non-Asian	38.5 (3.94)	46.4 (4.41)	0.011

4. Discussion

The Nothing Else snack prototype was least overall liked in comparison to the four commercial snack products, which was not surprising in view of its nutritional profile that was markedly different from the other bars, particularly in fibre content. Also the prototype was developed with low sugar and low fat contents to achieve a good nutrient profiling score (FSANZ, 2012). Both sugar and fat are perceived as mouthfeel enhancers and flavour carriers and were high in the top three commercial bars. Brand 1 had the highest fat content (31.6g per 100g) compared to all other samples and Brand 3 had the highest sugar content (35.8g per 100g). The Nothing Else bar had less fat content (17g per 100g) and the least sugar content (20g per 100g, Appendix). Sugar and fat are known to influence consumers' acceptance and liking of a food product. Drewnoski (1989) demonstrated that consumers have developed sensory preferences for fat and sugar compared to other macronutrients both in their adolescence and adult life. Studies on ice cream also revealed that higher sugar and fat contents were correlated with consumers' acceptance (Guinard, Zoumas-Morse, Panyam, & Kilara, 1996; Guinard et al., 1997).

Branding and packaging of a food has an influential effect on consumer perception and has become an increasingly important factor in the food marketing system. Consumer trust and loyalty enhance consumer perceived value of a food product (Kapferer, 2004). However, which part of brand generates consumers' beliefs is not fully clear (Fernqvist & Ekelund, 2014). While healthiness is important, the taste of a food still remains a higher priority for consumers: consumers purchase intent is mainly influenced by taste and price. For health conscious consumers, nutrition and health claims have positive effects on the hedonic rating (Villegas, Carbonell & Costtell, 2008; Carillo, Varela, & Fiszman, 2012). In general, older consumers are more concerned about health than younger consumers (Roininen et al., 1999). Azzurra and Paola (2009) reported that mature people aged $\geq$ 35 years with higher level of education were particularly interested in health-related features of nutrition and foods. However in some studies, no association between health interest and consciousness and the hedonic ratings has been demonstrated (Fernqvist & Ekelund, 2014). Although interindividual variation in responses was high, there is some evidence that age and ethnicity of participants influenced liking in this study. Non-Asian and older participants liked the Nothing Else prototype more than Asian and younger participants.

The findings of this study illustrate the relative effects of branding and nutrition information on informed liking of the Nothing Else snack prototype. In the present study, the presence of branding did not improve the ratings of consumer liking for the four commercial snack products. There is insufficient evidence that branding of the commercial products had positive effects on liking where the overt listing, front-of pack, of all the ingredients on and health related information were associated with improved overall liking of the Nothing Else bar. The Nothing Else bar uses eight ingredients that are perceived as natural. Furthermore, it presented the general health parameters such as a low glycaemic index, a good fibre content, and a low sodium content (Brown et al., 2015).

The main limitation of the current relatively small study was that the design of this analysis was unbalanced by age and ethnic groups. Furthermore, the participants were a convenience sample and not representative of the population. Two thirds of the participants were relatively young, aged 20 to 29 years. Also almost two thirds of the study population were Asian. A comparison between different age groups found that the older age group ($\geq$ 30 years) scored the sensory attributes of the Nothing Else bar higher and the most popular brand lower than that of the younger age group (20 to 29 years- data not shown). In addition, 'muesli-style' snack bars are not common snacks for Asian people who tended to rate the bars lower than Non-Asian people. We propose that this might be due to food neophobia (unfamiliarity to food) of muesli and oat-based products among the Asian population. A study undertaken by Chung et al. (2012) demonstrated the differences in liking for Korean-style salad dressings and beverages between USA and Korean consumers living in North American in relation to context and cultural factors. They concluded that non-Korean consumers showed food neophobia contributing to the disliking of Korean style salad dressing. Furthermore, the Nothing Else brand was new to most participants but familiarity of participants with the other established brands could have affected the scores of the informed test. Therefore, sensory trial participants should be purposively selected in a more rigorous way. Future work is required to understand the magnitude of the effects of branding in the target group of consumers.

The hypothesis that branding and health information would improve consumer liking was confirmed in this study. Although the mean liking score of each sensory attribute of the Nothing Else bar was different than that of the four commercial products, there was a meaningful improvement in consumer overall liking of the Nothing Else snack prototype when branding and nutrition information were associated with the product. Future trials need to look at intra-individual variability in response to the effect of repeated exposure to a food as a one-off taste trial does not predict how consumers may adapt and like a product more with repeated exposure (Stein, Nagai, Nakagawab, & Beauchamp, 2003), as shown in our sales trial (Brown et al., 2015).

5. Conclusion

This study has produced evidence that a healthier snack bar with clear front-of-pack labelling listing all the ingredients 'Nothing Else' was overall liked more than in the blind condition when the packaging was shown to consumers. This was not the case for other four commercial snack products. Our findings confirmed that the branding and health related nutrition information could improve consumer liking and brand perception particularly if backed by marketing.

Acknowledgments

The authors wish to sincerely thank Peter Tan, the director of AB Foods Ltd., Auckland, who provided funding and facilities for the development of the Nothing Else snack bar. Thanks to Dr Owen Young, Professor of Food Science at AUT, for valuable advice. We also wish to thank the laboratory staff in the School of Applied Science, AUT University for their technical assistance and support.

References

Azzurra, A., & Paola, P. (2009). *Consumers' behaviours and attitudes toward healthy food products: The case of Organic and Functional foods.* Paper prepared for presentation at the 113th EAAE Seminar "A resilient European food industry and food chain in a challenging world", Crete, Greece. Retrieved from http://ageconsearch.umn.edu/bitstream/57661/2/Annunziata.pdf

Bialkova, S., & van Trijp, H. (2010). What determines consumer attention to nutrition labels? *Food Quality and Preference, 21*(8), 1042-1051. http://dx.doi.org/10.1016/j.foodqual.2010.07.001

Bilman, E. M., van Trijp, J. C., & Renes, R. J. (2010). Consumer perceptions of satiety-related snack food decision making. *Appetite, 55*(3), 639-647. http://dx.doi.org/10.1016/j.appet.2010.09.020

Brown, D., Donaldson, B., Parsons, A., Macrae, D., Kelleher, J., Yan, M., & Rush, E. (2015). The Nothing Else: A case study. *Food and Nutrition Sciences, 6*, 332-338. http://dx.doi.org/10.2436/fns.2015.6033

Carillo, E., Varela, P., Salvador, A., & Fiszman, S. (2011). Main factors underling consumers' perception of enriched and reduced-calorie biscuits in tasting and non-tasting tests. *Journal of Sensory Studies, 26*, 85-95.

Carillo, E., Varela, P., & Fiszman, S. (2012). Effects of food package information and sensory characteristics on the perception of healthiness and the acceptability of enriched biscuits. *Food Research International*, *48*, 209-216. http://dx.doi.org/10.1016/j.foodres.2012.03.016

Chung, L., Chung, S. J., Kim, J. Y. Kim, K. O., O'Mahony, M., Vickers, Z., Cha, S., Ishii, R., Baures, K., & Kim, H-R. (2012). Comparing the liking for Korean style salad dressings and beverages between US and Korean consumers: Effects of sensory and non-sensory factors. *Food Quality and Preference, 26*, 105-118. http://dx.doi.org/10.1016/ j.foodqual.2012.03.011

Cohen, J. (1988). *Statistical power analysis for behavioural sciences* (2nd ed.). Hillsdale, NJ: Lawrence Erlbaum Associates.

Drewnowski, A. (1989). Sensory preferences for fat and sugar in adolescence and adult life. *Annals New York Academiy of Siemces, 561*, 243-250. http://dx.doi: 10.1111/j.1749-6632.1989. tb20986.x

Euromonitor International. (2012). *Snack bars in New Zealand*. Annual report.

Fernqvist, F., & Ekelund, L. (2014). Credence and the effect on consumer liking of food - A review. *Food Quality and Preference, 32*, 340-353. http://dx.doi.org/10.1016/j.foodqual.2013.10.005

Food Standards Australia New Zealand. (2012). *Nutrition, health and related claims.* Review report. Retrieved from http://www.foodstandards.govt.nz/code/proposals/pages/proposalp293nutritionhealthandrelatedclaims/default.aspx

Giacalone, D., Bredie, W. I. P., & Frøst, M. B. (2012). ''All-in-one test'' (Ai1): a rapid and easily applicable approach to consumer product testing. *Food Quality and Preference, 27*, 108-119. http://dx.doi.org/10.1016/jfoodqual.2012.09011

Guinard, J. X., Zoumas-Morse, C., Panyam, D., & Kilara, A. (1996). Effect of sugar and fat on the acceptability of vanilla ice cream. *Journal of Dairy Science, 79*(11), 1922-1927. http://dx.doi.org/10.3168/jds.S0022-0302(96)76561-X

Guinard, J. X., Zoumas-Morse, C., Mori, L., Uatoni, B., Panyam, D., & Kilara, A. (1997). Sugar and fat effects on sensory properties of ice cream. *Journal of Food Science, 62*(5), 1087-1094. http://dx.doi.org/10.1111/j.1365-2621.1997.tb15044.x

Kapferer, J. N. (2004). *The new strategic brand management* (3rd ed.). London: Kogan Page.

Lawless, H. T., & Heymann, H. (2010). *Sensory evaluation of food: principles and practices.* New York, NY: Springer.

Macfie, H. J., Bratchell, N., Greenhoff, K., & Vallis, L. V. (1989). Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. *Journal of Sensory Studies, 4*, 129-148. http://dx.doi.org/10.1111/j.1745-459X.1989.tb00463.x

Mueller, S., & Szolnoki, G. (2010). The relative influence of packaging, labelling, branding and sensory attributes on liking and purchase intent: Consumers differ in their responsiveness. *Food Quality and Preference*, 21(7), 774-783. http://dx.doi.org/10.1016/j.foodqual.2010.07.011

Provencher, V., Polivy, J., & Herman, C. P. (2009). Perceived healthiness of food. If it's healthy, you can eat more*! Appetite,* 52, 340-344. http://dx.doi.org/10.1016/j.appet.2008.11.005

Roininen, K., Lähteenmäki, L., & Tourila, H. (1999). Quantification of consumer attitudes to health and hedonic characteristics of food. *Appetite,* 33, 71-88.

Savige, G., MacFarlane, A., Ball, K., Worsley, A., & Crawford, D. (2007). Snacking behaviours of adolescent and their association with skipping meals. *International Journal of Behavioural Nutrition and Physical Activity, 4*, 36-44. http://dx.doi.org/10.1186/1479-5868-4-36

Schutz, H. G. (1999). Consumer data-sense and nonsense. *Food Quality and Preference, 10*, 245-251.

Sensory analysis (2014). *In Wikipedia, the free encyclopedia.* Retrieved January, 12, 2014, http://en.wikipedia.org/wiki/Sensory_analysis

Siró, I., kápolna, E., Kápolna, B., & Lugasi, A. (2008). Functional food. Product development, marketing and consumer acceptance - A review. *Appetite, 51*(3), 456-467. http://dx.doi: 10.1016/j.appet.2008.05.060

Steenhuis, I. H., Kroeze, W., Vyth, E. L., Valk, S., Verbauwen, R., & Seidell, J. C. (2010). The effects of using a nutrition logo on consumption and product evaluation of a sweet pastry. *Appetite, 55*(3), 707-709. http://dx.doi.org/10.1016/j.appet.2010.07.013

Stein, L. J., Nagai, H., Nakagawab, M., & Beauchamp, G. K. (2003). Effects of repeated exposure and health-related information on hedonic evaluation and acceptance of a bitter beverage. *Appetite, 40*, 119-129. http://dx.doi.org/10.1016/S0195-6663(02)00173-3

van Herpen, E., & Trijp, H. C. (2011). Front-of-pack nutrition labels. Their effect on attention and choices when consumers have varying goals and time constraints. *Appetite, 57*(1), 148-160. http://dx.doi.org/10.1016/j.appet.2011.04.011

Villegas, B., Carbonell, I., & Costell, E. (2008). Effects of product information and consumer attitudes on responses to milk and soybean vanilla beverage. *Journal of the Science of Food and Agriculture, 88*, 2426-2434. http://dx.doi.org/10.1002/jsfa.3347

Appendix

Table A-1. Nutritional information panel of the test products per 100g

Food	Energy (kJ)	Protein (g)	Fat total (g)	Saturated fat (g)	CHO (g)	Sugar (g)	Fibre (g)
Nothing Else*	1544	11.6	17.1	2.1	44.9	20.3	8.2
Brand 1	2160	14.2	31.6	6.7	40.2	23.8	5.5
Brand 2	1880	10.3	21.3	9.0	55.7	24.4	5.0
Brand 3	1610	6.5	14.1	7.4	60.1	35.8	4.8
Brand 4	1430	8.9	1.3	0.3	68	21.4	9.0

* Derived from food composition tables using FoodWorks version 7 (Xyris Pty Ltd., Australia).

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Salmonella Heidelberg Strain Responses to Essential Oil Components

Juliany Rivera Calo[1], Christopher A. Baker[1], Si Hong Park[1] & Steven C. Ricke[1]

[1] Center for Food Safety; Department of Food Science, 2650 N. Young Ave., University of Arkansas, Fayetteville, AR 72704, USA

Correspondence: Steven C. Ricke, Center for Food Safety; Department of Food Science, University of Arkansas, Fayetteville, AR 72704, USA. Tel: 1-479-575-4678. E-mail: sricke@uark.edu

Received: May 5, 2015 Accepted: June 28, 2015 Online Published: August 13, 2015

doi:10.5539/jfr.v4n5p73 URL: http://dx.doi.org/10.5539/jfr.v4n5p73

Abstract

Salmonella are one of the more prominent foodborne pathogens that represent a major health risk to humans. *Salmonella* serovar Heidelberg strains are increasingly becoming an important public health concern, since they have been identified as one of the primary *Salmonella* serovars responsible for human outbreaks. Over the years, *Salmonella* Heidelberg isolates have exhibited higher rates of resistance to multiple antimicrobial agents compared to other *Salmonella* serovars. Essential oils (EOs) have been widely used as alternatives to chemical-based antimicrobials. In the current research, five EOs were screened to determine their antimicrobial activity against 15 *S.* Heidelberg strains from different sources. Oils tested were R(+)-limonene, orange terpenes, cold compressed orange oil, trans-cinnamaldehyde and carvacrol. EOs were stabilized in nutrient broth by adding 0.15% (w/v) agar. Tube dilution assays and minimal inhibitory concentrations (MIC) were determined by observing color changes in samples during exposure to EOs. Carvacrol and *trans*-cinnamaldehyde completely inhibited the growth of *S.* Heidelberg strains, while R(+)-limonene and orange terpenes did not show any inhibitory activity against the strains tested. Cold compressed orange oil only inhibited growth of two of the strains exhibiting an MIC of 1%. All *S.* Heidelberg isolates evaluated exhibited similar responses to the respective EOs. The use of all natural antimicrobials such as specific EOs offers the potential to limit the majority of *S.* Heidelberg isolates that may occur in food production.

Keywords: essential oils, *Salmonella* Heidelberg; antimicrobials

1. Introduction

Foodborne illnesses continue to be one of the primary public health concerns in the United States (U. S.), and it has been estimated that over 1 million Americans contract *Salmonella* each year (Scallan et al., 2011). Annual costs for *Salmonella* control efforts are estimated to be $14.6 billion (Scharff, 2010; Heithoff et al., 2012). S*almonella* is not only a public health concern due to the number of cases per year, but many strains have developed resistance to antimicrobial agents (Kim et al., 2005; Foley & Lynne, 2008; Bajpai, Baek, & Kang, 2012) due to continued therapeutic use of antimicrobials in feed products (Su, Chiu, Chu, & Ou, 2004; Kim et al., 2005).

Salmonella enterica serovar Heidelberg (*S*. Heidelberg) ranks fourth among the top five serovars associated with human infections and is responsible for an estimated 84,000 illnesses in the U. S. annually (CDC, 2008; FDA, 2009; Foley et al., 2011; Han et al., 2011). *Salmonella* Heidelberg is one of the most commonly isolated serovars in the U.S. and Canada from clinical cases of salmonellosis, retail meats and livestock (Zhao et al., 2008; Hur, Jawale, & Lee, 2012). While most *Salmonella* infections are self-limiting and are resolved within a few days, *S.* Heidelberg tends to cause a significantly higher percentage of invasive infections (Vugia et al., 2004; Han et al., 2011). As a result, antimicrobial therapy is often necessary, making antimicrobial resistance a significant concern. Due to the tendency of *S.* Heidelberg to cause severe extra-intestinal infections (Wilmshurst & Sutcliffe, 1995) such as myocarditis and septicemia (Vugia et al., 2004), the occurrence of *S.* Heidelberg multidrug resistance strains is of extreme clinical importance. *Salmonella* Heidelberg strains exhibiting antimicrobial resistance have been isolated from humans, retail meats and food animals (Logue, Sherwood, Olah, Elijah, & Dockter, 2003; Nayak et al., 2004; Kaldhone et al., 2008; Zhao et al., 2008; Lynne, Kaldhone, David, White, & Foley, 2009; Oloya, Doetkott, & Khaitsa, 2009; Han et al., 2011). Studies suggest that poultry-associated *S.* Heidelberg strains harbor IncFIB, IncA/C, IncH2, and IncI1 plasmids, which may contain genes that confer resistance to several antibiotics such as tetracycline, kanamycin, streptomycin, and sulfonamides (Han et al., 2012). Because *S.*

Heidelberg is responsible for causing more invasive infections compared to other serovars, it is important to monitor its prevalence and resistance – novel and unique intervention strategies are a priority to reduce or eliminate its presence.

Aromatic plants and their extracts have been examined for their effectiveness in food safety and preservation applications (Fisher & Phillips, 2008). Essential oils, for example, exhibit antimicrobial properties that may make them suitable alternatives to antibiotics (Chaves et al., 2008). These potential attributes and an increasing demand for natural medicinal treatment options have brought attention to the use of EOs as potential antimicrobial alternatives (Fisher & Phillips, 2008; Solórzano-Santos & Miranda-Novales, 2012; Rivera Calo, Crandall, O'Bryan, & Ricke, 2015a). Essential oils derived as by-products in citrus processing have been screened for antimicrobial properties against common foodborne pathogens, and several of these compounds have exhibited antimicrobial properties (Dabbah, Edwards, & Moats, 1970; Rivera Calo et al., 2015a).

Alali et al. (2013) evaluated the use and application of various EOs in broiler drinking water to reduce ceca colonization and fecal shedding of *S.* Heidelberg. Although the EOs evaluated were not a sufficient means to limit *S.* Heidelberg isolates in ceca or reduce shedding, these authors reported a reduction of *S.* Heidelberg colonization in the crops of seeders as well as an increased weight gain in birds that were administered the EOs (Alali et al., 2013). This research was conducted to determine the efficacy of several EOs on *S.* Heidelberg isolates that originated from different sources.

2. Materials and Methods

2.1 Bacterial Strains Used in This Study

Fifteen different *S.* Heidelberg strains were evaluated (Table 1). Strains were isolated from multiple sources such as, turkey and ground turkey, poultry products, cattle, swine, poultry and poultry egg houses, and humans.

Table 1. *Salmonella* Heidelberg strains used in this study

Strain	Source	Reference
ARI-14	poultry products	In this study
SL 476	ground turkey	Fricke et al., 2011
SL 486	human	Fricke et al., 2011
692	chicken egg house	Lynne et al., 2009
945	human	Han et al., 2011
114	cattle	Lynne et al., 2009
163	turkey	Kaldhone et al., 2008
136	swine	Lynne et al., 2009
1148	human	Han et al., 2012
824	turkey	Kaldhone et al., 2008
130	chicken	Lynne et al., 2009
937	human	Han et al., 2011
118	cattle	Lynne et al., 2009
144	swine	Lynne et al., 2009
146	swine	Lynne et al., 2009

2.2 DNA Extraction

One colony of each *S.* Heidelberg strain was inoculated in 5 mL Luria Bertani (LB) broth and incubated at 37 °C, 190 rpm for 16 h in a C76 Water Bath Shaker (New Brunswick Scientific, Edison, NJ, USA). A one mL aliquot of bacterial cells was transferred to a microcentrifuge tube, and samples were centrifuged at 14,000 × *g* for 10 min and the corresponding supernatants were discarded. Genomic DNA was extracted with the Qiagen DNeasy Blood Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The isolated genomic DNA concentration and purity were measured using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE,

USA) and DNA samples were subsequently stored at -20 °C.

2.3 S. Heidelberg Confirmation by PCR

A conventional PCR assay was conducted with a MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA, USA). *Salmonella* Heidelberg specific primers were generated based on the type II restriction enzyme methylase subunit sequences of *S.* Heidelberg (Bronowski & Winstanley, 2009), and upon evaluation of the specificity for all 15 strains, each was determined to belong to the serovar Heidelberg (Table 2). Additionally, exclusivity assays were performed on different *Salmonella* serovars as well as other non-*Salmonella*. The total reaction (25 µL volume) was composed of 1 µL of template DNA, 1 µL of each primer (IDT, Coralville, IA, USA), 12.5 µL of SYBR Green (Cambrex Bioscience, Walkersville, MD, USA), and 9.5 µL of DNase-RNase free water. The PCR conditions consisted of pre-denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for 30 s, and a final extension cycle at 72 °C for 5 min. PCR products were confirmed on a 1.5% agarose gel and visualized on a transilluminator (Bio-Rad, Hercules).

Table 2. *Salmonella* Heidelberg specific primer pair used in this study

Primer	Sequence	Product	Reference
SH_SHP-2f	5'-GCATA GTTCC AAAGC ACGTT-3'	180	This study
SH_SHP-1r	5'-GCTCA ACATA AGGGA AGCAA-3'	180	This study

2.4 Essential Oils and Cultures

Each of the EOs evaluated (R(+)-limonene, orange terpenes, cold compressed orange oil, trans-cinnamaldehyde and carvacrol) were obtained from Sigma Aldrich (St. Louis, MO, USA). Fifteen *S.* Heidelberg strains from different sources were evaluated (Table 1). One colony of each strain was inoculated into Luria-Bertani (LB) broth and incubated at 42 °C, 190 rpm for 18 h. In addition, analyses were performed on *S.* Heidelberg strains incubated at 37 °C, 190 rpm for 18 h.

2.5 Modified Tube Dilution Assay

To maintain the EOs in a homogeneous mixture, 0.15% agar was added to nutrient broth (NB), boiled for 1 min and subsequently autoclaved. Once the media cooled to room temperature, triphenyl tetrazolium chloride (TTC) (1 %) was added to act as a growth indicator. As previously described by O'Bryan et al. (2008), serial dilutions were made by placing 10 mL of the NB with 0.15% agar (NBA) in the first tube and 5 mL in the remaining tubes for a total of four tubes. One hundred µL of the essential oil was added to the 1st (10 mL) tube for an initial 1% concentration. To perform serial dilutions, 5 mL of the emulsion was transferred to the next tube, and the procedure was repeated for a total of four dilutions. Five mL were removed from the last tube and discarded so that all tubes consisted of equal volumes (5 mL). All tubes were inoculated with 50 µL of an overnight culture of each *S.* Heidelberg strain, and the tubes were subsequently incubated for 24 h at 37 °C. A change in color from light yellow to pink/red indicated growth. Minimum inhibitory concentrations were determined as the lowest concentration of EOs that showed no color change in the medium, which was considered representative of the absence of detectable bacterial growth.

3. Statistical Analysis

MIC tests were repeated as three independent trials. Each mean ± standard deviation was determined using JMP Pro Software Version 11.0 (SAS Institute Inc., Cary, NC).

4. Results and Discussion

Salmonella Heidelberg specific primers (Table 2) were used to confirm that all 15 strains evaluated belong to *S.* Heidelberg (Figure 1). Park and Ricke (2015) developed a multiplex PCR assay to simultaneously detect *Salmonella* serovars Heidelberg, Enteriditis, and Typhimurium. The *S.* Heidelberg primers were generated for this study similar to Park and Ricke (2015), which involves BLAST alignment(s) and subsequent inclusivity and exclusivity assays for primer specificity confirmation. In addition to the potential detection applications, the *S.* Heidelberg specific primers used in this study suggest that although these isolates originated from different environments, there is a certain level of genetic uniformity among these *S.* Heidelberg isolates.

Minimum inhibitory concentrations were determined by observing tubes for any color change to pink or red (Table 3). For the *S.* Heidelberg strains grown at 42 °C, results indicate that *trans*-cinnamaldehyde and carvacrol

exhibited MICs of 131 μg/mL and 122 μg/mL, respectively, by completely inhibiting the growth of each *S.* Heidelberg strain, while orange terpenes and R(+)-limonene did not exhibit any inhibitory activity against any of these strains. Cold compressed orange oil only inhibited growth of two of the strains (945 and 114), with MIC's of 843 μg/mL. No relationship was observed between the source of the strain and the EOs tested. Similar results were obtained when strains were grown at 37 °C, although under this condition cold compressed orange oil showed no antimicrobial activity for all strains, while at 42 °C it inhibited strains 945 and 114 with an MIC of 843 μg/mL.

Table 3. Minimum inhibitory concentrations (μg/mL) of five EOs against *Salmonella* Heidelberg isolates

Isolate	Essential oils				
	carvacrol	R(+)-limonene	orange terpenes	orange oil	*trans*-cinnamaldehyde
SL 486	122 ± 0	No effect	No effect	No effect	131 ± 0
SL 476	122 ± 0	No effect	No effect	No effect	131 ± 0
ARI-14	122 ± 0	No effect	No effect	No effect	131 ± 0
692	122 ± 0	No effect	No effect	No effect	131 ± 0
945	122 ± 0	No effect	No effect	843 ± 0	131 ± 0
114	122 ± 0	No effect	No effect	843 ± 0	131 ± 0
163	122 ± 0	No effect	No effect	No effect	131 ± 0
136	122 ± 0	No effect	No effect	No effect	131 ± 0
1148	122 ± 0	No effect	No effect	No effect	131 ± 0
824	122 ± 0	No effect	No effect	No effect	131 ± 0
130	122 ± 0	No effect	No effect	No effect	131 ± 0
937	122 ± 0	No effect	No effect	No effect	131 ± 0
118	122 ± 0	No effect	No effect	No effect	131 ± 0
144	122 ± 0	No effect	No effect	No effect	131 ± 0
146	122 ± 0	No effect	No effect	No effect	131 ± 0

There are several indications that there may be differences in responses among *Salmonella* serovars and in some cases even among strains. This is critical to consider since overall recommendations for antimicrobial application to *Salmonella* can sometimes be made based on the responses of only a few serovars and/or strains. The growth responses of *S.* Typhimurium grown in spent media generated from several of the *S.* Heidelberg isolates used in this study have been evaluated (Rivera Calo, Park, Baker, & Ricke, 2015b). The spent media from twelve of the fifteen *S.* Heidelberg isolates evaluated in this study decreased the growth of *S.* Typhimurium (ATCC 14028), which suggests that *S.* Heidelberg isolates may compete in some manner with other *Salmonella* serovars (Rivera Calo et al., 2015b). Additionally, studies have shown the existence of differences between serovars as well as strains within the same serovar of *Salmonella enterica* can occur for certain antimicrobials. In their research, González et al. (2012) compared *hilA* gene expression in response to acid stress between different *Salmonella* serovars and strains. Results showed that there are serovar and strain differences in virulence gene expression and acid tolerance; regulation of *hilA* showed to be serovar and strain dependent as well as dependent on acid type (González et al., 2012). In an extensive genome analysis comparison between UK-1 and other *S.* Typhimurium strains, Luo et al. (2012) reported that virulence factors pertaining to one strain might increase or decrease virulence when present in a different strain. Shah et al. (2011) reported that isolates from *S.* Enteritidis that have been recovered from poultry or poultry environment are not equally pathogenic, nor do they have similar invasiveness.

Previous research has shown that different EOs exhibit antimicrobial activity pre- and post-harvest on *Salmonella.* The antimicrobial effect of EOs has been reported to be concentration dependent (Sivropoulou et al. 1996). Ravishankar et al. (2010) reported that at a 0.2% concentration, antimicrobials, carvacrol and cinnamaldehyde completely inactivated the antibiotic-resistant and -susceptible isolates of *S. enterica*. Zhou et al. (2007) investigated the antimicrobial activity of cinnamaldehyde, thymol and carvacrol individually and in

combination against *S.* Typhimurium, and reported the lowest concentrations of cinnamaldehyde, thymol and carvacrol inhibiting the growth of *S.* Typhimurium significantly were 200, 400 and 400 mg/L, respectively. When combined, cinnamaldehyde/thymol, cinnamaldehyde/carvacrol and thymol/carvacrol demonstrated that the concentration of cinnamaldehyde, thymol and carvacrol could be decreased from 200, 400 and 400 mg/L to 100, 100 and 100 mg/L, respectively (Zhou et al., 2007). In this study we used cinnamaldehyde in its *trans* isomer, which is the form that is present as a major component of bark extract of cinnamon (Kollanoor Johny et al., 2008).

The MIC method has previously been used to evaluate the effectiveness of orange EOs against different *Salmonella* serovars (Enteritidis, Seftenberg, Tennessee, Kentucky, Heidelberg, Montevideo, Michigan, Stanley, among others) (O'Bryan, Crandall, Chalova, & Ricke, 2008). These authors reported that three out of the seven citrus EOs evaluated (orange terpenes, d-limonene, and terpenes purified fractions from orange essence) exhibited antimicrobial activity, and that there was not a significant difference in the responses among the *Salmonella* serovars evaluated (O'Bryan et al., 2008). However, orange terpenes and d-limonene exhibited the most antimicrobial activity with a MIC of 1% for each serovar, and terpenes from orange essence exhibited MIC range from 0.125 to 0.5% (O'Bryan et al., 2008). In the current study, no effect was observed for any of the *S.* Heidelberg isolates subjected to orange terpenes. These contrasting observations may be due to the fact that O'Bryan et al. (2008) evaluated commercial essential oils and fractions (single fold d-limonene), which may contain additional antimicrobial properties that were not present in the essential oils evaluated in the current study. Additionally, despite the fact that limonene is one of the more well-known and characterized of the EOs from citrus products (Dabbah et al., 1970; Caccioni, Guizzardi, Biondi, Renda, & Ruberto, 1998) which can exert potent, broad-spectrum antimicrobial activity (Di Pasqua, Hoskins, Betts, & Mauriello, 2006), this essential oil, in the form of R(+)-limonene did not exhibit any antimicrobial activity in this study.

Essential oils have also been studied to have antimicrobial activity against *Salmonella* when used post-harvest. In their research, Alali et al. (2013) determined the effect of non-pharmaceuticals (a blend of organic acids, a blend of EOs, lactic acids, and a combination of levulinic acid and sodium dodecyl sulfate) on weight gain, feed conversion ratio, mortality of broilers and their ability to reduce colonization and fecal shedding of *S.* Heidelberg. Their results showed that the broilers that received the EOs had significantly increased weight gain and mortalities were lower compared to other treatments. *Salmonella* Heidelberg contamination in crops was significantly lower in challenged and unchallenged broilers that received EOs and lactic acids in drinking water, when compared to other treatments (Alali et al., 2013). Results of our study further supports that some EOs could be used as antimicrobial agents against the foodborne pathogen *Salmonella*. Further studies will be needed to confirm the antimicrobial effect of these EOs *in vivo* and which combinations represent optimal antimicrobial activity.

Hoffmann et al. (2014) compared the genomes of 44 S. Heidelberg strains, and identified at least 273 genes that varied among these strains. Uniform responses to various EOs were observed for each *S*. Heidelberg isolate in this study, which suggests that the inhibitory mechanism(s) of EOs result from a broad inhibitory action such as potentially a membrane effect. Additionally, the genetic diversity among *S.* Heidelberg isolates may not play a role in the efficacy of survival when exposed to EOs. Additional studies on *S*. Heidelberg and EOs may be necessary to identify the most appropriate pre- and post-harvest applications of essential oil for limiting *Salmonella* spp. in the food supply.

Acknowledgments

We thank Dr. Steven L. Foley, Division of Microbiology, NCTR, U.S. Food and Drug Administration, Jefferson, AR, and Dr. W. Florian Fricke, University of Maryland, School of Medicine, Baltimore, MD for providing Salmonella Heidelberg strains used in this study, and Dr. Young M. Kwon, Poultry Science Department, University of Arkansas, Fayetteville, AR for providing the EOs for the study. We also thank the Department of Food Science, University of Arkansas, Fayetteville, AR for providing graduate stipend support to authors J. R. C. and C. A. B, as well as the Michael Johnson Scholarship to C.A.B.

References

Alali, W. Q., Hofacre, C. L., Mathis, G. F., & Faltys, G. (2013). Effect of essential oil compound on shedding and colonization of *Salmonella enterica* serovar Heidelberg in broilers. *Poultry Science, 92*, 836-841. http://dx.doi.org/10.3382/ps.2012-02783

Alali, W. Q., Hofacre, C. L., Mathis, G. F., Faltys, G., Ricke, S. C., & Doyle, M. P. (2013). Effect of non-pharmaceutical compounds on shedding and colonization of *Salmonella enterica* serovar Heidelberg in broilers. *Food Control, 31*, 125-128. http://dx.doi.org/10.1016/j.foodcont.2012.10.001

Bajpai, V. K., Baek, K. H., & Kang, S. C. (2012). Control of *Salmonella* in foods by using essential oils: a review. *Food Research International, 45*, 722-734. http://dx.doi.org/10.1016/j.foodres.2011.04.052

Bronowski, C., & Winstanley, C. (2009). Identification and distribution of accessory genome DNA sequences from an invasive African isolate of *Salmonella* Heidelberg. *FEMS Microbiology Letters, 298*, 29-36. http://dx.doi.org/10.1111/j.1574-6968.2009.01697.x

Caccioni, D. R. L., Guizzardi, M., Biondi, D. M., Renda, A., & Ruberto, G. (1998). Relationship between volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum*. *International Journal of Food Microbiology, 43*, 73-79. http://dx.doi.org/10.1016/S0168-1605(98)00099-3

CDC. (2006). *Salmonella* Surveillance: Annual Summary, 2006. Atlanta, Georgia US Department of Health and Human Services, CDC. 2008. Retrieved February 23, 2015, from http://198.246.124.22/ncidod/dbmd/phlisdata/salmtab/2006/SalmonellaIntroduction2006.pdf

Chaves, A. V., He, M. L. Yang, W. Z., Hristov, A. N., McAllister, T. A., & Benchaar, C. (2008). Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria. *Canadian Journal of Animal Science, 88*, 117-122. http://dx.doi.org/10.4141/CJAS07061

Dabbah, R., Edwards, V. M., & Moats, W.A. (1970). Antimicrobial action of some citrus fruit oils on selected food-borne bacteria. *Applied Microbiology, 19*, 27-31.

Di Pasqua, R., Hoskins, N., Betts, G., & Mauriello, G. (2006). Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *Journal of Agriculture and Food Chemistry, 54*, 2745-2749. http://dx.doi.org/10.1021/jf0527221

FDA. (2009). National Antimicrobial Resistance Monitoring System-Enteric Bacteria (NARMS): 2006 Executive Report. U.S. Department of Health and Human Services, U.S. FDA. Accessed February 23, 2015. http://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM312360.pdf

Fisher, K., & Phillips, C. A. (2008). Potential antimicrobial uses of essential oils in food: is citrus the answer? *Trends in Food Science and Technology, 19*, 156-164. http://dx.doi.org/10.1016/j.tifs.2007.11.006

Foley, S. L., & Lynne, A. M. (2008). Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *Journal of Animal Science, 86*, E173-E187. http://dx.doi.org/10.2527/jas.2007-0447

Foley, S. L., Nayak, R. Hanning, I. B., Johnson, T. J., Han, J., & Ricke, S. C. (2011). Minireview: Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. *Applied and Environmental Microbiology, 77*, 4273-4279. http://dx.doi.org/10.1128/AEM.00598-11

Fricke, W. F., Mammel, M. K., McDermott, P. F., Tartera, C., White, D. G., LeClerc, J. E., … Cebula, T. A. (2011). Comparative genomics of 28 *Salmonella* enterica isolates: evidence for CRISPR-mediated adaptive sublineage evolution. *Journal of Bacteriology, 193*, 3556-3568. http://dx.doi.org/10.1128/JB.00297-11

González-Gil, F., Le Bolloch, A., Pendleton, S., Zhang, N., Wallis, A., & Hanning, I. (2012). Expression of *hilA* in response to mild acid stress in *Salmonella enterica* is serovar and strain dependent. *Journal of Food Science, 77*, M292-M296. http://dx.doi.org/10.1111/j.1750-3841.2012.02684.x

Han, J., David, D. E., Deck, J., Lynne, A. M., Kaldhone, P., Nayak, R., … Foley, S. L. (2011). Comparison of *Salmonella enterica* serovar Heidelberg isolates from human patients with those from animal and food sources. *Journal of Clinical Microbiology, 49*, 1130-1133. http://dx.doi.org/10.1128/JCM.01931-10

Han, J., Lynne, A. M., David, D. E., Tang, H. E., Xu, J., Nayak, R., … Foley, S. L. (2012). DNA sequence analysis of plasmids from multidrug resistant *Salmonella enterica* serotype Heidelerg isolates. *PLoS One, 7*, e51160. http://dx.doi.org/10.1371/journal.pone.0051160

Heithoff, D. M., Shimp, W. R., House, J. K., Xie, Y., Weimer, B. C., Sinsheimer, R. L., & Mahan, M. J. (2012). Intraspecies variation in the emergence of hyperinfectious bacterial strains in nature. *PLoS Pathogens, 8*, 1-17. http://dx.doi.org/10.1371/journal.ppat.1002647

Hoffmann, M., Zhao, S., Pettengill, J., Luo, Y., Monday, S. R., Abbot, J., … McDermott, P. F. (2014). Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype Heidelberg isolates from humans, retail meats, and animals. *Genome Biology and Evolution, 6*, 1046-1068.

http://dx.doi.org/10.1093/gbe/evu079

Hur, J., Jawale, C., & Lee, J. H. (2012). Antimicrobial resistance of *Salmonella* isolated from food animals: A review. *Food Research International, 45*, 819-830. http://dx.doi.org/10.1016/j.foodres.2011.05.014

Kaldhone, P., Nayak, R., Lynne, A. M., David, D. E., McDermott, P. F., Logue, C. M., & Foley, S. L. (2008). Characterization of *Salmonella enterica* serovar Heidelberg from turkey-associated sources. *Applied and Environmental Microbiology, 74*, 5038-5046. http://dx.doi.org/10.1128/AEM.00409-08

Kim, W. K., Karabasil, N., Bulajic, S., Dunkley, K. D., Callaway, T. R. Poole, T. L., … Nisbet, D. J. (2005). Comparison of spontaneous antibiotic resistance frequency of *Salmonella* Typhimurium growth in glucose amended continuous culture at slow and fast dilution rates. *Journal of Environmental Science and Health Part B, 40*, 475-484. http://dx.doi.org/10.1081/PFC-200047598

Kollanoor Johny, Darre, A. M. J., Hoagland, T. A., Schreiber, D. T., Donoghue, A. M., Donoghue, D. J., & Venkitanarayanan, K. (2008). Antibacterial effect of *trans*-cinnamaldehyde on *Salmonella* Enteritidis and *Campylobacter jejuni* in chicken drinking water. *Journal of Applied Poultry Research, 17*, 490-497. http://dx.doi.org/10.3382/japr.2008-00051

Logue, C. M., Sherwood, J. S. Olah, P. A., Elijah, L. M., & Dockter, M. R. (2003). The incidence of antimicrobial-resistant *Salmonella* spp. on freshly processed poultry from US Midwestern processing plants. *Journal of Applied Microbiology, 94*, 16-24. http://dx.doi.org/10.1046/j.1365-2672.2003.01815.x

Luo, Y., Kong, Q., Yang, J., Mitra, A., Golden, G., Wanda, S. Y., … Curtis III, R. (2012). Comparative genome analysis of the high pathogenicity *Salmonella* Typhimurium strain UK-1. *PLoS One, 7*, e40645. http://dx.doi.org/10.1371/journal.pone.0040645

Lynne A. M., Kaldhone, P., David, D., White, D. G., & Foley, S. L. (2009). Characterization of antimicrobial resistance in *Salmonella enterica* serotype Heidelberg isolated from food animals. *Foodborne Pathogens and Diseases, 6*, 207-215. http://dx.doi.org/10.1089/fpd.2008.0172

Nayak, R., Stewart, T., Wang, R. F., Lin, J., Cerniglia, C. E., & Kenney, P. B. (2004). Genetic diversity and virulence gene determinants of antibiotic-resistant *Salmonella* isolated from preharvest turkey production sources. *International Journal of Food Microbiology, 91*, 51-62. http://dx.doi.org/10.1016/S0168-1605(03)00330-1

O'Bryan, C. A., Crandall, P. G., Chalova, V. I., & Ricke, S. C. (2008). Orange essential oils antimicrobial activities against *Salmonella* spp. *Journal of Food Science, 73*, M264-M267. http://dx.doi.org/10.1111/j.1750-3841.2008.00790.x

Oloya, J., Doetkott, D., & Khaitsa, M. L. (2009). Antimicrobial drug resistance and molecular characterization of *Salmonella* isolated from domestic animals, humans, and meat products. *Foodborne Pathogens and Disease, 6*, 273-284. http://dx.doi.org/10.1089/fpd.2008.0134

Park, S. H., & Ricke, S. C. (2015). Development of multiplex PCR assay for simultaneous detection of *Salmonella* genus, *Salmonella* subspecies I, *Salm*. Enteritidis, *Salm*. Heidelberg and *Salm*. Typhimurium. *Journal of Applied Microbiology, 118*, 152-160. http://dx.doi.org/10.1111/jam.12678

Ravishankar, S., Zhu, L., Reyna-Granados, J., Law, B., Joens, L., & Friedman, M. (2010). Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. *Journal of Food Protection, 73*, 234-240.

Rivera Calo, J., Crandall, P. G., O'Bryan, C. A., & Ricke, S. C. (2015a). Essential oils as antimicrobials in food systems – A review. *Food Control, 54*, 111-119. http://dx.doi.org/10.1016/j.foodcont.2014.12.040

Rivera Calo, J., Park, S. H., Baker, C. A., & Ricke, S. C. (2015b). Specificity of *Salmonella* Typhimurium strain (ATCC 14028) growth responses to *Salmonella* serovar-generated spent media. *Journal of Environmental Science and Health Part B, 50*, 422-429. http://dx.doi.org/10.1080/03601234.2015.1011962

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M-A., Roy, S. L., … Griffin, P. M. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases, 17*, 7-15. http://dx.doi.org/10.3201/eid1707.110572

Scharff, R. L. (2010). Health-related costs from foodborne illness in the United States. Retrieved February 28, 2015, from http://publichealth.lacounty.gov/eh/docs/ReportPublication/HlthRelatedCostsFromFoodborneIllinessUS.pdf

Shah, D. H., Zhou, X., Addwebi, T., Davis, M. A., Orfe, L., Call, D. R., … Besser, T. E. (2011). Cell invasion of

poultry-associated *Salmonella enterica* serovar Enteritidis isolates is associated with pathogenicity, motility and proteins secreted by the type III secretion system. *Microbiology, 157*, 1428-1445. http://dx.doi.org/10.1099/mic.0.044461-0

Sivropoulou, A., Papanikolaou, E. Nikolaou, C. Kokkini, S. Lanaras, T., & Arsenakis, M. (1996). Antimicrobial and cytotoxic activities of *Origanum* essential oils. *Journal of Agricultural and Food Chemistry, 44*, 1202-1205. http://dx.doi.org/10.1021/jf950540t

Solórzano-Santos, F., & Miranda-Novales, M. G. (2012). Essential oils from aromatic herbs as antimicrobial agents. *Current Opinions in Biotechnology, 23*, 136-141. http://dx.doi.org/10.1016/j.copbio.2011.08.005

Su, L.-H., Chiu, C.-H., Chu, C., & Ou. J. T. (2004). Antimicrobial resistance in nontyphoid *Salmonella* serotypes: a global challenge. *Clinical Infectious Diseases, 39*, 546-551. http://dx.doi.org/10.1086/422726

Vugia, D. J., Samuel, M., Farley, M. M., Marcus, R., Shiferaw, B., Shallow, S., Smith, K., & Angulo, F. J. (2004). Invasive *Salmonella* infections in the United States, FoodNet, 1996-1999: incidence, serotype distribution, and outcome. *Clinical Infectious Diseases, 38*, S149-156. http://dx.doi.org/10.1086/381581

Watson, P. R., Paulin, S. M., Bland, A. P., Jones, P. W., & Wallis, P. S. (1995). Characterization of intestinal invasion by *Salmonella typhimurium* and *Salmonella dublin* and effect of a mutation in the invH gene. *Infection and Immunity, 63*, 2743-2754.

Wilmshurst, P., & Sutcliffe, H. (1995). Splenic abscess due to *Salmonella* Heidelberg. *Clinical Infectious Diseases, 21*, 1065-1066. http://dx.doi.org/10.1093/clinids/21.4.1065

Zhao, S., White, D. G., Friedman, S. L., Glenn, A., Blickenstaff, K., Ayers, K. L., … McDermott, P. F. (2008). Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006. *Applied and Environmental Microbiology, 74*, 6656-6662. http://dx.doi.org/10.1128/AEM.01249-08

Zhou, F., Ji, B., Zhang, H., Jiang, H., Yang, Z., Li, J., & Yan, W. (2007). The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella* Typhimurium. *Journal of Food Safety, 27*, 124-133. http://dx.doi.org/10.1111/j.1745-4565.2007.00064.x

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Occurrence of Aflatoxin in Some Food Commodities Commonly Consumed in Nigeria

I. O. Williams[1], S. A. Ugbaje[2], G. O. Igile[1] & O. O. Ekpe[3]

[1] Department of Biochemistry, University of Calabar, Calabar, Nigeria

[2] Microbiology Laboratory, National Agency for Food and Drug Administration and Control (NAFDAC), Port Harcourt, Nigeria

[3] Ministry of Agriculture and Natural Resources, Calabar, Cross River State, Nigeria

Correspondence: I. O. Williams, Department of Biochemistry, University of Calabar, Calabar, Nigeria. Tel: 234-803-501-8175. E-mail: imawills@yahoo.com

Received: February 26, 2015 Accepted: July 15, 2015 Online Published: August 27, 2015

doi:10.5539/jfr.v4n5p81 URL: http://dx.doi.org/10.5539/jfr.v4n5p81

Abstract

Aflatoxicosis is a public health problem in Nigeria like other tropical and sub-tropical regions of the world. Control of aflatoxin contamination requires thorough risk assessment, monitoring, quality control and empirical data. This study assayed total aflatoxin levels, identified and quantified four aflatoxin types in five food commodities commonly consumed in the six geopolitical zones of Nigeria. The food materials: *Zea mays, Colocynthis citrullus, Capsicum frutescens, Irvingia gabonensis* and *Arachis hypogea* were obtained from Watt market in Calabar urban. ELISA method was used for total aflatoxin, HPLC for aflatoxin types, AOAC for moisture. All (100%) the samples were contaminated with aflatoxin. Contamination was highest in *Irvingia gabonensis* (63.40 ± 1.79 µg/kg) and least in *Zea mays* (3.20 ± 0.12 µg/kg) ($p < 0.05$). Except for *Irvingia gabonensis* and *Colocynthis citrullus*, total aflatoxin was within safe intake level of the Nigerian regulatory authority (National Agency for Food and Drug Adminstration and Control {NAFDAC}). All four aflatoxin types occurred in *Irvingia gabonensis*, *Capsicum frutescens* and *Colocynthis citrullus*; none was detected in *Arachis hypogea*. AFB_1 contamination was highest in *Irvingia gabonensis* (11.71±0.10 µg/kg) followed by *Capsicum frutescens* (1.21 ± 0.01 µg/kg); AFB_2 ranged from 0.00 ± 0.00-2.43 ± 0.05 µg/kg, AFG_1 0.00 ± 0.00-3.73 ± 0.04 µg/kg, and AFG_2 0.00 ± 0.00-0.54 ± 0.01 µg/kg ($p < 0.05$). Only *Irvingia gabonensis* exceeded the limit of AFB_1 specified by NAFDAC for human foods. Moisture content varied widely (3.23 ± 0.03%-10.37 ± 0.19%). The trend in the occurrence of aflatoxins in the food samples was directly proportional ($r = 0.91$) to their moisture contents. Food commodities sold in Calabar carry potential health hazard. Improved handling through food processing, preservation and storage can minimize aflatoxins in foodstuffs and ensure sustainable quality of food supply.

Keywords: aflatoxicosis, carcinogenicity, food safety, mycotoxigenic fungi, NAFDAC

1. Introduction

The safety of food and feed has been a major concern of nations especially in recent years as more knowledge is gathered on the occurrence of natural toxins in food stuffs, fertilizers, animal feed and edible plant materials. Naturally occurring toxins have been characterized by the World Health Organization (WHO, 2002) as significant sources of food borne illnesses. Of the natural food toxins, the Food and Agriculture Organization (FAO) has estimated that mycotoxins (fungal toxins) alone contaminate about 25% of agricultural products worldwide resulting in huge losses for farmers (Smith, Solomons, Lewis, & Anderson, 1994; Wu, 2007). The biochemical properties of mycotoxins are diverse, and their toxic effects are exceedingly variable. Mycotoxins are carcinogenic, tremorogenic, haemorrhagic, genotoxic, teratogenic, nephrotoxic, hepatotoxic and immunotoxic (Refai, 1988; Hosseini & Bagheri, 2012).

Out of the about 300 mycotoxins so far known, aflatoxin is the most studied, because of its common occurrence, high potency and toxicity to man and animals (SP-IPM, 2009). Aflatoxins are associated with high incidences of liver cancer in Africa and elsewhere and are thought to exacerbate diseases such as hepatitis B virus-induced liver cancer and HIV/AIDS (Shepherd, 2008). Other health effects of aflatoxins in animals and humans include

reduced growth rate, weakened immune system and death (Eaton & Groopman, 1994). At least thirteen (13) different types of aflatoxins are produced in nature with aflatoxin B_1 considered as the most toxic and therefore of particular public health importance.

In recognition of the many dangers of aflatoxins, researchers and farmers around the world are seeking an understanding of how to manage the contamination dangers resulting from them. Meanwhile, policy makers are in the process of balancing food safety with food availability, a task requiring a thorough risk assessment and monitoring as well as empirical data. In view of the fact that foods from maize (*Zea mays*), melon seed (*Colocynthis citrullus*), ground nut (*Arachis hypogea*), bush mango (*Irvingia gabonensis)*and red pepper (*Caspsicum frutescens*) are consumed in a high rate in Nigeria, it is important to ensure that foods consumed are of premium quality of zero or minimal levels of aflatoxin and other contaminants. This is because people are as healthy as the food they eat. In addition to this, food adds to the economical income of man but unacceptable aflatoxin levels could pose a threat to this opportunity. This work was designed to assay for the aflatoxin contamination associated with some foodstuffs sold in Calabar urban with a view to enlightening the consumers on the need for proper food handling.

Figure 1. *Irvingia gabonensis*

Figure 2. *Caspsicum frutescens*

Figure 3. *Colocynthis citrullus*

Figure 4. *Arachis hypogea*

Figure 5. *Zea mays*

2. Materials and Methods

2.1 Collection of Food Materials and Preliminary Preparation of Samples

One (1) kg each of the five different food items: *Zea mays* (maize), *Colocynthis citrullus,* (melon seed), *Caspsicum frutescens* (red pepper)*, Irvingia gabonensis* (bush mango) and *Arachis hypogea* (groundnut) were purchased in their dried forms from five different shops in Watt Market in Calabar urban in January, 2013. The food materials were transported to the Research Laboratory in the Department of Biochemistry, University of Calabar where all extraneous materials were removed. Each food material type was ground into powder and thoroughly mixed using a milling machine (MF-10 Basic IKA Werke, USA). Two (2) kg of each powdered

sample was pooled and packaged in a sterile airtight self-adhesive polyethene pouch, labeled properly and stored in a glass chamber maintained under UV air sterilizer pending aflatoxin analysis and identification within 12 hours.

2.2 Determination of Moisture Content

The moisture content of each sample was determined using Automated Moisture Analyzer (Sartorius MA 150, Germany) as described by the Association of Official Analytical Chemists (AOAC, 2006). The method is based on loss of moisture upon drying at 105 ^{o}C.

2.3 Preparation of Samples for Total Aflatoxin Analysis

For the extraction of non-coloured samples, five hundred (500) g each of the samples of *Zea mays* and *Colocynthis citrullus* was weighed into a clean neogen cup, 1L of distilled water was added and covered tightly. The samples were allowed to settle after which the top layer of the extract was filtered through a Whatman No. 1 filter paper (150mm). The filtrate collected was then concentrated in a rotary evaporator (RE 300B Serial No. R000010551, UK) *in vacuo* at 40 ± 2 °C and used for total aflatoxin analysis.

For the extraction of naturally coloured samples, five hundred (500) g each of the composite samples of *Capsicum frutescens*, *Irvingia gabonensis* and *Arachis hypogea* was weighed into a clean neogen cup, 1.25 L of methanol/tween water (70:30) was added, covered tightly and placed on a laboratory shaker set at 250 rpm for 3minutes 30seconds. The samples were allowed to settle and the top layer of the extract filtered through a Whatman No. 1 filter paper (150mm). The filtrate collected was then concentrated in the rotary evaporator *in vacuo* at 40 ± 2 °C and used for total aflatoxin analysis.

2.4 Determination of Total Aflatoxin

Total aflatoxin content was determined using the AgraQuant assay kit (Romer Labs® Order No.COKAQ1100, Singapore). The AgraQuant assay kit procedure is based on a direct competitive enzyme-linked immunosorbent assay (ELISA) method as described by Ayar et al. (2007). The total aflatoxin concentration was read at 450 – 630 nm.

2.5 Preparation of Samples for Identification and Quantification of Individual Aflatoxins

Two hundred (200) g each of the samples was weighed into a 1L conical flask and 500 ml of 80% methanol was added and blended for 3 minutes. The homogenized sample was transferred into a 1 L conical flask and let to settle for 10 minutes. The supernatant layer was transferred into a 100ml centrifuge tube and centrifuged at 3000 rpm for 10minutes using a refrigerated centrifuge (Eppendorf AG 22331 Hamburg, Germany). The supernatant of the spun sample was transferred into a 500 ml separating funnel, 50 ml of 10% NaCl solution was added, followed by 50 ml hexane. The mixture was shaken for 1 minute and the organic layer discarded. To the aqueous layer collected, 50 ml of dichloromethane was added and shaken for 2 minutes. The dichloromethane layer was then passed through anhydrous sodium sulphate to remove the traces of moisture. The dichloromethane extraction was repeated twice and the fractions were combined and evaporated in the rotary evaporator under vacuum at 40 ± 2 °C. This was transferred into a 5ml volumetric flask and made up to the mark with dichloromethane. Two (2) ml was drawn into a vial and evaporated in the rotary evaporator at 40 ± 2 °C. The residue was then taken for HPLC analysis.

2.6 Identification and Quantification of Individual Aflatoxins

The residue obtained from the concentrated extract was each dissolved in 1ml mobile phase and injected into the High Performance Liquid Chromatography (HPLC) column. A sensitive UV detector set at 365 nm was used to detect the aflatoxin types, which were eluted as sharp peaks within 4-7 minutes. The concentration of each aflatoxin type (AFB_1, AFB_2, AFG_1 and AFG_2) was determined using the peak area ratios as described by AOAC (2006) and Herzallah (2009). The absorbance of each aflatoxin species was read at 365 nm.

2.7 *Statistical Analysis*

The data obtained were subjected to analyses using the one-way analysis of variance (ANOVA) in SPSS statistical package; LSD was used for multiple comparisons. Statistical significance was accepted at 5% probability level or less.

3. Results

The *Irvingia gabonensis* sample had the highest (10.37 ± 0.19%), while *Zea mays* had the least (3.23 ± 0.03%) moisture content ($p < 0.05$). Low moisture content was also recorded in *Colocynthis citrullus* and *Arachis hypogea* (4.05 ± 0.03% and 4.48 ± 0.01% respectively); *Capsicum frutescens* had a moderate (6.75 ± 0.08%)

level of moisture. The results also showed significantly different contents of total aflatoxin among the food commodities. Again, *Irvingia gabonensis* had the highest (63.40 ± 1.79 µg/kg), while *Zea mays* had the least (3.20 ± 0.12 µg/kg) total aflatoxin content. The total aflatoxin content in the other samples were: 8.00 ± 0.06 µg/kg in *Capsicum frutescens,* 6.10 ± 0.06 µg/kg in *Colocynthis citrullus* and 3.40 ± 0.01 µg/kg in *Arachis hypogea* (Table1).

The aflatoxin concentrations in the five different foodstuffs evaluated correlated directly (r = 0.91) with the moisture contents in the foodstuffs (Figure 6).

Aflatoxin B_1 (AFB_1) concentration was significantly higher ($p < 0.05$) in *Irvingia gabonensis* (11.71 ± 0.10 µg/kg) than the other four samples. AFB_1 was 1.21 ± 0.01 µg/kg in *Capsicum frutescens,* 0.82 ± 0.02 µg/kg in *Colocynthis citrullus* but not detected in the *Zea mays* and *Arachis hypogea* samples. Similarly, *Irvingia gabonensis* had the highest ($p < 0.05$) AFB_2 content (2.43±0.05). Other results for AFB_2 were: 0.34 ± 0.01 µg/kg in *Capsicum frutescens,* 0.05 ± 0.01 µg/kg in *Colocynthis citrullus*, but not detectable in *Zea mays and Arachis hypogea.* The AFG_1 was significantly ($p < 0.05$) higher in *Capsicum frutescens* (3.73 ± 0.04 µg/kg) than in the other samples (1.85 ± 0.03 µg/kg in *Irvingia gabonensis,* 0.39 ± 0.01 µg/kg in *Colocynthis citrullus,* and 0.19 ± 0.01µg/kg in *Zea mays*); *Arachis hypogea* did not show any detectable level of AFG_1. The AFG_2 was highest in *Zea mays* (0.54 ± 0.01 µg/kg) ($p < 0.05$) compared to the other four samples (0.20 ± 0.20 µg/kg in *Capsicum frutescens*, 0.14 ± 0.01 µg/kg in *Irvingia gabonensis* and 0.09 ± 0.00 µg/kg in *Colocynthis citrullus*). Again, AFG_2 was not detected in *Arachis hypogeal* (Figure 7).

Table 1. Moisture and total aflatoxin contents of the food samples

Food samples	Moisture (%)	Total AFs (µg/kg)
Zea mays	3.23 ± 0.03	3.20 ± 0.12
Colocynthis citrullus	4.05 ± 0.03*	6.10 ± 0.06*
Capsicum frutescens	6.75 ± 0.08^{*,a}	8.00 ± 0.06*
Irvingia gabonensis	10.37 ± 0.19^{*,a,b}	63.40 ± 1.79^{*,a,b}
Arachis hypogea	4.48 ± 0.01^{*,a,b}	3.40 ± 0.01^{a,b,c}

* = significantly different from *Z. mays* at $p < 0.05$; a = significantly different from *C. citrullus* seed at $p<0.05$ b = significantly different from *C. frutescens* at $p < 0.05$; c = significantly different from *I. gabonensis* at $p<0.05$ Values are expressed as mean ± SEM, n=3 (where SEM = standard error of mean).

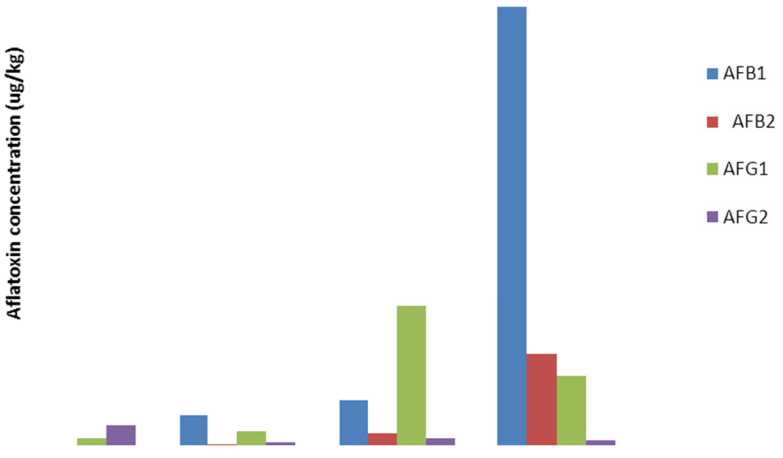


Figure 7. Aflatoxins B_1, B_2, G_1 & G_2 Concentration in the food samples

* = p<0.05 vs B_1; a = p<0.05 vs B_2; b = p<0.05 vs G_1; Values are expressed as mean ±SEM, n = 3 (where SEM = standard error of mean).

4. Discussion

This study assayed for the total aflatoxin content and concentration of four aflatoxin types (AFB_I, AFB_2, AFG_1, and AFG_2) in five locally available food commodities commonly used in food preparation in Nigeria. The food materials were: common maize (*Zea mays*), melon seed (*Colocynthis citrullus*), red pepper (*Capsicum frutescens*), bush mango (*Irvingia gabonensis*) and groundnut (*Arachis hypogea).* The results showed that all (100%) the food samples were contaminated with aflatoxin. It was further observed that except for bush mango and melon seeds, the levels of total aflatoxin in the food commodities were generally below the maximum allowable limits (10 µg/kg for pepper, and 4 µg/kg for others) specified by the European Commission (AESAN, 2011), which is also currently being used by the National Agency for Food and Drug Administration and Control (NAFDAC), in Nigeria. This observation agrees with the findings from other studies (Adebayo-Tayo, Onilude, Ogunjobi, Gbolagade, & Oladapo, 2006; Romagnoli, Meena, Gruppioni, & Bergamini, 2007; Russell & Peterson, 2007). However, levels of total aflatoxins below the levels found in the present study have also been reported in some foodstuffs (Zinedine et al., 2006).

The high levels of total aflatoxin found in some of the food materials used in this study may be attributed to some biotic and abiotic factors such as temperature, relative humidity, moisture content, food chemical composition, storage duration and insect attack. Studies by Simsek et al. (2002) showed that temperature favourable for aflatoxin production is 25-30 °C and relative humidity 97-99%. Also, Ross et al. (1979) had earlier reported that if both temperature (20-38^{o}C) and moisture (16-24%) are favourable for *Aspergillus flavus*, aflatoxin can be produced within 48 hours.

The level of moisture found in some of the food materials used in this study may be responsible for the growth of microorganisms with subsequent production of aflatoxins. Smith and Moss (1995) had shown that moisture determines whether microbes can colonize a substrate or not. The high moisture content in some of the food materials used in this study may be as a result of high humidity (>70%) and high temperature (>25%), which are characteristic of the tropical and sub-tropical regions of the world where Nigeria is located.

Also in this study, bush mango showed the highest level of total aflatoxin, followed by red pepper, melon seed and groundnut, while maize had the least. The trend in the occurrence of aflatoxins in these food samples is directly proportional to their moisture contents. It is therefore most probable that the differences in the moisture content of these food materials were responsible for the levels of total aflatoxin found in them. Maize which had the least moisture content also had the lowest total aflatoxin content. According to Sakai et al. (1984) and Schetzki and Ong (2001), substrate chemical composition is an important factor in aflatoxin contamination. Maize is a commodity with low lipid content while bush mango, melon seed and groundnut are oil seeds and contain high levels of lipid. It is relatively easier to dehydrate a food commodity with low lipid content than that with high lipid content. Hence, a higher level of moisture in the bush mango, melon seed and groundnut, leading to higher levels of total aflatoxins in them than in maize. In this study, a statistical correlation was found between aflatoxin concentration and moisture content of the food samples.

The results of this study also showed that AFB_1, AFB_2, AFG_1 and AFG_2 were unequally distributed in the five food samples examined. None of the aflatoxin types was detected in the groundnut, while the maize sample contained only AFG_1 and AFG_2. The bulk of the aflatoxin species were found in the bush mango and red pepper samples. This variation in occurrence e of the various aflatoxin types may be attributable to differences in the chemical composition of the food commodities used in the study. Such differences in food chemical composition leading to variation in aflatioxin content have been reported by Schatzki and Ong (2001). The food chemicals could interact with some environmental factors to bring about differences in the growth of microbes and in the subsequent formation of aflatoxins in the various commodities. These environmental factors include excessive moisture in the field, storage temperature, humidity, drought, variations in harvesting practices and insect infestations. These factors have been shown to determine the severity of mycotoxin contamination of foodstuffs (Hussein & Brassel, 2001).

It is however surprising that the groundnut samples used in this study did not show any detectable levels of the aflatoxin types assayed, especially AFB_1. A near similar observation was also made on the maize sample, which showed detectable levels of contamination only with the AFG_1 and AFG_2. Studies in other locations within and outside Nigeria have shown high levels of aflatoxin contamination in groundnut, the commonest being aflatoxin B_1 (Williams et al., 2004; Jimoh & Kolapo, 2008; Kamika, 2012; Oranusi &Olarewaju, 2013). Also, it has been demonstrated that maize is one of the most commonly contaminated foods with aflatoxins (Williams et al., 2004; Muthomi, Njenga, Gathumbi, & Cheminingwa, 2009; Oranusi & Olarewaju, 2013). For instance in Nairobi, Kenya, an outbreak of aflatoxicosis resulting in the death of 125 of the 317 (39.4%) cases reported was traced to

the contamination of maize and its products from retail shops (Muture & Ogana, 2005; Muthomi et al., 2009). However, Scussel and Baratto (1994) in their studies on the aflatoxin levels in grains including maize from Brazil did not find any detectable levels of the four aflatoxin types assayed in this work. These researchers attributed their findings to improved harvesting and storage practices, as well as weather during that particular year. It is most probable that these factors were responsible for the results obtained in this work moreso, since the various foodstuffs were collected in January, which in Calabar – Nigeria is a period of dry season when the relative humidity is generally low.

The high levels of contamination of bush mango with aflatoxins B_1, B_2, G_1 and G_2 observed in the present study agrees with the findings by Adebayo-Tayo et al. (2006) in some samples of bush mango (*Irvingia gabonensis*) examined in some markets in Akwa Ibom State, Nigeria. This was attributed to a number of factors including possible infection of the bush mango during cracking/ de-shelling to extract the cotyledons, drying, sorting as well as transportation. Akwa Ibom State shares a common boundary with Cross River State where this study took place, with similar climatic and socio-cultural features. It is therefore possible that all or some of these factors were responsible for the high levels of the aflatoxin types found in the bush mango samples in the present study.

Melon seed has also been reported (Bankole & Joda, 2004) to contain a level of AFB_1 similar to the results of this study. Also, this study obtained some levels of the four aflatoxin types in the red pepper. Studies by Aydin et al. (2007) had found aflatoxin B_1 in red pepper even though the levels in some of the samples collected were below the maximum tolerable limit. This could possibly be as a result of differences in the chemical composition of the food substrates. It is possible that different aflatoxin types have preference for certain foods depending on their chemical composition.

5. Conclusions

The results of our investigation demonstrate that the aflatoxigenic fungus (Aspergillus) aided by moisture and other factors is a common agent of contamination of foodstuffs marketed in Calabar. However, the levels of total aflatoxin in most of the food commodities are within acceptable limits and not all of them contain the B_1, B_2, G_1 or G_2 aflatoxins. The total and individual aflatoxin levels in the bush mango were generally above the maximum allowable limits of NAFDAC. Reduction of aflatoxin levels in food ingredients in Calabar and indeed Nigeria especially in bush mango should be a public health priority.

References

Adebayo-Tayo, B. C., Onilude, A. A., Ogunjobi,, A. A., Gbolagade, J. S., & Oladapo, M. O. (2006). Detection of fungi and aflatoxin in shelved bush mango seeds (*Irvingia* sp.) stored for sale in Uyo, Nigeria. *African Journal of Biotechnology, 5*, 1729-1732

AESAN. (2011). Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) in relation to the effect on the Spanish population of the derogation of national regulation on maximum allowed limits for aflatoxins B_1, B_2, G_1 & G_2 in food (pp. 1-16).

AOAC. (2006). Natural toxins. *In Official methods of analysis of AOAC International*. (18th ed., pp. 1-89). AOAC. International, Gaithersburg.

Aydin, A., Erkan, M. E., Baskaya, R., & Cfticioglu, G. (2007). Determination of aflatoxin B_1 in powdered red pepper. *Food Control, 18*, 1015-1018. http://dx.doi.org/10.1016/j.foodcont.2006.03.013

Ayar, A., Sert, D., & Con, A. A. (2007). Study on the occurrence of aflatoxin in raw milk due to feeds. *Journal of Food Safety*, 27, 199-207. http://dx.doi.org/10.1111/j.1745-4565.2007.00072.x

Bankole, S. A., & Joda, A. O. (2004). Effect of lemon grass (*Cymbopogon citratus*) powder and essential oil on mould deterioration and aflatoxin contamination of melon seeds (*Colocynthis citrullus* L.). *African Journal of Biotechnology, 3*(1), 52-59. http://dx.doi.org/10.5897/AJB2004.000-2009

Eaton, D. L., & Groopman, J. D. (1994). The toxicology of aflatoxins (pp. 392-426). New York: Academic Press. http://dx.doi.org/10.1016/B978-0-12-228255-3.50005-2

Herzallah, S. (2009). Determination of aflatoxins in eggs, milk, meat and meat products using HPLC fluorescent and UV detectors. *Food Chemistry, 114*(3), 1141-1146. http://dx.doi.org/10.1016/j.foodchem.2008.10.077

Hosseini, S., & Bagheri, R. (2012). Some major mycotoxins and their mycotoxicoses in nuts and dried fruits. *International Journal of Agronomy and Plant Production, 3*(5), 179-184.

Hussein, S. & Brassel, J. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals.

Toxicology, 167, 101-134. http://dx.doi.org/10.1016/S0300-483X(01)00471-1

Jimoh, K. O., & Kolapo, A. L. (2008). Mycoflora and aflatoxin production in market samples of some selected Nigerian foodstuffs. *Research Journal of Microbiology, 3*, 169-174. http://dx.doi.org/10.3923/jm.2008.169.174

Kamika, I. (2012). Determination of aflatoxins in peanut (*Arachis hypogea* L.) collected from Kinshasa, Democratic Republic of Congo and Pretoria, South Africa: a comparative review. Masters Degree Dissertation, University of South Africa.

Muthomi, J. W., Njenga, L. N., Gathumbi, J. K., & Cheminingwa, G. N. (2009). The occurrence of aflatoxins in maize and distribution of mycotoxin-producing fungi in Eastern Kenya. *Plant Pathology Journal, 8*(3), 113-119. http://dx.doi.org/10.3923/ppj.2009.113.119

Muture, B. N., & Ogana, G. (2005). Aflatoxin levels in maize and maize products during the 2004 food poisoning outbreak in Eastern Province of Kenya. *East African Medical Journal, 82*(6), 275-279. http://dx.doi.org/10.4314/eamj.v82i6.9296

Olayiwola, I. O., Oganah, B. C., Oguntona, C. R. B., Popoola, A. R., Sanni, S. A., & Sam-Wobo, S.O. (2013). *Discourse Journal of Agriculture and Food Sciences, 1*(5), 93-96.

Oranusi, S. U., & Olarewaju, S. A. (2013). Mycoflora and aflatoxin contamination of some foodstuffs. *International Journal of Biotechnology and Allied Fields, 1*(1), 9-18.

Romagnoli, B., Meena, V., Gruppioni, N., & Bergamini, C. (2007). Aflatoxins in spices, aromatic herbs, herb teas and medicinal plants marketed in Italy. *Food Control, 18*, 697-701. http://dx.doi.org/10.1016/j.foodcont.2006.02.020

Ross, I. J., Loewer, J. O., & White, G. M. (1979). Potential for aflatoxin development in low temperature drying systems. *American Society of Agricultural Engineers Paper 2*, 12-18.

Russell, R., & Peterson, M. (2007). Aflatoxin contamination in chilli from Pakistan. *Food Control, 18*, 817-820. http://dx.doi.org/10.1016/j.foodcont.2006.04.005

Sakai, T., Sugihara, K., & Kozuka, H. (1984). Growth and aflatoxin production of *Aspergillus parasiticus* in plant materials. *Journal of Hygienic Chemistry, 30*, 62-68. http://dx.doi.org/10.1248/jhs1956.30.2_62

Schatzki, T. F., & Ong, M. S. (2001). Dependence of aflatoxin in almonds on the type and amount of insect damage. *Journal of Agricultural and Food Chemistry, 49*, 4513-4519. http://dx.doi.org/10.1021/jf010585w

Scussel, V. M. & Baratto, W. R. (1994). Levels of aflatoxins in grains from Santa Catarina State, Southern Brazil. Proceedings of the 6th International working conference on stored-product protection (pp. 1051-1053).

Shepherd, G. S. (2008). Determination of mycotoxins in human food. *Chemical Society Review, 37*, 2468-2477. http://dx.doi.org/10.1039/b713084h

Simsek, O. Arici, M., & Demir, C. (2002). Mycoflora of hazelnut (*Corylus avellana* L.) and aflatoxin content in hazelnut kernels artificially infected with *Aspergillus parasiticus*. *Nahrung, 46*, 194-196. http://dx.doi.org/10.1002/1521-3803(20020501)46:3<194::AID-FOOD194>3.0.CO;2-D

Smith, J. E., & Moss, M. O. (1995). Mycotoxins Formation, Analysis and Significance (p. 148). Wiley & Sons, Chichester.

Smith, J. E., Solomons, G. L., Lewis, C. W., & Anderson, J. G. (1994). Mycotoxins in human nutrition and health. EC Directorate-General for Science, Research and Development, Agro-Industial Research Division, Brussels, Belgium.

SP-IPM. (2009). Advances in preventing and managing contaminants in food, feeds and the Environment. IPM Research Brief No. 7. SP-IPM Secretariat, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, pp. 40.

WHO. (2002). World Health Organisation Global strategy for food safety: safer food for better health, food safety programme, Geneva, Switzerland.

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., & Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences and interventions. *American Journal of Clinical Nutrition, 80*, 1106-1122.

Wu, F. (2007). Measuring the economic impacts of Fusarium toxins in animal feeds. *Animal Feed Science and Technology, 137*(3-4), 363-374. http://dx.doi.org/10.1016/j.anifeedsci.2007.06.010

Zinedine, A., Brera, C., Elakhdari, C., Catano, C., Debegnachi, F., Angelini, S., … Miraglia, M. (2006). Natural occurrence of mycotoxins in cereals and spices commercialized in Morocco. *Food Control, 17*, 868-874. http://dx.doi.org/10.1016/j.foodcont.2005.06.001

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Enhanced Antioxidant Capacity of Fresh Blueberries by Pulsed Light Treatment

Cheryl Rock[1], Senem Guner[2], Wade Yang[2], Liwei Gu[2], Susan Percival[2] & Esmeralda Salcido[1]

[1] California State University, United States

[2] University of Florida, United States

Correspondence: Cheryl Rock, California State University, United States. E-mail: cheryl.rock@csulb.edu

Received: February 1, 2015 Accepted: May 26, 2015 Online Published: August 31, 2015

doi:10.5539/jfr.v4n5p89 URL: http://dx.doi.org/10.5539/jfr.v4n5p89

Abstract

As a novel technology for food safety risk mitigation, pulsed light (PL) has been shown effective in surface decontamination of fresh blueberries in literature. However, little is known about the effects of PL on the antioxidant capacity and quality characteristics of fresh blueberries. Fresh blueberries from a local farm were treated with PL for 30, 60, 90 and 120 s. Results show that PL exposure enhanced the antioxidant activity (ORAC) and total phenolic content of fresh blueberries 50 and 48% respectively, relative to the control. Pulsed light also significantly increased the total anthocyanin contents, which may be due to the upregulation of Phenylalanine Ammonium Lyase (PAL) enzymes. There was no significant difference ($P \leq 0.05$) in the soluble solids, pH, titratable acidity, firmness, color and mass of the fresh blueberries within 120 s PL exposure. In conclusion, PL illumination enhanced the antioxidant capacity of fresh blueberries while maintaining other quality characteristics.

Keywords: nonthermal, phytochemical, anthocyanins, phenylalanine ammonium lyase, phenolics, flavonoids

1. Introduction

Blueberries (*Vaccinium corymbosum*) are a rich source of phenolic compounds, including quercetin, procyanidins and flavonoids (Sellappan et al., 2002). Anthocyanins are the major water-soluble flavonoids in blueberries, giving the red, purple and blue color to many fruits and vegetables (Espín et al., 2007). Additionally they are considered biologically active compounds exhibiting a wide range of health benefits, e.g., antioxidant (Cao et al., 1997), antifungal (Benkeblia, 2004), and anti-carcinogenic properties (Ames, 1983). It is well established that free radicals play an important role in the etiology of many diseases, such as cancer, vascular and neurodegenerative disease (Jacob, 1995). Espín et al. (2007) reported that anthocyanins and anthocyanin-rich berries or plant extracts exhibited a wide range of potential health benefits in both human and animal studies. These benefits are attributed to their free radical scavenging properties and their unique capacity in absorbing oxygen radicals, and chelating metal ions which are strong oxidizing agents of deoxyribonucleic acid (DNA) (Halliwell, 1995).

Among other fresh fruits, blueberries are regarded as the agricultural commodity rating the highest in antioxidant capacity, which warrants their potential health benefits and status as a functional food (U.S Highbush Blueberry Council, 2011). It has been estimated that U.S. per capita fresh blueberry consumption would increase 65% from estimated 0.756 kg in 2008 to 1.25 kg by 2015 (U.S Highbush Blueberry Council, 2009). This predicted increase in consumption is also accompanied with a demand for blueberry products with minimal changes in quality, nutritional profile and enhanced shelf life.

Pulsed light (PL) (100-1100 nm) is a novel food processing technology, which has shown effectiveness in decontaminating food surfaces, packaging materials and small berries and fruits including blueberries. To illustrate, Bialka and Demirci (2007) reported that maximum reductions in pathogenic bacteria such as *E. coli O157: H7* and *Salmonella* were 4.3 and 2.9 Log_{10} CFU/g respectively, in fresh blueberries using PL. Additionally, Krishnamurthy et al. (2008), have reported on the effects of continuous ultraviolet (UV) light (100-400 nm), primarily UV-C (100-290 nm), which has been predominantly used for its germicidal properties. However, the effects of PL on fresh blueberries antioxidant properties, is little known. The efficacy of PL can be attributed to its photothermal, photochemical and photophysical effects. We hypothesized that similar to UV light, PL exposure

might have enhancing effects on the antioxidant potency of fresh blueberries, besides its preservation on fruit quality. The most studied enzymes associated with secondary metabolite synthesis (anthocyanins and polyphenolics) in plants are PALs. Synthesis of these metabolites catalyzed by PAL is achieved through several pathways (Pentose Phosphate, Shikimate, Phenylpropanoid and Flavonoid), which may function synergistically (*Cao* et al., 2010) to counteract the damaging effects external stressors on cell components such as UV light. Therefore, the objective of this study was to investigate the effects of PL on the total antioxidant activity, polyphenolic contents, PAL enzyme, physiochemical attributes (pH, TA, SS), and quality (color, texture) of fresh blueberries.

2. Method

2.1 Sample Preparation

Early season (May - June) highbush blueberries were handpicked from a local farm (Gainesville, FL, USA) at commercial maturity stage from trees within the same grove. The fruits were stored at 4 °C for 1 day, and prior to PL treatment, they were sorted to remove impurities, washed and left to dry at ambient temperature (≈ 20-25 °C) for 1 day.

2.2 Pulsed Light Treatment

Blueberries (20 ±1 g) were placed in 70 mL aluminum dishes. Samples were transferred to the Xenon PL system model LH840 LMP HSG (Xenon Corporation, Wilmington, MA, USA) and treated at 3 pulses/s for 30, 60, 90 and 120 s at a distance of 13 cm from the lamp in stationary mode. The initial and final surface temperature of the samples was measured using a handheld infrared thermometer (Omega OS423-LS, Omega Technologies, Stamford, CT, USA).

2.3 Antioxidant Capacity

The blueberry samples were macerated using a homogenizer and extracted with a solvent mixture (20 mL) of acetone/water/acetic acid (70:29.9:0.1, v/v/v), followed by sonication (Zenith ultrasonic bath, 25 kHz, 1350 W, Zenith Manufacturing and Chemical Corp, Norwood, NJ, USA) for 30 min. The extracts were centrifuged (12,000 G [10,000 rpm], 45 min, 4 °C) and the supernatant was collected for oxygen radical absorbance capacity (ORAC) analysis as described by (Huang et al., 2002). Fifty µL ORAC phosphate buffer (PB) (75 mM ORAC-PB) and samples were added to a 96-well black plate (Fisher Scientific, Pittsburgh, PA, USA), followed by addition of 100 µL fluorescein (20 mM) solution. The mixture was incubated (37 °C, 10 min) before adding the peroxyl radical generator 2,2'azobis (2-amidinopropane) dihydrochloride (140 mM AAPH). The rate of fluorescence decay (485 nm excitation and 530 nm emissions for 1 min intervals for 40 min) was monitored by using a microplate reader (Spectra Max Gemini XPS Molecular Devices, Sunnyvale, CA, USA). The rate of florescent decay was then calculated using the area under the fluorescent decay using the Trolox standard curve. Antioxidant capacities were expressed as µmol trolox equivalents (TE)/g extracted samples.

2.4 Phenylalanine Ammonia Lyase (PAL) Enzyme

Under minimal light conditions, enzyme extraction was performed using untreated and PL treated blueberry samples (4 g) added to polyvinylpolypyrrolidone (PVP) (0.4 g) and homogenized into 16 ml of ice-cold borate buffer (50 mM borate; 400 µL/L β-mercaptoethanol; pH 8.5) (Velazquez et al., 2011). The homogenates were subsequently centrifuged (12,000 G [10,000 rpm], 30 min, 4 °C), and supernatants maintained at 4 °C and promptly assayed. To a 96 well clear plate, 235 µL borate buffer, 35 µL L-phenylalanine substrate solution (100 mM) and 80 µL of PAL extract were added. Using a microplate reader (Synergy HT, Biotek Instruments INC, Vermont, USA) spectrometric readings (290 nm) were taken before and after 1 h of incubation (40 °C) versus a reagent blank. The PAL activity (mg of t-cinnamic acid/ h) was calculated using cinnamic acid as a standard (0.02-0.10 mg/mL). L-Phenylalanine substrate solution was prepared in nanopure water before each assay.

2.5 Total Polyphenolics

Polyphenolic compounds were extracted as described by Kim *et al*. (2003). The sample mixture (20 g sample, 80% of 200 ml methanol, v/v) was sonicated for 30 min and centrifuged (12,000 G, 50 min, 4 °C). The solvent was evaporated using a Büchi Rotavapor 2025 (Gardner Denver Thomas, Inc., Niles, IL, USA) at 40 °C and the phenolic extracts were stored at -20 °C until further analysis. To a 96 well clear plate (Fisher Scientific, Pittsburgh, PA, USA), 12.5 µL of 2 M Folin-Ciocalteu phenol reagent was added to 50 µL of deionized distilled water (ddH_2O) and 12.5 µL of sample. After 5 min, 7% sodium carbonate (Na_2CO_3) solution (125 µL) was added to the mixture and incubated (90 min, 25 °C). Sample absorbance was measured at 750 nm using a microplate reader. A standard curve was developed using gallic acid and the concentration was expressed as mg gallic acid equivalents (GAE)/g fruit on a wet basis.

2.6 Total Flavonoids

A standard colorimetric assay (Kim et al., 2003) with slight modifications was used to quantify total flavonoid content. 25 µL of the sample along with 125 µL of ddH_2O was added to a 96 well clear plate. Subsequently, 7.5µL of 5% sodium nitrate ($NaNO_{2)}$ was added to the mixture and allowed to stand for 5 min. Fifteen microliters of 10% aluminum chloride ($AlCl_3$) was added to the mixture and incubated at ambient temperature for an additional 5 min. Following that, 50 µL of sodium hydroxide (1M, NaOH) was added to the mixture and immediately diluted by the addition of 27.5µL of ddH_2O. The absorbance of the mixture was measured at a wavelength of 510 nm against a reagent blank and compared to a catechin standard using a microplate reader. The total flavonoids was calculated as mg of catechin equivalents (CE)/g of fruit.

2.7 Total Anthocyanins

Two buffer systems (pH differential method) composed of potassium chloride (KCl) (pH 1.0, 0.025 M) and sodium acetate ($NaC_2H_3O_2$) (pH 4.5, 0.4 M) were used to perform anthocyanin analysis as described by Benvenuti et al. (2004). An aliquot of the blueberry extracts were simultaneously diluted (1:10) and adjusted to pH 1.0 and pH 4.5 using the respective buffers, and incubated at ambient temperature for 20 min. Absorbance was measured at each pH using a UV/VIS spectrophotometer (Beckman Coulter, Du 730, Life Sciences UV/VIS, Lawrence, KS, USA) at 510 and 700 nm respectively. Results were calculated using equations 1 and 2 below and expressed as mg cyanidin-3-glucoside (predominant anthocyanin) per g fruit using the corresponding MW (molecular weight [449.2]) and ε (molar absorptivity [26 900]). The acronyms A (absorbance) and DF (dilution factor) also denote the elements used in the equations below (Sellappan et al., 2002).

$$A = (A_{510\ nm}\text{-}A_{700\ nm})_{\ pH1.0} - (A_{510\ nm}\text{-}A_{700\ nm})_{\ pH4.5} \quad (1)$$

$$\text{Anthocyanins} = A \times MW \times DF \times 1000/(\varepsilon \times 1) \quad (2)$$

2.8 Color Analysis

The blueberry color was measured using a machine vision system (Nikon D200 digital camera housed in a light box [42.5 cm (W) × 61.0 cm (L) × 78.1 cm (H)] (Wallat 2002). The camera (focal light, 35 mm; polarization, 18.44 mm) was controlled by the LensEye software (Engineering and Cybersolutions Inc. Gainesville, FL, USA) and calibrated with a standard blue tile (L: 58.24; a*: -4.74; b*: -42.44) (Labsphere, North Sutton, NH, USA).

2.9 Texture Analysis

A texture analyzer (TA.XT Plus, Texture Technologies Corporation, Scarsdale, NY, USA) was utilized to evaluate the firmness of the blueberries by the compression test. The firmness was measured at the surface of the horizontally aligned blueberry to a total distance of 5 mm, using a TA-212 5/16" diameter probe (radius of curvature 13/64") and subjecting 50 kg of force a speed of 2.0 mm/s.

2.10 Soluble Solids (SS), pH and Titratable Acidity (TA)

Soluble solids were determined using a digital refractometer (Leica Mark II Abbe Refractometer, Buffalo, NY, USA) and expressed as $Brix^o$. The sample pH was measured using a pH meter (Fisher Scientific Accumet® Basic AB15/157, Pittsburgh, PA, USA). For TA, 10 g of blueberry samples was macerated into 50 mL of ddH_2O and titrated with sodium hydroxide (NaOH, 0.1 M) until a pH of 8.2 was obtained. The TA results were reported as % equivalent weight of malic acid/g fruit.

2.11 Statistical Analysis

The data obtained was analyzed using a statistical analysis system (SAS 9.1). Analysis of variance (one-way ANOVA) was performed and the significant differences in the means were separated using the Tukey's studentized range test. The data was tabulated as an average of triplicates ± standard deviation, and the level of significance was determined at $P \leq 0.05$.

3. Results and Discussion

3.1 Antioxidant Capacity

No significant differences ($P \leq 0.05$) were observed in ORAC values between control and PL treatments of 60 and 90 s. However, significant differences ($P \leq 0.05$) existed between control 30 and 120 s (Table 1). There was nearly a 75% increase in ORAC values (30 s) relative to control. Similarly, Wang et al. (2009) evaluated the antioxidant capacity and individual flavonoid compounds in blueberries exposed to UV-C illumination for 1, 5, 10 and 15 min. They found an increase in ORAC values compared to the control. Their values ranged from a low of 40.4 ± 3.2 (Control) to a high of 59.6 ± 2.0 µmol TE/g (UV-C illumination at 6.45 kJ m^{-2} which was lower than

our reported values. Our results indicated that PL is also capable of increasing the antioxidant content of blueberries, which was achieved at much shorter exposure times than those of UV-C illumination.

Table 1. Antioxidant capacity of pulsed light treated blueberries expressed as the oxygen radical absorbance capacity (ORAC)

Treatment	μmoL Trolox eq/g fresh weight
Control	119.2±4.82 b
PL 30 s	131.7±14.7 a
PL 60 s	121.4±14.7 b
PL 90 s	117.4± 5.14 b
PL 120 s	97.9±4.14 c

Means (in columns) with the same letter are not significantly different according to the Tukey's studentized Range Test $P \leq 0.05$. Data are expressed as mean ± standard deviation (SD).

3.2 Phenylalanine Ammonium Lyase (PAL)

In our study, the stimulatory effect of PL on PAL activity was investigated. The highest response was observed at a treatment time of 120 s (Figure 1). This may account for the increase in the antioxidant capacity and anthocyanin content of the blueberries as observed in our study. Previous research (Dixon & Paiva, 1995) indicates that PAL are photo induced by intense or UV light as a defensive response to attenuate its intensity which may damage photosynthetic cells. In this experiment, reduced enzymatic activity was observed at 90s. While an increase was expected as observed in blueberries treated with PL for 30 and 60s, the disparity in the enzyme activity could have been attributed to the enzymes biosynthesis pathways where tyrosine instead of phenylalanine could have been the main substrate resulting in another end product mainly coumaric acid instead of cinnamic acid (Cao et al., 2010).

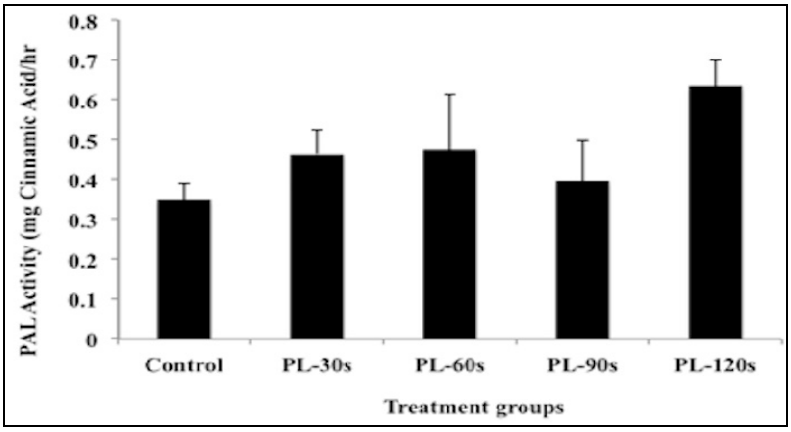


Figure 1. Stimulatory Effect of PL illumination on PAL activity

3.3 Total Phenolics

There was no significant difference ($P \leq 0.05$) in total phenolics between PL 30 s and control. A significant increase ($P \leq 0.05$) was found for PL 60 and 90 s compared to control (Table 2). The highest percentage increase relative to control was 48% at PL 90 s, indicating that PL had a significant ($P \leq 0.05$) enhancing effect on total phenolics within 90 s exposure. However, a decrease in total phenolics was observed at PL 120 s, which had a significantly ($P \leq 0.05$) lower value than PL 90 s, but comparable with control. Phenolic decrease in our experiments may be attributed to increased polyphenoloxidase (PPO) activity, which may have resulted in oxidation of these compounds as suggested by Moreno et al. (2007). According to Agarwal (2007), enzymes such as PPO function as antioxidants against oxidative stress, induced by the UV-B spectrum of PL, which may have upregulated their activity resulting in the oxidation of phenols to quinones. However, the fact that the total

phenolics at 120 s was not significantly different ($P \leq 0.05$) from control, suggested that PL did not have a degradative effect on the total phenolic content. Similar trends were observed by Wang et al. (2009). They found an increased level of total phenolics proportionate to the time of UV-C exposure. They reported a low of 3.12 ± 0.06 (control) to a high of 4.97 ± 0.09 mg GAE/g at the energy strength of 2.15 kJ m^{-2}.

3.4 Total Flavonoids

The flavonoid content significantly ($P \leq 0.05$) increased in all PL treated samples after 30 s, 60 s, 90 s and 120 s of PUV exposure as compared to the control (Table 2). Our values ranged from a low of 0.147 ± 0.01 mg/CE/g fruit (Control) to a high of 0.197 ± 0.02 mg/CE/g fruit (PUV 30s). However, although there was an increase in flavonoid content, there were no significant ($P \leq 0.05$) differences observed among the samples treated with PUV at 60s, 90s and 120s, showing that exposure after 30s did not significantly increase flavonoid content. The main underlying mechanism for this phenomenon was explained by Wang et al. (2009). They mentioned that at high doses of UV light might result in too much stress resulting in injury consequently inhibiting further flavonoid synthesis.

3.5 Total Anthocyanins

It was observed that PL stimulated an increase in blueberry anthocyanins, which ranged from a low of 0.738 mg/g ± 0.27 (control) to a high of 0.962 mg/g±0.24 (PL 120 s) (Table 2). Significant differences ($P \leq 0.05$) were observed in all PL treated samples relative to control, however, no significant differences were observed among the samples PL treated for 30, 60 and 90 s. As alluded to previously, PAL enzymes are associated with the biosynthesis of anthocyanins. This provides direct evidence as to why there was an increase in the anthocyanin content of blueberries as observed in our study. According to researchers (Heredia & Cineros-Zevallos, 2009), the upregulation of polyphenolic compounds such as anthocyanins may be a major defense mechanism inducible in blueberries due to PL illumination as an external stressor.

Table 2. Effect of pulsed light on total phenolics, flavonoids and anthocyanin content in blueberries

Treatment	Phenolics (mg/GAE/g Fruit)	Flavonoids (mg/CE/g Fruit)	Anthocyanins (mg/g Fruit)
Control	2.08±0.84 a	0.147±0.01 c	0.738±0.28 c
PUV 30 s	2.46±1.42 a	0.196±0.02 a	0.916±0.16 b
PUV 60 s	3.05±0.22 b	0.180±0.01 b	0.882±0.11 b
PUV 90 s	3.07±0.47 b	0.183±0.02 b	0.851±0.13 b
PUV 120 s	2.24±0.44 a	0.190±0.02 b	0.962±0.24 a

Means (in columns) with the same letter are not significantly different according to the Tukey's studentized Range Test $P \leq 0.05$. Data are expressed as mean ± standard deviation (SD).

3.6 Soluble Solids

Although the mean SS in blueberries ranged from a high of 11.67 °Brix±0.04 at PL 30 s to a low of 10.86 °Brix±0.04 at PL 60 s (Table 3), there was no significant difference ($P \leq 0.05$) of SS among all PL treatment groups compared to control. This may suggest that PL did not have an effect on SS metabolism, which may also vary according to blueberry cultivar. Perkins-Veazie et al. (2008) also reported that exposure to UV-C light did not affect SS content, but slight variations may be attributed to cultivar and storage conditions.

3.7 pH

A stable pH is very important to blueberries during processing and post-processing handling, as its hue and color is highly dependent on the pH change. This is because anthocyanin pigments may undergo reversible transformations with the change of pH (Tomas-Barberan & Espin, 2001). Table 3 shows the average pH values after the blueberries were PL treated compared to control. The pH value ranged from a low of 3.07 ± 0.02 at PL 90 s to a high of 3.21 ± 0.04 at PL 120 s. However, in general there was no significant difference ($P \leq 0.05$) in pH value among the PL treated blueberries and no difference either compared to control. This indicates that PL exposure up to 120 s did not have an effect on the pH of fresh blueberries. A similar trend was observed in a study performed by Perkins-Veazie et al. (2008). They reported that after the UV-C exposure (1-15 min, 8 cm from UV lamp), there was a slight increase in blueberry pH values, but there was no significant effect ($P \leq 0.05$).

3.8 Titratable Acidity

The TA values in Table 3 ranged between 0.40% ± 0.13 at PL 120 s and 0.47% ± 0.15 in the control. Our results were comparable with those of Perkins-Veazie et al. (2008), who reported 0.44 and 0.54% malic acid equivalents in the Collins and Bluecrop variety, respectively, after UV-C treatments. Our results showed no significant effect ($P \leq 0.05$) of PL on TA even after exposure for 120 s, which may imply that PL (within 120 s) had no degradative effects.

3.9 Texture Analysis

As an important rheological parameter for textural quality, firmness has been shown in literature as a standard trait to reflect the quality of fresh fruits and vegetables. As shown in Table 3, the firmness values of PL treated vs. untreated (control) blueberries showed no significant difference ($P \leq 0.05$). The firmness values ranged from a low 8.82 ± 0.05 Newtons (N) in blueberries PL treated for 120 s to a high of 10.29±0.04 N in untreated blueberries (control). Our results suggest that PL treatment in the time range tested did not have an effect on the firmness of the blueberries. In contrast, a study by Silva et al. (2005) investigating the variations in the physiochemical (pectin, pH, TA, fiber, skin toughness) observed differences in the firmness in several cultivars of blueberries. They found that the texture ranged from a high of 7.28 N to a low of 3.58 N in the Climax and Jersey cultivars, respectively. Their values were lower than those reported in our study, which may be attributed to varietal differences.

Table 3. Physiochemical properties of pulsed light treated blueberries.

Treatment	pH	Titratable acidity (% malic acid)	Soluble solids (°Brix)	Firmness (Newtons)
Control	3.16±0.06 a	0.48±0.15 a	11.6±0.5 a	10.30±0.03 a
PL 30 s	3.21±0.04 a	0.41±0.02 a	11.7±0.5 a	8.92±0.04 a
PL 60 s	3.13±0.04 a	0.41±0.11 a	10.9±0.2 a	9.32±0.05 a
PL 90 s	3.07±0.02 a	0.42±0.10 a	10.9±0.1 a	9.51±0.03 a
PL 120 s	3.21±0.03 a	0.40±0.13 a	11.4±0.4 a	8.83±0.05 a

Means (in columns) with same letter not significantly different according to the Tukey's Studentized Range Test $P \leq 0.05$. Data is expressed as mean ± standard deviation (SD).

3.10 Color Analysis

Significant differences ($P \leq 0.05$) were observed among all treatment groups in the degree of lightness (L*), greenness (a*), and blueness (b*) values compared to the control (Table 4). The L* values indicated that the blueberries darkened in color after prolonged PL exposures. No significant differences ($P \leq 0.05$) were observed between those treated for 30 s and 60 s relative to control. However, those treated for 90 and 120 s (L*=17.23 ± 1.94, 15.50 ± 1.75 respectively), were darkest in color as compared to control (L*=20.51 ± 1.93) and other treatment groups. Overall, our results showed that PL decreased b* values (i.e., more dark blue) of the samples as compared to the control. A similar result was observed by Moreno et al. (2007) who investigated the effects of ionizing radiation (0, 1.1, 1.6 and 3.2kGy) on blueberry color. With a higher dosage of irradiation, there was a decrease in the b* values resulting in darker fruit. They hypothesized that the darkening of the fruit color in the treated blueberries may be attributed to co-pigmentation where anthocyanins form complexes with flavonoid compounds, causing an increase in color intensity. As previously mentioned, other associative mechanisms during treatment could be the activation of enzymes (PAL) in the pentose phosphate, shikimate, phenylprenoid and flavonoid pathways, associated with the synthesis of anthocyanins that could act as UV screens to reduce the damaging effects of UV on the genetic materials in plant tissues (Tomas-Barberan & Espin, 2001). Our results showed that there was an increase in PAL enzyme activity, indicated by the accumulation of cinnamic acid after PL treatments (Figure 1). These results suggest that exposure to PL might have resulted in the darkening of the fruits.

Table 4. Effect of pulsed light treatment on blueberry color

Treatment	Lightness (L-)	Blueness (-b)	Greenness (-a)
Control	20.51±1.93 a	-7.29±0.93 c	-5.25±0.30 b
PL 30 s	20.40±1.56 a	-6.76±0.39 c	-5.46±0.29 b
PL 60 s	19.38±2.54 a	-6.12±1.12 b,c	-5.36±0.16 b
PL 90 s	17.23±1.94 b	-5.04±1.02 a,b	-5.30±0.17 b
PL 120 s	15.50±1.75 b	-4.35±0.51 a	-4.52±0.69 a

Means (in columns) with same letter not significantly different according to the Tukey's Studentized Range Test $P \leq 0.05$. Data are expressed as mean ± standard deviation (SD).

3.11 Temperature Rise

Novel technologies such as PL have been used as an alternative to thermal processing of foods (Oms-Oliu et al., 2010). It is well known that foods treated with high temperatures are susceptible to thermal degradation, which may result in undesirable changes to their organoleptic and physiochemical properties. For PL, when the exposure time is short (e.g., seconds), temperature rise during the treatment is low, because the photothermal effect of PL is minimal; however, prolonged exposure (e.g., minutes) would result in temperature increase of the product (Shriver et al., 2011; Yang et al., 2011) as the photothermal effect is intensified and is attributed to the infrared portion of the PL spectra.

According to Table 5, the surface temperature ranged from a low of 22.5 °C ± 0.7 in untreated (control) blueberries to a high of 35.5 °C ± 1.2 in PL treated blueberries for 120 s. It is noted that there was a few seconds delay before the temperature reading, when the sample was removed from the treatment chamber upon completion of the PL treatment, so the instantaneous surface temperature could possibly be a bit higher. It was observed that the surface temperature was not significantly different ($P \leq 0.05$) among control, PL 30 and 60, but significantly different after 90 s (Table 5).

Temperature rise to between 45 °C and 54 °C has been reported to improve sensory and nutritional quality of some horticulture products like tomato (Lurie & others, 2006; Rajchl et al., 2009) or low-temperature storage quality (Vlachonasios et al., 2001) without damage to the product. In contrast, the recorded surface temperature of blueberries after 120 s PL exposure generally did not exceed 54 °C, so there should not be any negative impact of the temperature rise on the blueberry quality, although no corresponding tests were conducted to verify such an inference in this study.

3.12 Weight Loss

Pulsed light treatment up to 120 s was found to cause no weight loss of the blueberries (Table 5). The initial mass was 20 ± 1 g for each of the samples, and no mass changes were detected on the balance (accurate to 1 g) for PL 30 to 120 s. Since the evaporation of water from the fruit to the environment is the predominant cause for weight loss during processing (Yang et al., 2010; Duan et al., 2011), the temperature of the product needs to be high enough to initiate the phase change of water from liquid to vapor, before it could escape from the food matrix. As mentioned earlier, the temperature rise of the fresh blueberries after PL treatments up to 120 s was not significant in this study. This explains why no mass changes were recorded in this study.

Table 5. Effect of pulsed light on the surface temperature and mass of fresh blueberries. The initial mass (control) was set to 20 g with triplicate samples

Treatment	Temperature (^{o}C)
Control	22.5±0.7 b
PL 30 s	23.0±0.4 b
PL 60 s	25.1±0.3 b
PL 90 s	32.7±0.7 a
PL 120 s	35.5±1.2 a

Means (in columns) with same letter not significantly different according to the Tukey's Studentized Range Test $P \leq 0.05$. Data are expressed as mean ± standard deviation (SD).

4. Conclusion

Pulsed light exposure up to 120 s had an enhancing effect on the ORAC and total phenolic content of fresh blueberries, without degradative effects on other quality characteristics. The ORAC values and total phenolic content increased by nearly 75% and 48%, respectively, relative to control. Anthocyanin production can be easily elicited and peaked within 30 s PL exposure and then level off for longer exposures up to 120 s. There was no significant difference ($P \leq 0.05$) in the SS, pH, TA, texture, color, and weight loss of the fresh blueberries up to 120 s PL exposure compared to control. The highest temperature rise was within 35.5 °C at 120 s PL exposure. The increase antioxidant capacity and increase in polyphenolics may be attributed to the stimulatory effect of PL on PAL.

References

Agarwal, S. 2007. Increased antioxidant activity in *cassia* seedlings under UV-Bradiation. *Biologia Plantarum, 51*(1), 157-160. http://dx.doi.org/10.1007/s10535-007-0030-z

Ames, B. N. (1983). Dietary Carcinogens and Anticarcinogens. *Science, 221*(4617), 1256-1264. http://dx.doi.org/10.1126/science.6351251

Benkeblia, N. (2004). Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium sativum*). *Lebensmittel-Wissenschaft und-Technologie, 37*(2), 263-268. http://dx.doi.org/10.1016/j.lwt.2003.09.001

Benvenuti, S., Pellati, F., Melegari, M., & Bertelli, D. (2004). Polyphenols, Anthocyanins, Ascorbic Acid, and Radical Scavenging Activity of *Rubus*, *Ribes* and *Aronia*. *Journal of Food Science, 69*(3), 164-169. http://dx.doi.org/10.1111/j.1365-2621.2004.tb13352.x

Bialka, K., & Demirci A. (2007). Decontamination of *Escherichia coli 0157:H7* and *Salmonella enterica* on Blueberries Using Ozone and Pulsed UV-Light. *Journal of Food Science, 72*(9), 391-396. http://dx.doi.org/10.1111/j.1750-3841.2007.00517.x

Cao, G., Sofic, E., & Prior, R. (1997). Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biology & Medicine, 22*(5), 749-760. http://dx.doi.org/10.1016/S0891-5849(96)00351-6

Cao, S., Hu, Z., Zheng, Y., & Lu, B. (2010). Effect of BTH on Anthocyanin Content and Activities Related to Enzymes in Strawberry after Harvest. *Journal of Agricultural Food Chemistry, 58*, 5801-5805. http://dx.doi.org/10.1021/jf100742v

Duan, J., Wu, R., Bernadine, S., & Zhao Y. (2011). Effect of edible coatings on the quality of fresh blueberries (Duke and Elliot) under commercial storage conditions. *Postharvest Biology and Technology, 59*, 71-79. http://dx.doi.org/10.1016/j.postharvbio.2010.08.006

Espín, J. C., García-Conesa, M. T., & Tomás-Barberán, F. A. (2007). Nutraceuticals: Facts and Fiction. *Phytochemistry, 68*, 2986-3008. http://dx.doi.org/10.1016/j.phytochem.2007.09.014

Dixon, R., & Paiva, N. (1995). Stress-Induced Phenylpropanoid Metabolism. *The plant cell, 7*, 1085-1097. http://dx.doi.org/10.2307/3870059

Halliwell, B. (1995). Antioxidant characterization: Methodology and mechanism. *Biochemical Pharmacology, 49*(10), 1341-1348. http://dx.doi.org/10.1016/0006-2952(95)00088-H

Heredia, J., & Cineros-Zevallos, L. (2009). The effect of exogenous ethylene and methyl jasmonate on PAL activity, phenolic profiles and antioxidant capacity of carrots under different wounding intensities. *Post Harvest Biology & Technology*, *51*, 242-249. http://dx.doi.org/10.1016/j.postharvbio.2008.07.001

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J., & Deemer, E. (2002). Development and Validation of Oxygen Radical Absorbance Capacity Assay for Lipophilic Antioxidants using Randomly Methylated B-Cyclodextrin as Solubility Enhancer. *Journal of Agricultural Food Chemistry, 50*, 4437-4444. http://dx.doi.org/10.1021/jf0201529

Jacob, R. A. (1995). The integrated antioxidant system. *Nutrition Research, 15*(5), 755-766. http://dx.doi.org/10.1016/0271-5317(95)00041-G

Krishnamurthy, K., Irudayaraj, J., Demirci, A., & Yang, W. (2008). UV Pasteurization of Food Materials. *Food Processing Operations Modelling: Design and Analysis*, 281-302.

Kim, D., Chun, O., Kim, Y., Moon, H., & Lee. C. (2003). Quantification of Phenolics and Their Antioxidant

Capacity in Fresh Plums. *Journal of Agricultural and Food Chemistry, 51*, 6509-6515. http://dx.doi.org/10.1021/jf0343074

Lurie, S., Laamim, M., Lapsker, Z., & Fallik, E. (2006). Heat treatment to decrease chilling injury in tomato fruit. Effects on lipids, pericarp lesions and fungal growth. *Physiologia Plantarum, 100*(2), 297-302. http://dx.doi.org/10.1034/j.1399-3054.1997.1000211.x

Moreno, M., Castell-Perez, M., Gomes, C., Da Silva, P., & Moreira, R. (2007). Quality of Electron Beam Irradiation of Blueberries at Medium Dose Levels (1.0 - 3.2kGy). *LWT-Food Science Technology, 40*(7), 1123-1132. http://dx.doi.org/10.1016/j.lwt.2006.08.012

Oms-Oliu, G., Martin-Belloso, O., & Solvia-Fortuny, R. (2010). Pulsed Light Treatments for Food Preservation. A Review. *Food Bioprocess Technology, 3*(1), 13-23. http://dx.doi.org/10.1007/s11947-008-0147-x

Perkins-Veazie, P., Collins, J., & Howard L. (2008). Blueberry Fruit Response to Postharvest Application of Ultraviolet Radiation. *Postharvest Biology and Technology, 47*, 280-285. http://dx.doi.org/10.1016/j.postharvbio.2007.08.002

Rajchl, A., Cizkova, H., Voldrich, M., Jiruskova, M., & Sevcik, R. (2009). Evaluation of shelf life and heat treatment of tomato products. *Czech Journal of Food Science, 27*, S130-133.

Sellappan, S., Akoh, C., & Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *Journal of Agricultural Food Chemistry, 50*(8), 2432-2438. http://dx.doi.org/10.1021/jf011097r

Shriver, S., Yang, W., Chung, S-Y., & Percival, S. (2011). Pulsed ultraviolet light reduces Immunoglobulin E binding to Atlantic white shrimp (*Litopenaeus setiferus*) extract. *International Journal of Environmental Research*. *Public Health, 8*, 2569-2583. http://dx.doi.org/10.3390/ijerph8072569

Silva, J., Marroquin, E., Matta, F., Garner, J., & Stojanovic, J. (2005). Physiochemical, Carbohydrate and Sensory Characteristics of Highbush and Rabbiteye Blueberry Cultivars. *Journal of the Science of Food and Agriculture, 85*(11), 1815-1821. http://dx.doi.org/10.1002/jsfa.2083

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Risk Factors and Control Measures for Bacterial Contamination in the Bovine Meat Chain: A Review on *Salmonella* and Pathogenic *E.coli*

Eugène Niyonzima[1,2], Martin Patrick Ongol[2], Anastase Kimonyo[2], Marianne Sindic[1]

[1]University of Liège – Gembloux Agro Bio – Tech. Laboratory of Agro-food Quality and Safety. Passage des Déportés 2, 5030 Gembloux, Belgium

[2]University of Rwanda – College of Agriculture, Animal Sciences and Veterinary Medicine, School of Food Science and Technology. PO Box 3900 Avenue de l'Armée, Kigali, Rwanda

Correspondence: Eugène Niyonzima, University of Liège – Gembloux Agro Bio – Tech. Laboratory of Agro-food Quality and Safety. Passage des Déportés 2, 5030 Gembloux, Belgium. Tel: 32-465-434-099. E-mail: eugeneniyo@yahoo.fr

Received: May 5, 2015 Accepted: July 30, 2015 Online Published: September 9, 2015

doi:10.5539/jfr.v4n5p98 URL: http://dx.doi.org/10.5539/jfr.v4n5p98

Abstract

Salmonella and pathogenic *Escherichia coli* are known to be the major bacterial agents responsible for human foodborne infections attributable to meat. A review of the specialized literature was carried out to identify the risk factors for bovine meat contamination by these pathogens from the cattle farm to meat consumption. Animal stress during transport to the slaughterhouse and the duration of the lairage period were identified as the key factors influencing the faecal excretion of *Salmonella* and pathogenic *E. coli* as well as cattle contamination prior to slaughter. At the abattoir level, hides and visceral contents appear to be the main sources of pathogenic bacteria that contaminate carcasses along the meat production chain. Finally, temperature abuses during distribution and meat contamination by infected handlers were found to be important contributors to the post-slaughter contamination of bovine meat. The findings of this study indicate that efficient management of human food borne infections attributable to bovine meat requires an integrated application of control measures involving all actors along the meat chain, namely slaughterhouses, meat processing plants, distributors and consumers.

Keywords: bovine meat, *Salmonella*, pathogenic *E. coli*, safety, risk factors

1. Introduction

Meat is consumed in different parts of the world as a source of animal proteins (Food and Agriculture Organization, 2013) and its chemical composition is favourable for the proliferation of a wide range of microbial populations which makes raw meat to be one of the vehicles of foodborne infections in humans (Doulgeraki, Ercolini, Villani, & Nychas, 2012; Scallan et al., 2011). The actual number of foodborne infections attributable to meat is difficult to assess accurately, principally because only a small proportion of illness cases is officially reported especially in developing countries. On the other hand, even within the reported cases, only a limited number allow identification of the food vehicle. Data from outbreaks constitute an interesting source of information to associate foodborne illness cases to their respective food vehicles and causal agents (Scallan et al., 2011). Greig and Ravel (2009), by using outbreak data published internationally from 1996 to 2005, noted that 12.7 % of reported foodborne outbreaks were attributable to bovine meat while 10.5 and 4.6 % were associated with chicken and pork, respectively. According to the same authors, *Salmonella* and pathogenic *Escherichia coli*, respectively, were identified as the causal agents in 32.9 and 34.6 % of foodborne outbreaks of bacterial origin attributable to beef.

Several studies have addressed the sources and potential control measures of bovine meat contamination by *Salmonella* and pathogenic *E. coli* at different stages of the meat chain i.e. primary production (Barkocy-Gallagher et al., 2003; Millemann, 2008), animal transportation to the slaughterhouse (Arthur et al., 2007; Barham et al., 2002) ; slaughtering operations (Antic et al., 2010); further processing (Carney et al., 2006; Scanga et al., 2000), distribution (Haileselassie, Taddele, Adhana, Kalayou, & Tadesse, 2013); cooking (Juneja, Eblen, & Ransom, 2001); however literature on bovine meat contamination and possible control measures considering the entire meat chain is still limited, probably because of the length and the complexity of the chain.

The contamination of meat by microbial pathogens can occur at any stage of the meat chain (Duffy, Cummins, Nally, O' Brien, & Butler, 2006; Rhoades, Duffy, & Koutsoumanis, 2009). Furthermore, the prevention or mastery of meat contaminations can be carried out at a stage of the chain different from the stages at which the contamination has occurred (Chen et al., 2012). Therefore, the food chain approach constitutes an efficient method to control bacterial contaminations of meat at consumption. The objective of this study was to review the existing knowledge on sources and risk factors for bovine meat bacterial contamination and provide an up to date view on control measures of the same by using a meat chain approach. The focus was put on *Salmonella* and pathogenic *E. coli,* as they are reported to be the leading causes of foodborne bacterial infections attributable to bovine meat (Greig & Ravel, 2009).

The literature search was undertaken first by reviewing literature in databases of peer-reviewed scientific publications, namely Scopus, PubMed and Google Scholar, using the following key words: cattle, bovine, beef, meat, safety, abattoir, slaughter, slaughterhouse, salmonella, salmonellosis, Escherichia coli, microbial (bacterial) contamination, hygiene, risk factors and distribution. Only articles in English or French were retained. On the other hand, books and other official publications dealing with the subject were consulted.

In this paper, an overview of the prevalences of *Salmonella* and pathogenic *E. coli* in bovine meat was carried out before tackling their risk factors along the bovine meat chain and discussing their respective control measures.

2. *Salmonella* and Pathogenic *E. coli* in Bovine Meat

Contaminated bovine meat is considered to be one of the sources of foodborne *Salmonella* and pathogenic *E. coli* infections in humans. The reported prevalence of *Salmonella* and pathogenic *E. coli* in bovine meat and products thereof varies from one product to another, but wide variability is also observed amongst different countries (Tables 1 and 2). The prevalences are globally lower in bovine carcasses at the slaughterhouse level and higher in meat cuts and minced beef at retail (EFSA and ECDC, 2013b; Stevens et al., 2006). This could be associated with bacterial contamination of meat that can occur during the transport of bovine carcasses from the slaughterhouse to the meat processing units, during cutting and mincing operations within meat processing plants and/or during the marketing of bovine meat in retail outlets. Niyonzima et al., (2013) reported a 2.2 log cfu increase in *E. coli* load between the slaughtering and marketing of beef at a commercial abattoir in Kigali city (Rwanda). Similarly, an increase in the prevalence and concentration of *Salmonella* and *E. coli* during the cutting and mincing of bovine meat is generally reported in meat processing plants (Hassanein, Fathi, Ali, & El-malek, 2011; Rhoades et al., 2009; Scanga et al., 2000). The variations in *Salmonella* and pathogenic *E. coli* prevalence amongst different countries could be attributed to a number of factors (including the farming systems and practices, slaughtering practices and post slaughter handling of meat as well as the general hygiene at different stages of the meat chain); which differ from one country to another. Higher prevalences are principally observed in developing countries, where poor hygienic conditions during slaughtering and meat handling are generally reported (Gashe & Mpuchane, 2000; Hassanein et al., 2011; Magwira, Gashe, & Collison, 2005; Stevens et al., 2006), whereas lower prevalence are mostly observed in developed countries where good hygienic practices are reported to be strictly followed and monitored along the meat chain (EFSA and ECDC, 2013; Vipham et al., 2012; Bosilevac et al., 2009).

The reported prevalence in different countries would be, however, not comparable because of differences in the sampling strategy and the analytical methods used. In some studies the number of analyzed samples amounted to thousands (Bosilevac et al., 2009; EFSA and ECDC, 2013b) , whereas in others only a very limited number of samples was analysed (Gashe & Mpuchane, 2000; Temellİ, Eyİgör, & Anar, 2012). Differences were also observed in sampling methodology, where the surface swabbed on bovine carcasses to detect pathogens or the weight of the meat samples analysed varied between different studies. In the studies conducted in European Union countries for example, the surface area covered by a carcass swab was reported to vary from 100 to 600 cm^2, while the weight of the meat sample analysed varied from 1 to 25 g (EFSA and ECDC, 2013b, 2014). Additionally, the analytical methods used to detect *Salmonella* and pathogenic *E. coli* in meat and meat products differed from one study to another. For *Salmonella,* a culturing method including a pre-enrichment phase in buffered peptone water, a selective enrichment and isolation followed by biochemical confirmation of isolates was the predominant method used (Bosilevac et al., 2009; Tafida et al., 2013). However, in other studies other detection methods such as PCR were used alone or in combination with a culturing method (Hassanein et al., 2011; Vipham et al., 2012). The same trend was observed in the methodology used to detect verotoxinogenic *E. coli* in meat and meat products (Temellİ et al., 2012). The prevalence of *Salmonella* or pathogenic *E. coli* in faeces, on hides or on bovine carcasses was reported to be higher when a PCR-based method was used than when the pathogen was detected by conventional culturing methods (Barkocy-Gallagher et al., 2003; Mainil &

Daube, 2005). This would be due to the fact that PCR methods consider the bacterial DNA and take into account all the bacterial cells, whether living or dead; whereas the culture method only consider living bacterial cells (Johansson et al., 2000).

Even if differences in the sampling strategy and analytical methods used in different studies do not allow an accurate comparison of the prevalence of pathogenic *E. coli* and *Salmonella* in meat amongst different countries, it appears that these two pathogens are detectable worldwide in significant proportions in meat in general, and particularly in bovine meat. According to the EFSA and ECDC report (2014) on zoonoses, data collected in 2012 from nine European Union member states showed prevalences of 1.3 and 0.1%, respectively, for verocytotoxigenic *E. coli* (VTEC) and VTEC O157 in fresh bovine meat. The prevalence of VTEC in meat from animal species other than bovines in the EU was not estimated, probably because of the non-representativeness of the data available. However, the prevalence of VTEC in different Member States in 2011 was reported to be higher in bovine meat compared to meat from other animal species.This could be probably due to the fact that the enteric carriage of pathogenic *E. coli* is mostly observed in cattle than in other animal species (Mainil & Daube, 2005). In Ireland, VTEC was detected in 1% of 291 bovine carcass samples, while no positive finding was reported from 134 sheep carcass samples (EFSA and ECDC, 2013b).

Table 1. The prevalence of *Salmonella* in fresh bovine meat

Product	% of positive samples	Number of tested samples	Country	References
Beef carcasses	42.8	236	Senegal	(Stevens et al., 2006)
	0.2	1275	Australia	(Phillips et al., 2001)
	6	250	Mexico	(Narvaez-Bravo et al., 2013)
	0	53	Poland	(EFSA and ECDC, 2014)
Butcher shop beef	20	25	Egypt	(Hassanein et al., 2011)
	9.9	354	Botswana	(Gashe & Mpuchane, 2000)
	2.4	370	Nigeria	(Tafida et al., 2013)
	1.02	2885	USA	(Vipham et al., 2012)
	0.8	274	France	(EFSA and ECDC, 2014)
	0.3	747	Germany	(EFSA and ECDC, 2014)
	1.1	117	Hungry	(EFSA and ECDC, 2014)
	0	26	Italy	(EFSA and ECDC, 2014)
	0.9	649	Netherland	(EFSA and ECDC, 2014)
Ground beef	20	25	Botswana	(Gashe & Mpuchane, 2000)
	11	88	Mexico	(Heredia et al., 2001)
	4.2	4136	USA	(Bosilevac et al., 2009)

A comparable observation was reported in the Czech Republic, where 0.3% of 1159 bovine carcasses were reported to be positive for VTEC while not a single positive sample was found in 1395 pig carcasses (EFSA and ECDC, 2013b). At the retail level, the Netherlands reported 0.3% of 702 bovine meat samples were positive for VTEC while no positive sample was found from 86 sheep meat samples (EFSA and ECDC, 2013b). In contrast, a higher prevalence of VTEC was reported in Spain, where 2.9% of 34 poultry samples were found to be positive for VTEC against a prevalence of 0.0% (n=45) in bovine meat (EFSA and ECDC, 2013b).

Table 2. The prevalence of pathogenic *E. coli* in fresh bovine meat

Product	% of positive samples	Number of tested samples	Country	References
Beef carcasses	0.4	250	Mexico	(Narvaez-Bravo et al., 2013)
	0.9	453	Belgium	(EFSA and ECDC, 2014)
	1.3	622	Czech Rep.	(EFSA and ECDC, 2014)
	5.7	315	Germany	(EFSA and ECDC, 2014)
	0	203	Romania	(EFSA and ECDC, 2014)
Butcher shop meat	10	20	Turkey	(Temellİ et al., 2012)
	11.1	27	Egypt	(Mohammed et al., 2014)
	5.22	134	Botswana	(Magwira et al., 2005)
	1.8	492	Germany	(EFSA and ECDC, 2013b)
	0	45	Spain	(EFSA and ECDC, 2013b)
	3.2	555	Netherland	(EFSA and ECDC, 2014)
Ground beef	3.76	133	Botswana	(Magwira et al., 2005)
	3.85	52	Turkey	(Temellİ et al., 2012)
	16.7	30	Egypt	(Mohammed et al., 2014)
	3.8	479	Germany	(EFSA and ECDC, 2013b)

The prevalence of *Salmonella* in bovine meat has been found to be low compared to meat from other animal species. In the European Union, during 2012, the prevalence of *Salmonella* in bovine meat and products thereof was reported to be 0.2% whereas in pig and broiler meat it was estimated to be 0.7 and 4.1%, respectively (EFSA and ECDC, 2014). The highest *Salmonella* prevalence observed in poultry meat could be attributed to the colonization of the reproductive tract of infected subjects by the pathogen that may increase the probability of *Salmonella* dissemination on carcasses under preparation through cross contamination (Gast, Guraya, Guard-Bouldin, Holt, & Moore, 2007). Although the prevalence of *Salmonella* in bovine meat seems to be relatively low, contaminated bovine meat remains a significant risk for *Salmonella* infection in humans, particularly for people consuming more beef than meat from other animal species. Additionally, the high protein and fat content of foods such as meat was reported to protect the bacterium against the gastric acidity (Birk et al., 2012; Blaser & Newman, 1982; Kothary & Babu, 2001). This suggests that the consumption of contaminated meat, even with a limited number of pathogens, would present a significant risk of infection and/or intestinal colonization in humans.

As for other bacterial pathogens, the minimum number of *Salmonella* capable of causing illness, is difficult to determine as it depends on a number of factors including (but not limited to) the food matrix, the host susceptibility and the virulence factors of the pathogen (McEntire, Acheson, Siemens, Eilert, & Robach, 2014). However, recent studies using outbreak data indicate that doses as low as 36 colony forming units can cause illness in humans (Teunis et al., 2010). This infective dose would be qualified as “low “comparatively to foodborne pathogens such as *Vibrio cholerae* that require doses as high as 10^4-10^8 cells to cause infection in humans (Kothary & Babu, 2001). The infective dose for pathogenic *E coli* is also known to be “low”. Coia (1998) reported contamination levels as low as 2 organisms per 25 grams in food and environmental samples incriminated in VTEC O157 outbreaks. Because of the low infective dose, the contamination limit for these pathogens has been fixed to the absence in 25g of meat preparations intended to be eaten raw (European Commission, 2005).

It is assumed that the level of microbial contamination of meat at the end consumer stage is function of contaminations acquired during different stages of meat preparation. Therefore, reducing the prevalence of foodborne infections such as *Salmonella* and verotoxinogenic *E. coli* attributable to bovine meat in humans requires integrated control measures involving all actors in the bovine meat chain from primary production to the final consumer.

3. Bacterial Contamination of Bovine Meat along the Production Chain

3.1 Preslaughter Contamination of Live Cattle

Salmonella infection is commonly reported in different animal species. Considering their adaptation to hosts, *Salmonella* serotypes are grouped in three categories: namely serotypes only pathogenic for humans like *S.* Typhi and *S.* Paratyphi; serotypes adapted to animal species such as *S.* Gallinarum, *S.* Dublin, *S.* Abortusequi, *S.* Abortusovis and *S.* Choleraesuis which are pathogenic for poultry, cattle, horses, sheep, and pigs respectively; and finally ubiquitous serovars like *S.* Typhimurium and *S.* Enteritidis adapted to humans and other animal species (Jay et al., 2005). In cattle, *Salmonella* infection can be clinically manifested by a wide range of symptoms including diarrhoea and possible dysentery, joint infections, pneumonia as well as abortions (Millemann, 2008). However, bovines may also carry *Salmonella* in their gastro-intestinal tract without any clinical symptom of the disease. In the latter case bovines are called asymptomatic carriers. In both infected and asymptomatic carriers, *Salmonella* can be excreted through the faeces for a relatively long period. Gopinath et al. (2012) reported that the faecal shedding of *Salmonella* in cattle may last up to 400 days.

As with *Salmonella*, asymptomatic carriage and faecal shedding of pathogenic *E. coli* are common in bovines of all ages; but clinical manifestations of the disease are mainly observed in young calves with 2 weeks to 2 months of age with diarrhoea as the main symptom (Alexa, Konstantinova, & Sramkova-Zajakova, 2011; Millemann, 2008). The duration of faecal shedding in cattle can last up to 19 weeks (Khaitsa et al., 2003). On a clinical basis, pathogenic *E. coli* strains are grouped in 3 classes namely those rarely associated to diseases either in animals or in humans (i.e VTEC-2), strains associated to disease in both animals and humans (i.e EHEC-2) and finally strains such as EHEC-1 and VTEC-1 reported to be highly infectious for humans but rarely in animals (Mainil & Daube, 2005).

The faecal shedding of *Salmonella* and pathogenic *E. coli* constitutes an important factor of cattle contamination. In fact, pathogens excreted in the faeces may contaminate the environment through which other cattle can acquire contamination and carry the bacteria in their digestive tract and/or on their hides (Rhoades et al., 2009). The contamination of live cattle destined for slaughter may occur at the farm level, during the transportation of bovines to the slaughterhouse or during the lairage period in the abattoir.

At the farm level, contaminated feed and water have been reported to be the main sources of *Salmonella* and pathogenic *E. coli* infections in cattle (Millemann, 2008). However, dissemination of the infection within the herd is mainly attributable to faecal excretion of the pathogens. The prevalence of pathogenic *E. coli* and *Salmonella* is generally reported to be higher on cattle hides than in the faeces. This is due to the fact that a single animal shedding the pathogen in its faeces may contaminate the hides of many other animals in the herd, either directly or via the ground and lairage fixtures (Small et al., 2002). In a study conducted on 200 steers and heifers in a large feed yard, Barham et al. (2002) reported an *E. coli* O157 prevalence of 18% on hides while its prevalence in faeces was as low as 9.5%. A similar relationship was reported by Barkocy-Gallagher et al. (2003), who detected *E. coli* O157:H7 on 60.6% of cattle presented for slaughter, while the faecal prevalence was 5.9%. As with VTEC, *Salmonella* prevalence was reported to be higher on cattle hides than in faeces. Barkocy-Gallagher et al. (2003) reported a *Salmonella* prevalence of 71% on the hides of feedlot cattle while a prevalence of only 4.3% was recorded from faecal samples of the same group.

The control of pathogenic *E. coli* and *Salmonella* infections on cattle farms includes the treatment of all carriers and infected subjects but also limiting the spread and severity of the disease. When the infection is identified early in the herd and few animals are affected, their isolation is an important measure to consider. Furthermore, faecal dejections from infected animals should be managed in a manner to avoid contamination of feed, water or livestock equipments. Antibiotic therapy, especially in subjects affected by salmonellosis, should be used cautiously as the emergence of *Salmonella* strains resistant to antibiotics commonly used in veterinary medicine is reported to be increasing (EFSA and ECDC, 2013a; Stevens et al., 2006). The treatment of *E. coli* and *Salmonella* infections in cattle herds has been thoroughly reviewed by Millemann (2008) and is not further developed in this paper.

The faecal shedding of pathogens from asymptomatic carriers constitutes a serious obstacle on the control of *Salmonella* and pathogenic *E. coli* infections in cattle; as shedders are not clinically identifiable and in most of times, not subjected to treatment. Traditionally, asymptomatic carriers can be detected through the culture of multiple faecal samples collected from suspected shedders during a relatively long period (Gopinath et al., 2012; Guy, Tremblay, Beausoleil, Harel, & Champagne, 2014). However, this approach presents a disadvantage of being logistically difficult to conduct and inefficient especially in carriers where the faecal shedding of *Salmonella* or pathogenic *E. coli* is intermittent (Edrington et al., 2004; Fitzgerald et al., 2003). As

an alternative to the cultural methods, serological methods that consist in the detection of antibodies specifically directed against some antigens expressed by the pathogen exist. An example is the measurement of immunoglobulins directed against O-antigens from *Salmonella* Dublin in the blood that was reported to be used as an indicator of *Salmonella* infection in cattle (Robertsson, 1984). However, further studies indicate that serological tests are indicative on the current and/or previous infection status of the subjects but not on their shedding status (Olopoenia & King, 2000). Therefore, considering the importance of the detection of shedders in the control of *Salmonella* and pathogenic *E. coli* infections in cattle farms and the weaknesses of the existing methods, it is recommended to develop more sensitive methods to detect shedding animals in the herd. Meanwhile, one should consider an approach consisting of serological screening followed up by a faecal culture of all seropositive animals to detect active carriers (Nielsen, 2013).

Animal stress is known to induce high levels of secretion of *Salmonella* and pathogenic *E. coli* in cattle faeces and increase the probability of contaminating healthy animals (Gopinath et al., 2012; Mainil & Daube, 2005). During their transport to the slaughterhouse cattle may be subjected to a number of stresses, including high stocking densities, long transport duration, abnormal temperatures, noise pollution and changes in the general environment that can significantly increase the number of shedders. Cattle can also be infected by pathogenic microorganisms from a contaminated truck that has not been properly cleaned and disinfected or by direct contact with infected animals embedded in the same truck. Similarly, contaminated transport trucks can be a source of infection for slaughterhouses and farms initially free of *Salmonella* or pathogenic *E. coli*. At the slaughterhouse level, cattle are kept in lairage before killing them. In Europe and the United States, cattle are generally slaughtered on the day of their arrival to the abattoir, while in other countries they are usually slaughtered the day after. In the latter case, the period of lairage allows animals to rest, rehydrate and recover from the stress of transport (Ferguson & Warner, 2008). During the period of lairage, cattle can be subjected to these same stress factors that increase the risk of contamination. On the other hand, in most cases the lairage is only cleaned at the end of the day and is therefore a potential source of contamination for cattle that can acquire an infection from contaminated animals or a soiled environment (Beach, Murano, & Acuff, 2002). Different authors have reported significant increases in pathogen prevalences on cattle hides during their transport and in the lairage period in the slaughterhouse. In a study conducted on 286 cattle, Arthur et al. (2007) reported that the prevalence of *E. coli* O157:H7 on hides increased from 50.3 to 94.4% between the time the cattle were loaded onto tractor-trailers at the feedlot and the time their hides were removed in the slaughterhouse. Similarly, Barham et al. (2002) reported an increase in *Salmonella* prevalence on cattle hides (from 6 to 89%) during the transport and lairage of 200 cattle, whereas the prevalence of *Salmonella* in the faeces of the same group increased from 18 to 46%.

A number of measures could contribute significantly to reducing the risk of bacterial contamination of cattle destined for slaughter in the preslaughter environment. At the farm level, cattle destined for slaughter should be clean and dry with no visible dirt on their hides (Antic et al., 2010). Any practice that can generate animal stress during transport, such as mixing cattle from different farms and over loading trucks should be avoided (Small & Buncic, 2009). Likewise, trucks should be cleaned and disinfected after each transport of cattle (Swanson & Morrow-Tesch, 2001). At the abattoir, the lairage period should be kept to a strict minimum. Heavily contaminated animals must be separated from the others and the lairage pens must be cleaned and disinfected at the end of each slaughtering day and monitored by visual and bacteriological control (Wong et al., 2002).

3.2 Contamination During the Slaughtering Process

In the abattoir, the cattle slaughtering process includes successive steps, namely: stunning, sticking, skinning, evisceration, carcass splitting, refrigeration and eventually cutting and deboning (Figure 1) that can contribute significantly to the overall microbial load of bovine carcasses and meat cuts. In this section, sources, risk factors and control measures for meat contamination by *Salmonella* and pathogenic *E. coli* throughout the cattle slaughtering process are reviewed and discussed.

3.2.1 Cattle Stunning

Stunning is an operation that aims to render animals destined for slaughter unconscious prior to sticking and bleeding. It allows suffering by the animals to be minimised during the slaughtering process, especially the sticking. Beside animal welfare considerations, stunning also makes the throat-slitting less hazardous for the operator (Food and Agriculture Organization, 2006). Although chemical and electrical stunning methods are allowed in domestic ungulates, mechanical stunning is the most commonly used stunning method in cattle (Gregory et al., 2000). The devices used for mechanical stunning can be of a penetrating or non-penetrating type. A number of studies have been conducted to address possible microbial contamination of meat during the stunning process. In one experimental study, Buncic et al. (2002) demonstrated that the use of a penetrating

captive bolt (PCB) in sheep presents a risk of microbial contamination for stunned animals through the stun wound. Marked organisms (*E. coli* K12 or *Ps. fluorescens*) were inoculated into the brains of sheep through the stun wound immediately after stunning by a cartridge-operated, penetrative captive bolt pistol. The marked organisms were found in blood, liver, lungs, spleen and lymph nodes and on the surface of inoculated animals. When the same pistol was then used to stun subsequent healthy sheep, marked organisms were found in the blood of 30% to 40% of the animal carcasses. Similar findings were reported by Daly et al. (2002) after inoculation of a marker strain of *Ps. fluorescens* into the central nervous system of cattle. Prendergast et al. reported dispersion of central nervous system tissues when a PCB was used for animal stunning.

Although the contamination of bovine carcasses by microorganisms introduced into the central nervous system during the stunning process by penetrating devices has been demonstrated experimentally, further studies are needed to assess the risk of such contaminations under commercial conditions. During the mentioned studies (Buncic et al., 2002; Daly et al., 2002), the levels of bacteria inoculated experimentally into the brain were relatively higher comparatively to the levels of bacteria commonly reported in slaughterhouses; suggesting that the risk of transmitting pathogens through the stun wound would be much lower under commercial conditions. However, as it known that *Salmonella* and pathogenic *E. coli* require low infective doses (Blaser & Newman, 1982; Coia, 1998) the risk should be considered as significant. Beside the possible contamination of cattle via the contaminated stunning gun, different authors reported regular cross contamination of hides in the stun box between stunned animals consecutively fallen in the same box via contaminated surfaces (Small & Buncic, 2009; Small et al., 2002) highlighting the need of a proper sanitation of the stun box.

As a control measure for food safety issues associated with the use of penetrating stunning devices in cattle, alternate stunning methods should be considered. The use of non-penetrating guns appears to be a good alternative. Nevertheless, potential problems associated with this type of gun, such as the frequent recovery before sticking, need to be resolved (EFSA, 2004). The use of electrical stunning seems to be another safer option (Anil et al., 2001). This method is used in different countries, namely New Zealand, Australia and the United Kingdom (Wotton, Gregory, Whittington, & Parkman, 2000); however, its high cost and some doubts about animal welfare associated with the ineffective use of this method need to be addressed (Heim, Löpfe, Mumford, & Speedy, 2007). Furthermore, the possibility of cleaning and disinfecting the stun box after stunning each animal should be considered to avoid cross contamination of hides from faecally soiled surfaces during the stunning process.

3.2.2 Cattle Sticking

Sticking is an operation that consists of severing the major blood vessels of the animal in order to extract the maximum amount of circulating blood during bleeding. Two sticking methods are generally used in cattle: thoracic and cervical sticking. Thoracic sticking includes a section of major blood vessels from the heart and allows rapid and complete bleeding, whereas during cervical sticking only vessels in the neck (carotid arteries and jugular veins) are cut and bleeding out is slower (Food and Agriculture Organization, 2006). The stick wound constitutes a channel that can allow the introduction of microbial contaminants into the carcass. The main source of contamination is the sticking knife, which can contaminate carcasses by direct transfer of bacteria from the transpierced skin but also by cross contamination if the knife is not sterilized between successive sticking operations.

In an experimental study (Mackey & Derrick, 1979), it was demonstrated that microbial contamination of bovine carcasses can occur during sticking. Marked strains of *E. coli*, *Cl. perfringens* and *Bacillus thuringiensis* were placed on a sticking knife before use. After the sticking operations, marked organisms were isolated from the internal organs, namely the heart, lung, spleen, liver and kidneys and from muscles. However, even if the potential for meat contamination from the sticking knife has been shown under laboratory conditions, the risk of such transfers, especially for pathogens like *Salmonella* and pathogenic *E. coli*, under commercial conditions seems to be quite low. Mackey and Derrick (1979) reported that in order to induce contamination of the deep tissues of a carcass a large inoculum of 10^{10} to 10^{12} bacteria was required, whereas the actual level of contamination generally encountered in slaughterhouses is many orders of magnitude less. In a study conducted on bovine hides at a beef slaughter plant in Ireland, hide contamination by *E. coli* O 157 was reported to be as low as 100 cfu per 100 cm^2 or less in 90.8% of 109 cattle(O'Brien et al., 2005). Comparable findings were reported in the USA, where 62.7% of 124 cattle were found to carry fewer than 100 cfu/100 cm^2 of *E. coli* O 157 (Rhoades et al., 2009). The concentration of *Salmonella* on cattle hides is also known to be relatively low. In a study conducted on 100 cattle at slaughter, Fegan et al. (2005) reported a prevalence of 68% with the highest concentration being 4.8 MPN per cm^2. Nevertheless, contaminated knives remain an important source of localised microbial contamination of the sticking wound (Rheault et al., 1999). Additionally, the sticking wound

can be contaminated by microorganisms from the environment, especially when exsanguination is performed on animals lying on the ground.

In order to avoid/prevent microbial contamination of bovine carcasses through the sticking wound, cattle should be bled out in a suspended position to prevent contamination from the slaughtering environment (Food and Agriculture Organization, 2006); two separate knives should be used for sticking (one for the skin and another for muscles) and they should be decontaminated in hot water at 82°C or by another method with equivalent effect after being used (Eustace et al., 2007); and finally, the sticking site should be trimmed if any microbial contamination is suspected (Rheault et al., 1999).

3.2.3 Hide Decontamination Treatments

Cattle hides constitute one of the main sources of carcass contamination by bacterial pathogens such as *Salmonella* and pathogenic *E. coli,* whereas the contamination of hides is generally acquired from faeces of colonised animals or indirectly from the soiled environment (Arthur et al., 2010). The contamination of carcasses from soiled hides occurs during the skinning process. A number of intervention strategies to reduce the bacterial load on cattle hides and consequently reduce the risk of carcass contamination during skinning operations, have been addressed by various authors. These include physical, chemical and biological treatments applied alone or in combination. In this section major hide decontamination treatments are reviewed and their effect on reducing the bacteriological load on cattle hides is discussed.

The reported physical decontamination treatments include hide washing with cold or hot water, steam sprayings and cattle dehairing. Washing cattle hides with water has been found to remove dirt from the hides but seemed to have a minimal effect on the bacterial load of treated hides. The study by Mies et al. (2004) showed that washing cattle with cold water for 2 minutes did not permit significant reductions in aerobic bacteria, coliforms and *E. coli* on the hides. However, raising the water temperature from 15 to 60 °C reduced the load of aerobic bacteria by 0.5 logarithmic units (Bosilevac et al., 2005).

The effect of steam sprayings in decontaminating cattle hides was studied under laboratory conditions by McEvoy et al. (2003). These authors, by treating cattle hide pieces with steam at subatmospheric pressure during 1 to 20 seconds, reported reductions in total viable bacteria on treated hides of 2.9 to 3.9 logarithmic units after a treatment at 80°C, while similar treatments at 75°C reduced total viable bacteria counts by only 1.9 to 2.6 log units. In another study, reductions of inoculated *E. coli* O157 by 4.2 to 6.0 log units were reported after spaying cattle hides by steam at 80°C during 10 to 20 seconds (McEvoy, Doherty, Sheridan, Blair, & McDowell, 2001).

Dehairing cattle can be carried out by clipping the hide or using chemicals. The study by Small et al.(2005) showed that dehairing cattle hides with a clipper does not reduce the aerobic bacterial load on the hides, probably because of dust generation and subsequent dispersal of the bacteria. However, treating previously clipped hides with other physical or chemical hide decontamination methods was found to afford bacterial load reductions significantly higher than these obtained on unclipped hides (Baird et al., 2006). The use of chemical dehairing has been studied by Castillo et al. (1998). These authors, using a solution of sodium sulphide, water rinses, and hydrogen peroxide under laboratory conditions, achieved significant reductions in *E. coli* O157:H7 and *S.* Typhimurium previously inoculated on bovine hides (more than 4 logarithmic units). However, in a study conducted on 240 cattle in a commercial beef processing plant, Nou et al. (2003), using a similar method on cattle immediately after stunning, reported a reduction in *E. coli* O157 prevalence on the treated cattle hides from 88 to 67% without any significant reduction in aerobic bacteria or *Enterobacteriacea* populations.

A wide range of chemical antimicrobials have reportedly been used in hide decontamination treatments. These include organic acids (Mies et al., 2004), commercial detergents and disinfectants (Baird et al., 2006; Small et al., 2005), ozonated and electrolysed water (Bosilevac et al., 2005), and combinations of different chemicals (Carlson et al., 2008). However, their efficacy in reducing the bacterial load on cattle hides has been found to be dependent on a number of experimental factors such as the mode of application, the product concentration and temperature, the duration of exposure and the target microbial species. Limited studies have addressed the effect of chemical antimicrobials on pathogens such as *Salmonella* and *E. coli* present on cattle hides destined to slaughter. Nevertheless, organic acids appear to be the most studied group of chemical (Loretz, Stephan, & Zweifel, 2011). The effect of organic acid sprays in reducing *Salmonella* load on cattle hides was studied by Mies et al. (2004). These authors sprayed cattle hides with different concentrations (2 to 6%) of acetic and lactic acids and noted reductions in *Salmonella* Typhimurium previously inoculated on the hides of 2.4 to 4.8 and 1.3 to 5.1 logarithmic units, respectively. However, treating live cattle with a lactic acid solution (0.5%) during 1 minute did not reduce the proportion of *Salmonella*-positive hide samples. In another study, a reduction average

of 2 log units in *Salmonella* and *E. coli* O157 loads was reported on previously inoculated cattle hides using lactic and acetic acid (10%, 55°C) sprays (Carlson et al., 2008).

Although a variety of biological treatments are reported to be used in carcass decontamination, bacteriophages constitute the only biological treatment reported to be used in hide decontamination (Bolder, 1997; Chen et al., 2012). Some bacteriophages targeted to bacteriological pathogens namely *Salmonella* and *E. coli* O157:H7 have been already approved in United States for cattle hide decontamination, however the possibility of their utilization under commercial conditions is still being investigated (Loretz et al., 2011)

Apart from reducing the bacterial load on hides and possibly improving carcass microbiological quality, some hide decontamination interventions were found to present some disadvantages. It has been reported that treatments with water or steam increase the humidity on the surface of the treated hides (Loretz et al., 2011). This makes the skinning operations more difficult for the operator and may increase the risk of carcass contamination from the hide, especially when hide removal is carried out manually. Antic et al. (2010) reported that microbial contamination of bovine carcases during skinning was more likely to occur when the animal hide was wet. Steam treatments were also found to deteriorate the commercial quality of hides (McEvoy et al., 2003). Furthermore, animal and operator welfare problems, namely eye and skin irritation as well as corrosion of slaughtering equipment, have reportedly been associated with the use of chemical antimicrobials, particularly organic acids (Chen et al., 2012; Mies et al., 2004).

Hide decontamination, treatments appear to be an important strategy that can significantly reduce the risk of carcass contamination from soiled hides during the skinning process. However, considering existing data, it is difficult to accurately appraise their effect under normal slaughtering conditions as most of available informations derive from experimental studies. Additionally, very limited number of studied treatments concerned bacterial pathogens such as *Salmonella* or pathogenic *E. coli*. It is therefore imperative to conduct further studies to assess the effects of these interventions on major bacterial pathogens under practical slaughtering conditions. Another issue is to identify the optimal moment in the slaughtering process at which the hide decontamination treatment should be carried out under commercial conditions. The moment between stunning and sticking would be appropriate provided that the animal's unconsciousness lasts until the hide decontamination process ends. Alternatively, the moment after sticking but before hide removal would be used. In the latter case, appropriate measures should be taken so as not to contaminate the sticking wound during the hide decontamination process.

3.2.4 Cattle Skinning

The skinning stage is one of the slaughtering steps where microbial contamination of bovine carcasses is most likely to occur. This is due to the fact that the hide is, in most cases, heavily populated by a wide range of microorganisms that can be transferred to carcasses during skinning operations (Loretz et al., 2011). Bacterial pathogens such as *Salmonella* and *E. coli* O157 are also commonly isolated from hides of cattle destined to slaughter (Barham et al., 2002; Barkocy-Gallagher et al., 2003). During the skinning process, carcass contamination may occur through direct contact between the carcass and the hide or indirectly through equipment or operators contaminated by hides. Carcass contamination by airborne transfer is also possible (Antic et al., 2010).

Cattle hide removal can be carried out either manually or mechanically by means of a hide puller. The advantage of manual hide removal relies mainly in its low financial investment in equipment, but it has been found to present several disadvantages in terms of slaughter productivity and meat hygiene (Food and Agriculture Organization, 2006). These include the requirement for a very high skill level for effective hide removal without damaging both hide and carcass; the difficulty of the task and the time consumed even for a skilled operator; and a high risk of contaminating the carcass with microorganisms from the hide during the skinning process. On the other side, mechanical skinning by means of a hide puller seems to require less manual contact with the hide and consequently minimizes the risk of carcass contamination by microorganisms from operators and slaughtering equipments. Additionally, it increases the productivity of the slaughterhouse and improves the value of the hides by damaging them less. The disadvantages of mechanical skinning include the high cost of the equipment and fractures of the spinal column sometimes associated with the use of a downward hide puller.

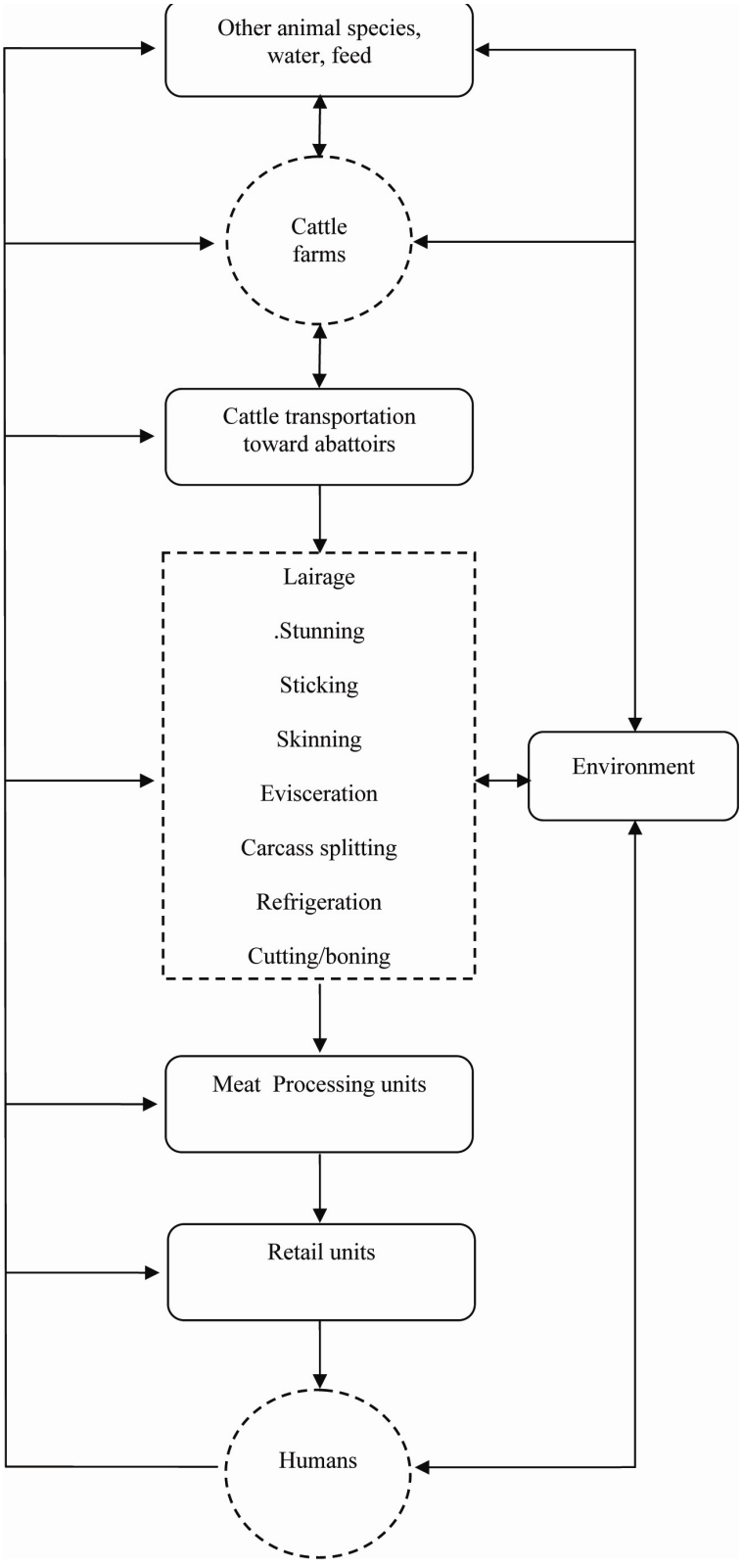


Figure 1. Potential sources and pathways for microbial contamination of bovine meat (Adapted from: Food and Agriculture Organization, 2006 and Millemann, 2008)

Peer-reviewed studies addressing the quantification of microorganisms transferred from hides to carcasses during the skinning indicate that, under commercial conditions, dressed bovine carcasses carry a very small proportion (ranging from 1.6 to 0.003%) of the hide microflora (Arthur et al., 2004; Bacon et al., 2000). Another

study showed that only 0.5 to 0.00002% of the hide microflora is transferred to dressed bovine carcass via direct contact (Antic et al., 2010) highlighting the importance of other transmission pathways such as indirect contamination via knives and/or hands or airborne transfers. Nevertheless, even if the reported hide-to-meat microbial transmission rates appear to be relatively low, it should be noted that the risk associated to these transmissions is still significant. In fact, carcass contamination from hides occurs regularly under commercial slaughtering conditions and the reported bacterial loads on hides are so high that proportions as low as less than 1% would constitute levels of many logarithmic units (Loretz et al., 2011). By summarizing data from numerous studies published internationally, Antic et al. (2010) reported bacterial contamination levels of 6–10 log cfu/cm^2 and 4.5–8 log cfu/cm^2 respectively on visually dirty and clean hides from cattle destined to slaughter.

The control of carcass bacterial contamination from hides during skinning operations consists basically in preventing hide-to-meat contaminations through process hygiene means and/or the elimination microbial contaminants from hides before skinning operations by adequate treatments. Concerning the process hygiene, several studies have reported an association between the hide cleanliness and the microbiological status of dressed carcasses (McEvoy et al., 2000; McCleery et al., 2008). Thus, in many countries (including but not limited to Australia, Ireland, Finland, Norway and United Kingdom) Good Hygienic Practice programs in cattle dressing are based on the cleanliness of cattle hides. In these countries, only cattle with clean hides are slaughtered under normal conditions whereas dirty animals are either cleaned (and allowed to dry before slaughtering) or are slaughtered separately under special conditions as they are considered to present a high risk for cross contaminations (McEvoy et al., 2000). A recent study conducted in Norwegian abattoirs (Hauge, Nafstad, Røtterud, & Nesbakken, 2012) confirmed that, under commercial conditions, carcasses from clean animals present levels of hygiene indicator bacteria (total aerobic bacteria and *E. coli* counts) significantly lower than these from dirty animals. Although the cleanliness of cattle hides prior to skinning presents considerable beneficial effects on the bacteriological status of dressed carcasses, it should be noted however, that these effects are not absolute. In fact, it is known that pathogenic bacteria such as *E. coli* O157 are commonly isolated from visually clean hides (Nastasijevic et al., 2008). Therefore, the selection of cattle with clean hides for slaughter should be combined with other good hygienic practices including hygiene for staff and skinning equipments as well good manufacturing practices particularly an immediate carcass trimming when any carcass contamination is suspected (Kiermeier et al., 2006; J J Sheridan, 1998). The elimination of bacterial contaminants from hides prior to skinning constitutes a promising alternative to consider. However, as presented in previous sections of the present paper, most of the existing informations on the effects of hide decontamination treatments derive from experimental studies. Further studies are therefore still needed to accurately appraise the effects of these treatments under commercial slaughtering conditions.

3.2.5 Evisceration

As the skinning step, evisceration constitutes a critical slaughtering stage where microbial contamination of carcasses is most likely to occur. The gastro-intestinal tract of cattle is naturally colonised by microorganisms that may be transferred to carcasses during the evisceration process (McEvoy et al., 2000). Additionally, bacterial pathogens such as *Salmonella* and *E. coli* are also frequently isolated in faeces of cattle destined to slaughter highlighting their probable presence in the digestive tract of the same animals (Rhoades et al., 2009). During the evisceration process, carcass contamination occurs by direct contact between the carcass and the gastro-intestinal contents or indirectly through soiled slaughtering equipments and staff. Contaminations may also occur during the removal of pharynx, tonsil and tongue as they are reported to be heavily contaminated by various microbial contaminants (Sheridan, 1998; Wheatley, Giotis, & Mckevitt, 2014).

Several peer-reviewed studies indicate a significant increase of bacterial loads on carcasses during the evisceration process; however the degree of increase varies from one study to another. The observed variation could be attributed to a number of factors including the differences in experimental designs and the process hygiene that differ from one slaughterhouse to another. For example, an average increase of 0.7 logcfu/cm^2 in *Enterobacteriaceae* counts was reported during the evisceration of lamb carcasses in 4 Irish abattoirs (Sierra, Sheridan, & McGuire, 1997); whereas in Rwanda increases of 3 and 1.3 log cfu/g were respectively observed in total aerobic bacteria and *E. coli* counts during the evisceration of cattle at a commercial abattoir (Niyonzima et al., 2013). Another Irish study reported an increase of 2-4 log in *Enterobacteriaceae* populations during the evisceration of pork carcasses (Wheatley et al., 2014).

The control of carcass bacterial contaminations during the evisceration process relies mainly on Good Slaughtering Practices. The techniques mostly used include the “bunging” and the “rodding”. The bunging or bung tying consists in sealing the rectum and covering it with a plastic bag in order to reduce the spread of faecal material from the rectum to the carcass; whereas the rodding corresponds to sealing the oesophagus to avoid the

spread of its content onto the carcass (McEvoy, Sheridan, Blair, & McDowell, 2004). These techniques are effective in reducing the risk of bacterial transfers during the evisceration. Nesbakken et al. (1994) reported that bunging reduced significantly the occurrence of *Yersinia enterocolitica* on pig carcasses. Furthermore, the introduction of that technique in Norwegian pork abattoirs resulted in decreasing the incidence of Yersiniosis by 25% in the population (Sheridan, 1998). Similarly, special attention must also be paid to the training of staff on Good Hygienic Practices as well as on the sanitation of slaughtering equipments particularly knives to minimize the risk of cross contaminations. Bolton et al. (2002) recommend sanitizing knives by a two-knife system that consists in the utilization of one knife while the other is being sanitized in hot water at 82°C or above.

Despite the reported increases in bacterial load on carcasses during their evisceration, some authors indicate that the existing measures including rectum and oesophagus sealing, intact removal of visceral contents and an appropriate training of staff in Good Hygienic Practices could reduce the risk of carcass contamination from viscera to the point where they do not contribute significantly to the overall contamination of the carcass (McEvoy et al., 2000; Wheatley et al., 2014).

3.2.6 Carcass Splitting

The carcass splitting stage is not generally considered as a major source of contamination (Wong et al., 2002). However, the splitting saw as well as other slaughtering equipment can be contaminated with pathogenic bacteria such as *Salmonella* and *E. coli* and may contribute to their spread to several carcasses. In a study conducted in 4 European countries, Hald et al. (2003) reported that 9.4% of 384 carcass splitter machines were contaminated with *Salmonella* during the slaughtering process. In addition, Warriner et al. (2002) demonstrated that *E. coli* and potential enteric pathogens can be transferred between pork carcasses through the splitting saw. Therefore, cleaning and disinfection of the splitting saw should be carried out after splitting each carcass in order to reduce the risk of cross contaminations. The European regulations recommend disinfecting the splitting saw after splitting each animal using water at 82°C or above or using another method with an equivalent effect (European Commission, 2004).

Although adherence to Good Hygiene Practices in abattoirs improves the microbiological quality of the meat significantly, it is generally recognized that contamination of meat is unavoidable during the cattle slaughtering process (McCann et al., 2006). Therefore, carcass decontamination before refrigeration appears as a corrective measure to restore the bacterial load of carcasses to the acceptable range.

3.2.7 Carcass Decontamination Treatments

Various treatments including physical, chemical and biological methods applied alone or in combination have been identified to reduce the levels of bacterial load on carcasses. In this section majors carcass decontamination treatments as well as their respective effects on the bacterial load of carcasses are discussed.

The physical decontamination treatments mostly reported for carcasses include hot water washes and application of steam. These treatments are generally carried out in special cabinets where carcasses are splayed with water or steam at controlled pressure and temperature. Carcass sprays with hot water were found to lower significantly the bacterial load on treated carcasses. However, the reduction rates reported were found to be dependent of experimental factors such as the temperature, pressure and the duration of the treatment (Loretz et al., 2011). The effectiveness of carcass decontamination by hot water was demonstrated by Bosilevac et al. (2006). These authors conducted a study in a commercial abattoir and reported 2.7 log reductions in both aerobic plate counts and *Enterobacteriaceae* counts on pre-evisceration bovine carcasses washed in a cabinet with water at 74°C for 5.5 seconds. The prevalence of *E. coli* O157 was also reduced by 81% in treated carcasses. The decontamination of carcasses with steam was reported to yield bacterial reductions comparable to these obtained with hot water spays. However, the treatments with steam presents an advantage of reaching cavities and crevices of carcasses that are generally inaccessible to hot water (Hugas & Tsigarida, 2008). One of the side effects reportedly associated with carcass decontamination treatments by steam or hot water is the change in the carcass colour after a prolonged treatment. McCann et al. (2006) reported a cooked appearance on the surface of carcasses having undergone a steam decontamination treatment of 10 seconds or longer. Furthermore, weight gain resulting from water absorption by treated carcasses generally reported in hot water decontamination treatments may be perceived as a fraud by meat consumers (EFSA, 2010).

Organic acid sprays, namely acetic, citric and lactic acids are the most-reported chemical decontamination methods used on beef carcasses. They are known to reduce the number and prevalence of food borne pathogens and the microbial load on meat carcasses (Huffman, 2002), but their efficacy depends on the type of meat tissue, the type and load of initial microbial contamination, as well as the pH, concentration and temperature of the organic acid solution (Hugas & Tsigarida, 2008). Various studies conducted under laboratory conditions showed

that spraying inoculated bovine carcasses with acetic or citric acid yielded bacterial reductions varying between 0.7 and 4.9 logs for aerobic bacteria, non pathogenic *E. coli*, *E. coli* O157:H7 and *Salmonella* (Loretz et al., 2011). However, lower reductions are generally reported in studies conducted under commercial conditions. This could be due to lower acid concentrations used in respect to meat quality and staff welfare considerations (Chen et al., 2012). By spraying acetic acid (2.5%) to bovine carcasses prior to chilling Algino et al. (2007) reported reductions of coliforms, *Enterobacteiaceae* and *E. coli* levels ranging from 0.6 to 1.4 logs. In a study by Barboza de Martinez et al. (2002) spraying carcasses at the end of slaughter by lactic acid (1.5%) yielded reductions of 0.5, 1.8 and 0.6 logs, respectively, for aerobic bacteria, coliforms and *E. coli*. In another study, lactic acid (2%; 42°C) spraying of pre-eviscerated bovine carcasses was reported to reduce the prevalence of *E. coli* O157:H7 by 35% as well as aerobic bacteria and *Enterobacteriaceae* counts by respectively 1.6 and 1.0 logs (Bosilevac et al., 2006). Other chemicals such as chlorine, trisodium phosphate, acidified sodium chlorite and peroxyacids are also used for meat decontamination but to a lesser extent. Generally, the use of these substances leads to 1–1.5 log reductions in foodborne pathogens such as *Salmonella* and *E. coli* O157 (Hugas & Tsigarida, 2008). A number of drawbacks have however been reportedly associated to the chemical decontamination of carcases especially by organic acids. These include staff welfare problems such as eye or skin irritations and the corrosion of slaughtering equipments (Chen et al., 2012; Mies et al., 2004).

Reported biological treatments for meat decontamination include the use of bacteriocins and bacteriophages. Bacteriocins are anti-microbial proteinaceous compounds produced by some bacteria. The most widely known bacteriocin is nisin, which is produced by *Lactobacillus lactis* subsp. *lactis* and is effective against Gram-positive bacteria. Nisin is used as a preservative agent in foods like cheese but its use in carcass decontamination has been limited by a number of factors, namely its deficient inhibitory effect on Gram-negative bacteria, low level of production *in vivo* and likely inactivation of its effect due to interactions with other food components (Bolder, 1997; Chen et al., 2012). Nevertheless, combinations of nisin and other treatments have been reported to reduce microbial contamination on carcasses. Barboza de Martinez et al. (2002) reported that a combination of nisin and lactic acid sprays under commercial conditions reduced aerobic bacteria, coliforms and *E. coli* populations on carcasses by 2.0, 2.2, and more than 1.0 log, respectively, whereas treatment with nisin alone reduced bacterial levels by less than 0.2 log. The use of bacteriophages has also been reported to present a number of benefits as an alternative biocontrol method. These include their high host specificity and lack of effect on the organoleptic qualities of the food as well as their ability to survive under commercial processing procedures (Hugas & Tsigarida, 2008). However, their use in food decontamination is still limited by factors such as the potential development of resistance in targeted bacteria (Chen et al., 2012). The use of *E. coli* O157:H7 and *Salmonella* targeted bacteriophages for cattle hide decontamination has already been approved in the USA. However, further investigations to address their efficacy under long-term commercial conditions and their possible utilization for carcass decontamination are still required (Loretz et al., 2011).

Although obtaining bovine carcasses free of pathogenic bacteria and with low microbial contamination appears to be a shared goal of all countries, carcass decontamination policies vary from one country to another. In the USA, for example, a number of carcass decontamination treatments are allowed and commonly used in cattle slaughterhouses. These include physical interventions such as hot water or steam spraying and chemical treatment with organic acids, namely lactic and acetic acids (Chen et al., 2012). Contrary to this, in Europe important efforts have been put into the application of Good Manufacturing Practices throughout the entire meat production line, and for many years carcass decontamination treatments in the European Union were limited to the use of clean or potable water. The current European regulation (European Commission, 2004) allows the use of substances other than water for the removal of surface bacterial contamination from meat; however, the European Food Safety Authority (EFSA) must provide a chemical and microbiological risk assessment before the European commission authorizes the use of such substances (Hugas & Tsigarida, 2008). Currently, no chemical substance has yet been approved for decontamination of beef carcasses within the European Union (EFSA, 2014a).

Carcass decontamination treatments constitute a potential control measure to reduce the levels of bacterial and pathogen loads on carcasses. However, available informations indicate a wide variability in bacterial reduction yields as most of data results from studies conducted under different conditions. Thus, studies to compare the effectiveness of different treatments under the same conditions would be of valuable importance to identify the cost-effective interventions to be used in cattle slaughterhouses. Furthermore, the risk, in some abattoir, to rely only on the carcass decontamination step and abandon existing good hygienic and manufacturing practices in previous slaughtering stages need to be considered before the adoption of such interventions.

3.2.8 Carcass Refrigeration

Apart from meat maturation purposes, refrigeration of carcasses after the slaughtering process is performed to inhibit the growth of spoilage and/or pathogenic bacteria that could still be on the carcasses and consequently increase their shelf-life (Dave & Ghaly, 2011). Carcass refrigeration is generally carried in two phases including the rapid chilling phase consisting in rapidly reducing the carcass temperature and a second phase of cold storage intended to maintain the low temperature of carcasses. Different methods of carcass chilling were thoughtfully reviewed by Savell et al. (2005) and are not further developed in this paper. In commercial slaughterhouses, carcasses are generally chilled for 48-72 hours before their transfer in the boning hall. Nevertheless, the duration of carcass chilling may be extended beyond 72 hours to improve the quality of meat. This process is referred to as aging (EFSA, 2014b). The inhibition of bacterial growth on carcasses at refrigeration temperatures is a consequence of low-temperature stress undergone by microorganism. In fact, as the temperature decreases, the bacterial lag phase extends whereas the growth rate decreases and the ultimate cell numbers may decrease (Beales, 2004; N J Russell et al., 1995).

Although bacterial growth on carcasses is known to be inhibited at refrigeration temperatures (Korsak, Clinquart, & Daube, 2004; Russell, 2002), several published studies indicate increases in levels of bacterial loads on refrigerated carcasses. In a study conducted in a commercial abattoir, Bolton et al. (2002) reported an increase in total viable bacterial counts from 3.8 to 4.5 log cfu/cm^2 on carcasses at the refrigeration stage. Another study conducted on poultry carcasses showed that after 9 days of storage, *Salmonella* loads were slightly reduced (by less than 1 log unit) on carcasses refrigerated at 2 and 6°C whereas in carcasses refrigerated at 8°C *Salmonella* number increased by 1.5 log units (Jiménez, Tiburzi, Salsi, Moguilevsky, & Pirovani, 2009).

Microbial growth on carcasses under refrigeration is mainly attributable to the temperatures of chilling equipments that are not sufficiently low to inhibit the microbial growth and/or to intermittent rupture of the cold chain. In fact, bacterial pathogens such as *Salmonella* may multiply to hazardous levels during periods of temperature abuse (Delhalle et al., 2009; Wong et al., 2002). Thus, slaughterhouses should be equipped with chilling equipments capable to rapidly decrease and maintain low the temperature of carcasses during the entire refrigeration period. In European Union countries for example, the carcass temperature must be decreased to maximum 7°C in the first 24 hours of refrigeration (European Commission, 2004). Additionally, trough cross-contaminations, microorganisms present on carcasses under refrigeration or on chilling equipments may get disseminated to other carcasses and proliferate when the environment become favourable to their growth (for example during temperature abuses). Published studies indicate that bacterial pathogens can survive on surfaces of refrigerators (Jackson, Blair, McDowell, Kennedy, & Bolton, 2007) or on chilling evaporators (Evans, Russell, James, & Corry, 2004) and pose a cross-contamination risk to the refrigerated foods. This highlights the need for a regular cleaning and disinfection of chilling rooms and/or equipments in the slaughterhouse.

The refrigeration of carcasses constitutes a determinant slaughtering stage influencing the final bacterial load on carcasses. However, despite the exiting control measures (including adequate chilling equipments, the regular monitoring of the temperature of carcasses and the mastery of cross-contaminations through an effective appliance to Good Hygienic Practices) that have proven their effectiveness in significantly reducing the risk of bacterial/pathogen growth on carcasses under refrigeration (Delhalle et al., 2009); in some slaughterhouses, bacterial growth is still being reported on refrigerated carcasses probably due to the failure in applying adequate control measures. It is thus imperative for slaughterhouses to deploy all material, technical and financial means required to control the bacterial growth on carcasses at this critical stage of slaughter.

3.3 Post Slaughter Contamination of Bovine Meat

The post slaughter section of the meat chain comprises a series of sub-stages (including cutting/boning, transportation of carcasses or meat cuts, meat storage, manufacture of meat products, retail, and eventually cooking) at which contamination of meat may occur. In this section majors sources of post slaughter meat contamination by pathogenic bacteria and their respective control measures are reviewed.

At the end of the slaughtering process carcasses are generally cut in special meat pieces (cutting) and separated from bones (boning) for industrial and commercial utilisations. The cutting and boning of carcasses may take place in the slaughterhouse or in specialised plants. The cutting and boning operations are generally performed on refrigerated carcasses however boning of non refrigerated carcasses (hot boning) is also possible (Røtterud et al., 2006). Even if hot boning presents a number of advantages including a reduced cost and fewer requirements in chilling equipments and space (Pinto Neto, Beraquet, & Cardoso, 2013) it is rarely used in European countries. This is mainly due to a possible proliferation of pathogenic bacteria on processed meat and a reduced shelf life of subsequent vacuum packed meat (Yang, Balamurugan, & Gill, 2011). A recent report from the European Food

Safety Authority indicate that the surface temperature of boned beef cuts from chilled carcasses decreases to 8°C in few hours whereas the temperature of hot boned and vacuum packed meat pieces may remain at 25°C for many hours; creating favourable conditions for the proliferation of spoilage and pathogenic microorganisms (EFSA, 2014b). Data from published studies indicate that bacterial/pathogen loads on carcasses may significantly increase during the cutting/boning operations even in slaughterhouses where cold boning is practicised. In a study conducted in an Irish beef abattoir, McEvoy et al. (2004) reported increases of 2.3 and 2.1 logcfu/cm^2 respectively for total viable bacteria and *Enterobacteriaceae* counts on the inside round of carcasses during the cutting/boning operations. Similar increases were also reported in *E. coli* numbers during the boning of beef carcasses (Gill, McGinnis, & Bryant, 2001). Increases in bacterial/pathogen numbers following the cutting and boning operations could be associated to cross-contaminations. During these operations, carcasses or meat pieces of various origins and different contamination levels are handled in close proximity, creating numerous opportunities for cross-contamination or spread of pathogenic bacteria (Wong et al., 2002). Various origins of microbial contamination during cutting/boning were reported in literature. These include carcasses or meat pieces to be processed (McEvoy et al., 2004); meat cutting/boning equipments such as knives, meat conveyors or cutting boards (Gill, Badoni, & McGinnis, 1999; Gill et al., 2001; Jiménez et al., 2009) and soiled surfaces or operators (Sheridan, Lynch, & Harrington, 1992). One of the measures to prevent cross contaminations resulting from contaminated carcasses or meat pieces would be to identify the most contaminated raw materials and to process them separately preferably at the end of the production (Koohmaraie et al., 2012). Contaminations from equipments can be mastered through a regular cleaning and disinfection of equipments and surfaces whereas effective training of staff on Good Hygienic Practices could help in preventing contaminations from personnel (Delhalle et al., 2009). The working temperature constitutes another factor influencing bacterial growth on meat during the cutting/boning operations. In fact, during these operations the temperature of processed meat increases and this would favour the growth of existing microbial populations. It is therefore recommended to carry out boning operations under refrigerated conditions. In some commercial abattoirs, the boning hall is refrigerated at 10-12°C (EFSA, 2014b; McEvoy et al., 2004).

Although temperature abuse is reportedly the main factor associated to bacterial load increases on carcasses or meat pieces during transportation and storage (Delhalle et al., 2009; Wong et al., 2002), cross-contaminations originating from chilling equipments or personnel are also significant contributors to the ultimate bacterial load of meat (Evans et al., 2004; Jackson et al., 2007; Sheridan et al., 1992). Thus, it is imperative to prevent cross contamination through an effective and regular sanitization of chilling rooms and meat transportation vehicles. Additionally, personnel involved in the loading of meat transportation vehicles should be educated in good hygiene practices. On the other side, the regular monitoring of temperatures in chilling rooms and meat transportation vehicles could contribute significantly in reducing the risk of temperature abuses occurring during meat storage and transportation (Savell et al., 2005). Recent published studies indicate that meat transportation vehicles with a chilling capacity comparable to the one of conventional chilling rooms are commercially available. Most of these vehicles are designed for longer distances and can decrease the core temperature of bovine carcasses from approximately 20°C at the loading time to 7°C or lower after 48 hours. Additionally, these vehicles are equipped with apparatus to continuously monitor the temperature of meat during the transportation (EFSA, 2014b).

As in previous stages of the meat chain, cross-contamination from equipments, personnel or the working environment are likely to occur during the manufacture of meat products if appropriate control measures are not effectively applied (Roels et al., 1997; Wong et al., 2002). Nevertheless, microbial contaminants from incorporated non-meat ingredients as well as contaminations associated to the manufacture method used, appear to be specific to this particular stage of the meat chain. The grinding of meat for example, would result in the dissemination of microorganisms previously localised at the surface of meat pieces in the entire batch of minced meat (Gould et al., 2011). As the meat temperature increases during grinding operations due to friction movements, these microorganisms may proliferate in relatively short period resulting in bacterial number increases in minced meat (Heinz & Hautzinger, 2007). A number of preservative treatments (including thermal interventions, smoking, curing etc.) are commonly used in meat processing to enhance the bacteriological stability of meat products and consequently increase their shelf-life. These interventions, generally based on the control of the temperature, pH, water activity, microbial competition/interaction and oxido-reduction potential, were found to significantly reduce the bacterial/pathogen load in meat (Chen et al., 2012; Hugas & Tsigarida, 2008; Loretz et al., 2011). However, as their preservative effects depend also on the initial bacterial numbers in meat; it is crucial for meat processors to assure that the used raw materials are of good microbiological quality (Wong et al., 2002). Another important measure would be to decide the fate of raw materials according to their microbiological quality. In some commercial meat processing plants, heavily contaminated raw materials are

generally reserved for the manufacture of meat products destined to undergo a heat treatment (Koohmaraie et al., 2012; McCleery et al., 2008; McEvoy et al., 2000; Nastasijevic et al., 2008).

At the retail level, temperature conditions are reportedly an important factor influencing the final microbiological quality and safety of meat products (Delhalle et al., 2009). Several published studies indicate a wide variation in meat product temperatures within retail cabinets but most of the data are simply indicative of the product temperature at the time and place of the study and do not address conditions that would dynamically influence the temperature changes (Nychas, Skandamis, Tassou, & Koutsoumanis, 2008). Nevertheless, it is recognised that displayed meat products in retail cabinet must be at temperatures sufficiently low (generally below 4°C) to inhibit the growth of spoilage or pathogenic microorganisms (Wong et al., 2002). It is therefore imperative to regularly monitor the temperature of display cases to prevent temperature abuses during meat storage. Furthermore, appropriate control measures must be applied to prevent cross contaminations from equipments, personnel or working environment in retail establishment where meat processing activities such as cutting or grinding are carried out (Gould et al., 2011). The retail level represents an important stage of the meat chain in regard with the final quality and safety of meat products as it constitutes the last "check point" where contaminated products can be identified before their consumption particularly for ready-to-eat meat products (Wong et al., 2002).

The last section of the meat chain related to the transportation, storage and cooking by the consumer appears to be less studied although it is the most important in regards with the food safety aspects of meat products. This is due to the difficulties in collecting data concerning the mode and the duration of meat transportation toward the consumer's household, temperature conditions in domestic refrigerator and freezers, durations of storage before consumption as well as consumer's cooking habits (Nychas et al., 2008). However, it is recognised that the cooking stage is the last line of defence of consumers against *Salmonella* and pathogenic *E. coli* infections attributable to bovine meat (Korsak et al., 2004; Mainil & Daube, 2005). These pathogens are generally destroyed in foods at conventional pasteurisation temperatures. In beef, *Salmonella* is reported to have a decimal reduction time (D-value) of 0.53 minutes (z=5°C) at 65°C (Juneja et al., 2001; Korsak et al., 2004) whereas *E. coli* O157:O7 presents a D-value of 0.39 minutes (z=6°C) at the same temperature (Juneja, Snyder, & Marmer, 1997). It is therefore recommended to cook meat until the internal temperature reaches a minimal temperature of 70°C to assure a thermal destruction of these pathogens in meat as most of the reported *Salmonella* and *E. coli* O157:H7 outbreaks attributable to meat were found to be associated to the ingestion of raw or undercooked meat products (Abong'o & Momba, 2009; Greig & Ravel, 2009; Roels et al., 1997).

4. Conclusion

Despite a large number of control measures along the meat chain, meat contaminations by *Salmonella* and pathogenic *E-coli* remain a serious public health problem in humans. In the pre-slaughter stages of the meat chain, difficulties in identifying asymptomatic shedders constitute the main obstacle to the control of the infection spread in live animals. Further studies are therefore needed to identify cost-effective techniques and approaches to diagnose asymptomatic carriers in cattle herd before animal transportation to abattoirs. During the slaughtering process, the skinning and evisceration operations appears to be the most critical stages for carcass contamination. Thus Good Manufacturing Practices in accordance with HACCP principles must be strictly applied in commercial slaughterhouses to reduce the risk of carcass contamination at those specific stages. The decontamination of carcasses has also shown a potential in reducing pathogen numbers on carcasses prior to chilling, even if its utilisation in some countries is still limited by a number of factors including the cost of installations, the commercial quality of treated carcasses as well as the risk of relying only on the carcass decontamination step and reduce efforts devoted to Good Hygiene and Manufacturing Practices in previous slaughtering stages. Along post-slaughter stages of the chain, handling, time and temperature are the main factors influencing the microbial contamination of meat. Therefore application of appropriate GMP and GHP by meat processing plants is of great importance to prevent cross-contaminations during cutting/boning, processing, transportation and retail of meat products. Similarly, the cold chain must be respected at all stages of meat distribution. Although all stages of the bovine meat chain are of significant relevance in regard to the ultimate bacterial contamination of meat, the cooking step constitutes the most important stage to assure the safety of beef at consumption. In fact, the cooking step is the last stage of the meat chain at which *Salmonella* and pathogenic *E. coli* can be completely destroyed.

References

Abong'o, B. O., & Momba, M. N. B. (2009). Prevalence and characterization of Escherichia coli O157:H7 isolates from meat and meat products sold in Amathole District, Eastern Cape Province of South Africa. *Food Microbiology, 26*(2), 173-6. http://dx.doi.org/10.1016/j.fm.2008.10.001

Alexa, P., Konstantinova, L., & Sramkova-Zajakova, Z. (2011). Faecal shedding of verotoxigenic Escherichia coli in cattle in the Czech Republic. *Veterinarni Medicina, 56*(4), 149-155.

Algino, R. J., Ingham, S. C., & Zhu, J. (2007). Survey of antimicrobial effects of beef carcass intervention treatments in very small state-inspected slaughter plants. *Journal of Food Science, 72*(5), M173-9. http://dx.doi.org/10.1111/j.1750-3841.2007.00386.x

Anil, M. H., Love, S., Helps, C. R., Mckinstry, J. L., Brown, S. N., Philips, A., … Harbour, D. (2001). Jugular venous emboli of brain tissue induced in sheep by the use of captive bolt guns. *The Veterinary Record, 148*, 619-620.

Antic, D., Blagojevic, B., Ducic, M., Nastasijevic, I., Mitrovic, R., & Buncic, S. (2010). Distribution of microflora on cattle hides and its transmission to meat via direct contact. *Food Control, 21*(7), 1025-1029. http://dx.doi.org/10.1016/j.foodcont.2009.12.022

Arthur, T. M., Bosilevac, J. M., Brichta-Harhay, D. M., Guerini, M. N., Kalchayanand, N., Shackelford, S. D., … Koohmaraie, M. (2007). Transportation and lairage environment effects on prevalence, numbers, and diversity of Escherichia coli O157:H7 on hides and carcasses of beef cattle at processing. *Journal of Food Protection, 70*(2), 280-6.

Arthur, T. M., Bosilevac, J. M., Nou, X., Shackelford, S. D., Wheeler, T. L., Kent, M. P., … Koohmaraie, M. (2004). Escherichia coli O157 prevalence and enumeration of aerobic bacteria, Enterobacteriaceae, and Escherichia coli O157 at various steps in commercial beef processing plants. *Journal of Food Protection, 67*(4), 658-65.

Arthur, T. M., Brichta-Harhay, D. M., Bosilevac, J. M., Kalchayanand, N., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2010). Super shedding of Escherichia coli O157:H7 by cattle and the impact on beef carcass contamination. *Meat Science, 86*(1), 32-7. http://dx.doi.org/10.1016/j.meatsci.2010.04.019

Bacon, R. T., Belk, K. E., Sofos, J. N., Clayton, R. P., Reagan, J. O., & Smith, G. C. (2000). Microbial Populations on Animal Hides and Beef Carcasses at Different Stages of Slaughter in Plants Employing Multiple-Sequential Interventions for Decontamination. *Journal of Food Protection, 63*, 1080-1086.

Baird, B. E., Lucia, L. M., Acuff, G. R., Harris, K. B., & Savell, J. W. (2006). Beef hide antimicrobial interventions as a means of reducing bacterial contamination. *Meat Science, 73*(2), 245-248. http://dx.doi.org/10.1016/j.meatsci.2005.11.023

Barboza de Martinez, Y., Ferrer, K., & Salas, E. M. (2002). Combined effects of lactic acid and nisin solution in reducing levels of microbiological contamination in red meat carcasses. *Journal of Food Protection, 65*, 1780-1783.

Barham, A. R., Barham, B. L., Johnson, A. K., Allen, D. M., Blanton, J. R., & Miller, M. F. (2002). Effects of the transportation of beef cattle from the feedyard to the packing plant on prevalence levels of Escherichia coli O157 and Salmonella spp. *Journal of Food Protection, 65*(2), 280-283.

Barkocy-Gallagher, G. a, Arthur, T. M., Rivera-Betancourt, M., Nou, X., Shackelford, S. D., Wheeler, T. L., & Koohmaraie, M. (2003). Seasonal prevalence of Shiga toxin-producing Escherichia coli, including O157:H7 and non-O157 serotypes, and Salmonella in commercial beef processing plants. *Journal of Food Protection, 66*(11), 1978-86.

Beach, J. C., Murano, E. A., & Acuff, G. R. (2002). Prevalence of Salmonella and Campylobacter in beef cattle from transport to slaughter. *Journal of Food Protection, 65*(11), 1687-1693.

Beales, N. (2004). Adaptation of Microorganisms to Cold Temperatures, Weak Acid Preservatives, Low pH, and Osmotic Stress: A Review. *Comprehensive Reviews in Food Science and Food Safety, 3*(1), 1-20. http://dx.doi.org/10.1111/j.1541-4337.2004.tb00057.x

Birk, T., Kristensen, K., Harboe, A., Hansen, T. B., Ingmer, H., De Jonge, R., … Aabo, S. (2012). Dietary Proteins Extend the Survival of <I>Salmonella</I> Dublin in a Gastric Acid Environment. *Journal of Food Protection, 75*(2), 353-358. http://dx.doi.org/10.4315/0362-028X.JFP-11-132

Blaser, M. J., & Newman, L. S. (1982). A review of human salmonellosis: I. Infective dose. *Reviews of Infectious Diseases, 4*(6), 1096-106.

Bolder, N. (1997). Decontamination of meat and poultry carcasses. *Trends in Food Science and Technology, 8*, 221-227.

Bolton, D. J., Pearce, R. a, Sheridan, J. J., Blair, I. S., McDowell, D. a, & Harrington, D. (2002). Washing and chilling as critical control points in pork slaughter hazard analysis and critical control point (HACCP) systems. *Journal of Applied Microbiology, 92*(5), 893-902.

Bosilevac, J. M., Guerini, M. N., Kalchayanand, N., & Koohmaraie, M. (2009). Prevalence and characterization of salmonellae in commercial ground beef in the United States. *Applied and Environmental Microbiology, 75*(7), 1892-1900. http://dx.doi.org/10.1128/AEM.02530-08

Bosilevac, J. M., Nou, X., Barkocy-Gallagher, G. a, Arthur, T. M., & Koohmaraie, M. (2006). Treatments using hot water instead of lactic acid reduce levels of aerobic bacteria and Enterobacteriaceae and reduce the prevalence of Escherichia coil O157:H7 on preevisceration beef carcasses. *Journal of Food Protection, 69*(8), 1808-13.

Bosilevac, J. M., Shackelford, S. D., Brichta, D. M., & Koohmaraie, M. (2005). Efficacy of ozonated and electrolyzed oxidative waters to decontaminate hides of cattle before slaughter. *Journal of Food Protection, 68*(7), 1393-1398.

Buncic, S., McKinstry, J., Reid, C. –., & Anil, M. . (2002). Spread of microbial contamination associated with penetrative captive bolt stunning of food animals. *Food Control, 13*, 425-430. http://dx.doi.org/10.1016/S0956-7135(01)00054-8

Carlson, B. A., Geornaras, I., Yoon, Y., Scanga, J. a, Sofos, J. N., Smith, G. C., & Belk, K. E. (2008). Studies to evaluate chemicals and conditions with low-pressure applications for reducing microbial counts on cattle hides. *Journal of Food Protection, 71*(7), 1343-1348.

Carlson, B. A., Ruby, J., Smith, G. C., Sofos, J. N., Bellinger, G. R., Warren-Serna, W., … Belk, K. E. (2008). Comparison of antimicrobial efficacy of multiple beef hide decontamination strategies to reduce levels of Escherichia coli O157 : H7 and Salmonella . *Journal of Food Protectionn, 71*(11), 2223-7.

Carney, E., O'Brien, S. B., Sheridan, J. J., McDowell, D. a, Blair, I. S., & Duffy, G. (2006). Prevalence and level of Escherichia coli O157 on beef trimmings, carcasses and boned head meat at a beef slaughter plant. *Food Microbiology, 23*(1), 52-9. http://dx.doi.org/10.1016/j.fm.2004.12.001

Castillo, A., Dickson, J. S., Clayton, R. P., Lucia, L. M., & Acuff, G. R. (1998). Chemical dehairing of bovine skin to reduce pathogenic bacteria and bacteria of fecal origin. *Journal of Food Protection, 61*(5), 623-625.

Chen, J. H., Ren, Y., Seow, J., Liu, T., Bang, W. S., & Yuk, H. G. (2012). Intervention Technologies for Ensuring Microbiological Safety of Meat: Current and Future Trends. *Comprehensive Reviews in Food Science and Food Safety, 11*, 119-132. http://dx.doi.org/10.1111/j.1541-4337.2011.00177.x

Coia, J. E. (1998). Clinical, microbiological and epidemiological aspects of Escherichia coli O157 infection. *FEMS Immunology and Medical Microbiology, 20*(1), 1-9.

Daly, D. J., Prendergast, D. M., Sheridan, J. J., Blair, I. S., & Mcdowell, D. A. (2002). Use of a Marker Organism To Model the Spread of Central Nervous System Tissue in Cattle and the Abattoir Environment during Commercial Stunning and Carcass Dressing. *Applied and Environmental Microbiology, 68*(2), 791-798. http://dx.doi.org/10.1128/AEM.68.2.791

Dave, D., & Ghaly, A. E. (2011). Meat Spoilage Mechanisms and Preservation Techniques : A Critical Review. *American Journal of Agricultural and Biological Sciences, 6*(4), 486-510.

Delhalle, L., Saegerman, C., Farnir, F., Korsak, N., Maes, D., Messens, W., … Daube, G. (2009). Salmonella surveillance and control at post-harvest in the Belgian pork meat chain. *Food Microbiology, 26*, 265-71. http://dx.doi.org/10.1016/j.fm.2008.12.009

Doulgeraki, A. I., Ercolini, D., Villani, F., & Nychas, G.-J. E. (2012). Spoilage microbiota associated to the storage of raw meat in different conditions. *International Journal of Food Microbiology, 157*(2), 130-41. http://dx.doi.org/10.1016/j.ijfoodmicro.2012.05.020

Duffy, G., Cummins, E., Nally, P., O' Brien, S., & Butler, F. (2006). A review of quantitative microbial risk assessment in the management of Escherichia coli O157:H7 on beef. *Meat Science, 74*(1), 76-88. http://dx.doi.org/10.1016/j.meatsci.2006.04.011

Edrington, T. S., Hurne, M. E., Looper, M. L., Schultz, C. L., Fitzgerald, a. C., Callaway, T. R., … Nisbet, D. J. (2004). Variation in the faecal shedding of Salmonella and E. coli O157:H7 in lactating dairy cattle and examination of Salmonella genotypes using pulsed-field gel electrophoresis. *Letters in Applied Microbiology, 38*(5), 366-372. http://dx.doi.org/10.1111/j.1472-765X.2004.01495.x

EFSA. (2004). *Welfare aspects of animal Stunning and killing methods: Scientific Report of the Scientific Panel for Animal Health and Welfare.* Retrieved May 26, 2014, from www.efsa.europa.eu/en/scdocs/doc/45.pdf

EFSA. (2010). Scientific Opinion on the safety and efficacy of using recycled hot water as a decontamination technique for meat carcasses. *EFSA Journal, 8*(9), 1-69. http://dx.doi.org/10.2903/j.efsa.2010.1827.

EFSA. (2014a). Decontamination of carcasses. *Decontamination of carcasses*. Retrieved May 26, 2014, from http://www.efsa.europa.eu/en/topics/topic/decontamination.htm

EFSA. (2014b). Scientific Opinion on the public health risks related to the maintenance of the cold chain during storage and transport of meat. Part 1 (meat of domestic ungulates). *EFSA Journal, 12*(3), 1-81. http://dx.doi.org/10.2903/j.efsa.2014.3601

EFSA and ECDC. (2013a). The European Union Summary Report on antimicrobial resistance in Antimicrobial resistance in zoonotic and indicator bacteria from humans , animals and food in the European Union in 2011. *EFSA Journal, 11*(5), 3196. http://dx.doi.org/10.2903/j.efsa.2013.3196

EFSA and ECDC. (2013b). The European Union Summary Report on Trends and Sources of Zoonoses , Trends and Sources of Zoonoses , Zoonotic Agents and Food-borne Outbreaks in 2011. *EFSA Journal, 11*(4), 3129. http://dx.doi.org/10.2903/j.efsa.2013.3129

EFSA and ECDC. (2014). The European Union Summary Report on Trends and Sources of Zoonoses , Zoonotic Agents and Food-borne Outbreaks in 2012. *EFSA Journal, 12*(2), 3547. http://dx.doi.org/10.2903/j.efsa.2014.3547

European Commission. (2004). Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. *Official Journal of the European Union*, *L139*(30 April 2004), 22-82.

European Commission. (2005). Regulation No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, *L338*, 1-26.

Eustace, I., Midgley, J., Giarrusso, C., Laurent, C., Jenson, I., & Sumner, J. (2007). An alternative process for cleaning knives used on meat slaughter floors. *International Journal of Food Microbiology, 113*(1), 23-27. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.06.034

Evans, J. a., Russell, S. L., James, C., & Corry, J. E. L. (2004). Microbial contamination of food refrigeration equipment. *Journal of Food Engineering, 62*(3), 225-232. http://dx.doi.org/10.1016/S0260-8774(03)00235-8

Fegan, N., Vanderlinde, P., Higgs, G., & Desmarchelier, P. (2005). A study of the prevalence and enumeration of Salmonella enterica in cattle and on carcasses during processing. *Journal of Food Protection, 68*(6), 1147-53.

Ferguson, D. M., & Warner, R. D. (2008). Have we underestimated the impact of pre slaughter stress on meat quality in ruminants ? *Meat Science, 80*(1), 12-19. http://dx.doi.org/10.1016/j.meatsci.2008.05.004.

Fitzgerald, a. C., Edrington, T. S., Looper, M. L., Callaway, T. R., Genovese, K. J., Bischoff, K. M., … Nisbet, D. J. (2003). Antimicrobial susceptibility and factors affecting the shedding of E. coli O157:H7 and Salmonella in dairy cattle. *Letters in Applied Microbiology, 37*(5), 392-398. http://dx.doi.org/10.1046/j.1472-765X.2003.01417.x

Food and Agriculture Organization. (2006). Manipulations avant l'abattage, méthodes d' étourdissement et d' abattage. In FAO (Ed.), *Bonnes Pratiques pour l'industrie de viandes* (2nd ed., pp. 1-20). Rome: Food and Agriculture Organization, Fondation Internationale Carrefour.

Food and Agriculture Organization. (2013). *Food Outlook: Biannual report on global food markets* (GIEWS.). Rome Italy: Trade and Market Division - FAO. Retrieved from http://www.fao.org/Giews/english/fo/index.htm

Gashe, B. A., & Mpuchane, S. (2000). Prevalence of Salmonellae on Beef Products at Butcheries and their Antibiotic Resistance Profiles. *Journal of Food Science, 65*(5), 880-883. http://dx.doi.org/10.1111/j.1365-2621.2000.tb13604.x

Gast, R. K., Guraya, R., Guard-Bouldin, J., Holt, P. S., & Moore, R. W. (2007). Colonization of specific regions of the reproductive tract and deposition at different locations inside eggs laid by hens infected with Salmonella enteritidis or Salmonella heidelberg. *Avian Diseases, 51*(1), 40-44.

Gill, C. O., Badoni, M., & McGinnis, J. C. (1999). Assessment of the adequacy of cleaning of equipment used for breaking beef carcasses. *International Journal of Food Microbiology, 46*(1), 1-8. http://dx.doi.org/10.1016/S0168-1605(98)00181-0

Gill, C. O., McGinnis, J. C., & Bryant, J. (2001). Contamination of beef chucks with Escherichia coli during carcass breaking. *Journal of Food Protection, 64*(11), 1824–1827. http://dx.doi.org/10.1016/S0963-9969(00)00026-0

Gopinath, S., Carden, S., & Monack, D. (2012). Shedding light on Salmonella carriers. *Trends in Microbiology*, *20*(7), 320–327. http://dx.doi.org/10.1016/j.tim.2012.04.004

Gould, L. H., Seys, S., Everstine, K., Norton, D., Ripley, D., Reimann, D., … Selman, C. a. (2011). Recordkeeping practices of beef grinding activities at retail establishments. *Journal of Food Protection, 74*(6), 1022-1024. http://dx.doi.org/10.4315/0362-028X.JFP-10-370

Gregory, N., & Shaw, F. (2000). Penetrating Captive Bolt Stunning and Exsanguination of Cattle in Abattoirs. *Journal of Applied Animal Welfare Science, 3*(3), 215-230. http://dx.doi.org/10.1207/S15327604JAWS0303

Greig, J. D., & Ravel, A. (2009). Analysis of foodborne outbreak data reported internationally for source attribution. *International Journal of Food Microbiology, 130*(2), 77-87. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.12.031

Guy, R. A., Tremblay, D., Beausoleil, L., Harel, J., & Champagne, M. J. (2014). Quantification of E. coli O157 and STEC in feces of farm animals using direct multiplex real time PCR (qPCR) and a modified most probable number assay comprised of immunomagnetic bead separation and qPCR detection. *Journal of Microbiological Methods, 99*(1), 44-53. http://dx.doi.org/10.1016/j.mimet.2014.02.002

Haileselassie, M., Taddele, H., Adhana, K., Kalayou, S., & Tadesse, G. (2013). Food safety knowledge and practices of abattoir and butchery shops and the microbial profile of meat in Mekelle City, Ethiopia. *Asian Pacific Journal of Tropical Biomedicine, 3*(5), 407-12. http://dx.doi.org/10.1016/S2221-1691(13)60085-4

Hald, T., Wingstrand, A., Swanenburg, M., Von Altrock, A., & Thorberg, B. M. (2003). The occurrence and epidemiology of Salmonella in European pig slaughterhouses. *Epidemiol. Infect., 131*, 1187-1203.

Hassanein, R., Fathi, S., Ali, H., & El-malek, A. M. A. (2011). Detection and identification of Salmonella species in minced beef and chicken meats by using Multiplex PCR in Assiut city. *Veterinary World, 4*(1), 5-11.

Hauge, S. J., Nafstad, O., Røtterud, O.-J., & Nesbakken, T. (2012). The hygienic impact of categorisation of cattle by hide cleanliness in the abattoir. *Food Control, 27*(1), 100-107. http://dx.doi.org/10.1016/j.foodcont.2012.03.004

Heim, D., Löpfe, J., Mumford, E., & Speedy, A. (2007). Transmissible spongiform encephalopathies management at the slaughterhouse. In *Management of transmissible spongiform encephalopathies in meat production* (pp. 39-44). Rome Italy: Food and Agriculture Organization.

Heinz, G., & Hautzinger, P. (2007). Principle of meat processing technology. In *Meat Processing Technology for Small to Medium Scale Producers*.

Heredia, N., García, S., Rojas, G., & Salazar, L. (2001). Microbiological condition of ground meat retailed in Monterrey, Mexico. *Journal of Food Protection, 64*(8), 1249-1251.

Huffman, R. D. (2002). Current and future technologies for the decontamination of carcasses and fresh meat. *Meat Science, 62*(3), 285-294.

Hugas, M., & Tsigarida, E. (2008). Pros and cons of carcass decontamination: The role of the European Food Safety Authority. *Meat Science, 78*(1-2), 43-52. http://dx.doi.org/10.1016/j.meatsci.2007.09.001

Jackson, V., Blair, I. S., McDowell, D. A., Kennedy, J., & Bolton, D. J. (2007). The incidence of significant foodborne pathogens in domestic refrigerators. *Food Control, 18*, 346-351.

Jay, M. J., Loessner, J. M., & Golden, A. D. (2005). Food Borne Diseases. In F. S. T. Series (Ed.), *Modern Food Microbiology* (7th ed., pp. 619-650). California: Springer US. http://dx.doi.org/10.1007/b100840

Jiménez, S. M., Tiburzi, M. C., Salsi, M. S., Moguilevsky, M. a, & Pirovani, M. E. (2009). Survival of Salmonella on refrigerated chicken carcasses and subsequent transfer to cutting board. *Letters in Applied Microbiology, 48*(6), 687-91. http://dx.doi.org/10.1111/j.1472-765X.2009.02596.x

Johansson, A., Berglund, L., Eriksson, U., Göransson, I., Wollin, R., Forsman, M., … Sjöstedt, A. (2000). Comparative analysis of PCR versus culture for diagnosis of ulceroglandular tularemia. *Journal of Clinical Microbiology, 38*(1), 22-26.

Juneja, V. K., Eblen, B. S., & Ransom, G. M. (2001). Thermal Inactivation of Salmonella spp . in Chicken Broth, Beef, Pork, Turkey, and Chicken: Determination of D- and Z-values. *Journal of Food Science, 66*(1), 146-152. http://dx.doi.org/10.1111/j.1365-2621.2001.tb15597.x

Juneja, V. K., Snyder, O. P., & Marmer, B. S. (1997). Thermal Destruction of Escherichia coli 0157 : H7 in beef and chicken: determination of D- and z-values. *International Journal of Food Microbiology, 35*, 231-237.

Khaitsa, M. L., Smith, D. R., Stoner, J. a, Parkhurst, a M., Hinkley, S., Klopfenstein, T. J., & Moxley, R. a. (2003). Incidence, duration, and prevalence of Escherichia coli O157:H7 fecal shedding by feedlot cattle during the finishing period. *Journal of Food Protection, 66*(11), 1972-1977.

Kiermeier, A., Bobbitt, J., Vanderlinde, P., Higgs, G., Pointon, A., & Sumner, J. (2006). Use of routine beef carcase Escherichia coli monitoring data to investigate the relationship between hygiene status of incoming stock and processing efficacy. *International Journal of Food Microbiology, 111*(3), 263-269. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.05.006

Koohmaraie, M., Scanga, J., De La Zerda, M., Koohmaraie, B., Tapay, L., & Beskhlebnaya, V. (2012). Tracking the sources of salmonella in ground beef produced from nonfed cattle. *Journal of Food Protection, 75*(8), 1464-1468. http://dx.doi.org/10.4315/0362-028X.JFP-11-540.Tracking

Korsak, N., Clinquart, A., & Daube, G. (2004). Salmonella spp . dans les denrées alimentaires d ' origine animale : un réel problème de santé publique ? *Ann. Méd. Vét, 148*, 174-193.

Kothary, M. H., & Babu, U. S. (2001). Infective dose of foodborne pathogens in volunteers: A review. *Journal of Food Safety, 21*, 49-73.

Loretz, M., Stephan, R., & Zweifel, C. (2011). Antibacterial activity of decontamination treatments for cattle hides and beef carcasses. *Food Control, 22*, 347-359. http://dx.doi.org/10.1016/j.foodcont.2010.09.004

Mackey, B. M., & Derrick, C. M. (1979). Contamination of the Deep Tissues of Carcasses by Bacteria Present on the Slaughter Instruments or in the Gut. *Journal of Applied Bacteriology, 46*(2), 355-366. http://dx.doi.org/10.1111/j.1365-2672.1979.tb00832.x

Magwira, C. a, Gashe, B. a, & Collison, E. K. (2005). Prevalence and antibiotic resistance profiles of Escherichia coli O157:H7 in beef products from retail outlets in Gaborone, Botswana. *Journal of Food Protection, 68*(2), 403–6.

Mainil, J. G., & Daube, G. (2005). Verotoxigenic Escherichia coli from animals, humans and foods: who's who? *Journal of Applied Microbiology, 98*(6), 1332-44. http://dx.doi.org/10.1111/j.1365-2672.2005.02653.x

McCann, M. S., Sheridan, J. J., McDowell, D. A., & Blair, I. S. (2006). Effects of steam pasteurisation on Salmonella Typhimurium DT104 and Escherichia coli O157:H7 surface inoculated onto beef, pork and chicken. *Journal of Food Engineering, 76*(1), 32-40. http://dx.doi.org/10.1016/j.jfoodeng.2005.05.024

McCleery, D. R., Stirling, J. M. E., McIvor, K., & Patterson, M. F. (2008). Effect of ante- and postmortem hide clipping on the microbiological quality and safety and ultimate pH value of beef carcasses in an EC-approved abattoir. *Journal of Applied Microbiology, 104*(5), 1471–9. http://dx.doi.org/10.1111/j.1365-2672.2007.03670.x

McEntire, J., Acheson, D., Siemens, A., Eilert, S., & Robach, M. (2014). The Public Health Value of Reducing Salmonella Levels in Raw Meat and Poultry. *Food Protection Trends, 34*(6), 386-392.

McEvoy, J. M., Doherty, A. ., Sheridan, J. J., Blair, I. S., & McDowell, D. A. (2001). Use of steam condensing at subatmospheric pressures to reduce Escherichia coli O157:H7 numbers on bovine hide. *Journal of Food Protection, 64*(11), 1655-1660.

McEvoy, J. M., Doherty, A. M., Finnerty, M., Sheridan, J. J., McGuire, L., Blair, I. S., … Harrington, D. (2000). The relationship between hide cleanliness and bacterial numbers on beef carcasses at a commercial abattoir. *Letters in Applied Microbiology, 30*(5), 390-395.

McEvoy, J. M., Doherty, A. M., Sheridan, J. J., Bailey, D. G., Blair, I. S., & McDowell, D. A. (2003). The effects of treating bovine hide with steam at subatmospheric pressure on bacterial numbers and leather quality. *Letters in Applied Microbiology, 37*(4), 344-348. http://dx.doi.org/10.1046/j.1472-765X.2003.01406.x

McEvoy, J. M., Sheridan, J. J., Blair, I. S., & McDowell, D. A. (2004). Microbial contamination on beef in relation to hygiene assessment based on criteria used in EU Decision 2001/471/EC. *International Journal of Food Microbiology*, *92*(2), 217–225. http://dx.doi.org/10.1016/j.ijfoodmicro.2003.09.010

Mies, P. D., Covington, B. R., Harris, K. B., Lucia, L. M., Acuff, G. R., & Savell, J. W. (2004). Decontamination of cattle hides prior to slaughter using washes with and without antimicrobial agents. *Journal of Food Protection, 67*(3), 579-582.

Millemann, Y. (2008). Les Salmonelloses et les collibacilloses septicémiques. In J. Gourreau (Ed.), *Maladies des bovins* (4th ed., pp. 56–67). France: Edition France Agricole.

Mohammed, M. A., Sallam, K. I., Eldaly, E. A. Z., Ahdy, A. M., & Tamura, T. (2014). Occurrence, serotypes and virulence genes of non-O157 Shiga toxin-producing Escherichia coli in fresh beef, ground beef, and beef burger. *Food Control, 37*, 182-187. http://dx.doi.org/10.1016/j.foodcont.2013.09.035

Narvaez-Bravo, C., Miller, M. F., Jackson, T., Jackson, S., Rodas-Gonzalez, a, Pond, K., … Brashears, M. M. (2013). Salmonella and Escherichia coli O157:H7 prevalence in cattle and on carcasses in a vertically integrated feedlot and harvest plant in Mexico. *Journal of Food Protection, 76*(5), 786-95. http://dx.doi.org/10.4315/0362-028X.JFP-12-079

Nastasijevic, I., Mitrovic, R., & Buncic, S. (2008). Occurrence of Escherichia coli O157 on hides of slaughtered cattle. *Letters in Applied Microbiology, 46*(1), 126-31. http://dx.doi.org/10.1111/j.1472-765X.2007.02270.x

Nesbakken, T., Nerbrink, E., Rotterud, O. J., & Borch, E. (1994). Reduction of Yersinia entercolitica and Listeria spp. on pig carcasses by enclosure of the rectum during slaughter. *International Journal of Food Microbiology, 23*(2), 197-208. http://dx.doi.org/10.1016/0168-1605(94)90052-3

Nielsen, L. R. (2013). Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of Salmonella Dublin in cattle. *Veterinary Microbiology, 162*(1), 1-9. http://dx.doi.org/10.1016/j.vetmic.2012.08.003

Niyonzima, E., Bora, D., & Ongol, M. P. (2013). Assessment of beef meat microbial contamination during skinning , dressing , transportation and marketing at a commercial abattoir in Kigali city , Rwanda. *Pakistan Journal of Food Science, 23*(3), 133-138.

Nou, X., Rivera-Betancourt, M., Bosilevac, J. M., Wheeler, T. L., Shackelford, S. D., Gwartney, B. L., … Koohmaraie, M. (2003). Effect of chemical dehairing on the prevalence of Escherichia coli O157:H7 and the levels of aerobic bacteria and Enterobacteriaceae on carcasses in a commercial beef processing plant. *Journal of Food Protectionn, 66*(11), 2005–2009.

Nychas, G.-J. E., Skandamis, P. N., Tassou, C. C., & Koutsoumanis, K. P. (2008). Meat spoilage during distribution. *Meat Science, 78*(1-2), 77-89. http://dx.doi.org/10.1016/j.meatsci.2007.06.020

O'Brien, S. B., Duffy, G., Carney, E., Sheridan, J. J., Mcdowell, D. A., & Blair, I. . (2005). Prevalence and numbers of Escherichia coli O157 on bovine hides at a beef slaughter plant. *Journal of Food Protection*, *68*(4), 660–5.

Olopoenia, L. a, & King, a L. (2000). Widal agglutination test - 100 years later: still plagued by controversy. *Postgraduate Medical Journal*, *76*(892), 80–84. http://dx.doi.org/10.1136/pmj.76.892.80

Phillips, D., Sumner, J., Alexander, J. F., & Dutton, K. M. (2001). Microbiological quality of Australian beef. *Journal of Food Protection*, *64*(5), 692–696. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11348002

Pinto Neto, M., Beraquet, N. J., & Cardoso, S. (2013). Effect of chilling methods and hot-boning on quality parameters of M.longissimus lumborum from Bos indicus Nelore steer. *Meat Science, 93*(2), 201-206. http://dx.doi.org/10.1016/j.foodcont.2005.10.018

Prendergast, D. M., Sheridan, J. J., Daly, D. J., McDowell, D. A., & Blair, I. S. (2003). Dissemination of central nervous system tissue from the brain and spinal cord of cattle after captive bolt stunning and carcass splitting. *Meat Science, 65*(4), 1201-1209. http://dx.doi.org/10.1016/S0309-1740(03)00026-3

Rheault, N., & Quessy, S. (1999). Evaluation de la contamination microbienne des plaies de saignee lors du processus d ' abattage des porcs. *Canadian Veterinary Journal, 40*, 261-264.

Rhoades, J. R., Duffy, G., & Koutsoumanis, K. (2009). Prevalence and concentration of verocytotoxigenic Escherichia coli, Salmonella enterica and Listeria monocytogenes in the beef production chain: a review. *Food Microbiology, 26*(4), 357-76. http://dx.doi.org/10.1016/j.fm.2008.10.012

Robertsson, J. A. (1984). Humoral antibody responses to experimental and spontaneous Salmonella infections in cattle measured by ELISA. *Journal of Veterinary Medicine Serie B, 31*, 367-380. http://dx.doi.org/10.1111/j.1439-0450.1984.tb01314.x

Roels, T. H., Frazak, P. a, Kazmierczak, J. J., Mackenzie, W. R., Proctor, M. E., Kurzynski, T. a, & Davis, J. P. (1997). Incomplete sanitation of a meat grinder and ingestion of raw ground beef: contributing factors to a large outbreak of Salmonella typhimurium infection. *Epidemiology and Infection, 119*(2), 127-134. http://dx.doi.org/10.1017/S0950268897007851

Røtterud, O. J., Helps, C. R., Hillman, T. J., Fisher, A. V, Harbour, D., Anil, H., & Nesbakken, T. (2006). Hot boning of intact carcasses: a procedure to avoid central nervous system self-contamination in beef and beef products. *Journal of Food Protection, 69*(2), 405–411.

Russell, N. J. (2002). Bacterial membranes: The effects of chill storage and food processing. An overview. *International Journal of Food Microbiology, 79*(1-2), 27-34. http://dx.doi.org/10.1016/S0168-1605(02)00176-9

Russell, N. J., Evans, R. I., ter Steeg, P. F., Hellemons, J., Verheul, a, & Abee, T. (1995). Membranes as a target for stress adaptation. *International Journal of Food Microbiology, 28*(2), 255-261. http://dx.doi.org/10.1016/0168-1605(95)00061-5

Savell, J. W., Mueller, S. L., & Baird, B. E. (2005). The chilling of carcasses. *Meat Science*, *70*, 449-459. http://dx.doi.org/10.1016/j.meatsci.2004.06.027

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M.-A., Roy, S. L., … Griffin, P. M. (2011). Foodborne Illness Acquired in the United States—Major Pathogens. *Emerging Infectious Diseases, 17*(1), 7–15. http://dx.doi.org/10.3201/eid1701.P11101

Scanga, J. A., Grona, A. D., Belk, K. E., Sofos, J. N., Bellinger, G. R., & Smith, G. C. (2000). Microbiological contamination of raw beef trimmings and ground beef. *Meat Science, 56*(2), 145-52.

Sheridan, J. J. (1998). Sources of contamination during slaughter and measures for control. *Journal of Food Safety*, *18*, 321–339.

Sheridan, J. J., Lynch, B., & Harrington, D. (1992). The effect of boning and plant cleaning on the contamination of beef cuts in a commercial boning hall. *Meat Science*. http://dx.doi.org/10.1016/0309-1740(92)90105-D

Sierra, M. L., Sheridan, J. J., & McGuire, L. (1997). Microbial quality of lamb carcasses during processing and the acridine orange direct count technique (a modified DEFT) for rapid enumeration of total viable counts. *International Journal of Food Microbiology, 36*(1), 61-67. http://dx.doi.org/10.1016/S0168-1605(96)01247-0

Small, A., & Buncic, S. (2009). Potential for the cross-contamination of the hides of cattle while they are held in lairage. *The Veterinary Record, 164*(9), 260-265. http://dx.doi.org/10.1136/vr.164.9.260

Small, A., Reid, C. A., Avery, S. M., Karabasil, N., Crowley, C., & Buncic, S. (2002). Potential for the spread of Escherichia coli O157, Salmonella, and Campylobacter in the lairage environment at abattoirs. *Journal of Food Protection, 65*(6), 931-6.

Small, A., Wells-Burr, B., & Buncic, S. (2005). An evaluation of selected methods for the decontamination of cattle hides prior to skinning. *Meat Science*, *69*(2), 263–268. http://dx.doi.org/10.1016/j.meatsci.2004.07.005

Stevens, A., Kaboré, Y., Perrier-Gros-Claude, J.-D., Millemann, Y., Brisabois, A., Catteau, M., … Dufour, B. (2006). Prevalence and antibiotic-resistance of Salmonella isolated from beef sampled from the slaughterhouse and from retailers in Dakar (Senegal). *International Journal of Food Microbiology, 110*(2), 178–186. http://dx.doi.org/10.1016/j.ijfoodmicro.2006.04.018

Swanson, J. C., & Morrow-Tesch, J. (2001). Cattle transport : Historical , research , and future perspectives. *Journal of Animal Science*, *79*, 102–109.

Tafida, S. Y., Kabir, J., Kwaga, J. K. P., Bello, M., Umoh, V. J., Yakubu, S. E., … Hendriksen, R. (2013a). Occurrence of Salmonella in retail beef and related meat products in Zaria, Nigeria. *Food Control, 32*(1), 119–124. http://dx.doi.org/10.1016/j.foodcont.2012.11.005

Tafida, S. Y., Kabir, J., Kwaga, J. K. P., Bello, M., Umoh, V. J., Yakubu, S. E., … Hendriksen, R. (2013b). Occurrence of Salmonella in retail beef and related meat products in Zaria, Nigeria. *Food Control, 32*, 119-124. http://dx.doi.org/10.1016/j.foodcont.2012.11.005

Temellİ, S., Eyİgör, A., & Anar, Ş. (2012). Prevalence of Escherichia coli O157 in red meat and meat products determined by VIDAS ECPT and LightCycler PCR. *Turk. J. Vet. Anim. Sci, 36*(3), 305-310. http://dx.doi.org/10.3906/vet-1107-38

Teunis, P. F. M., Kasuga, F., Fazil, A., Ogden, I. D., Rotariu, O., & Strachan, N. J. C. (2010). Dose-response modeling of Salmonella using outbreak data. *International Journal of Food Microbiology, 144*(2), 243-249. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.09.026

Vipham, J., Brashears, M., Loneragan, G., Echeverry, A., Brooks, J., Chaney, W., & Miller, M. (2012). Salmonella and Campylobacter baseline in retail ground beef and whole-muscle cuts purchased during 2010 in the United States. *Journal of Food Protection, 75*(12), 2110-2115. http://dx.doi.org/10.4315/0362-028X.JFP-12-077.Salmonella

Warriner, K., Tg, A., Kaur, S., & Ce, D. (2002). Cross-contamination of carcasses and equipment during pork processing. *Journal of Applied Microbiology, 93*(1), 169-77.

Wheatley, P., Giotis, E. S., & Mckevitt, A. I. (2014). Effects of slaughtering operations on carcass contamination in an Irish pork production plant. *Irish Veterinary Journal, 67*(1), 1-6. http://dx.doi.org/10.1186/2046-0481-67-1

Wong, L. F., Hald, T., Wolf, P. J. Van Der, & Swanenburg, M. (2002). Epidemiology and control measures for Salmonella in pigs and pork. *Livestock Production Science, 76*, 215-222.

Wotton, S. B., Gregory, N. G., Whittington, P. E., & Parkman, I. D. (2000). Electrical stunning of cattle. *The Veterinary Record, 147*(24), 681–684.

Yang, X., Balamurugan, S., & Gill, C. O. (2011). Effects on the development of blown pack spoilage of the initial numbers of Clostridium estertheticum spores and Leuconostoc mesenteroides on vacuum packed beef. *Meat Science, 88*(3), 361-367. http://dx.doi.org/10.1016/j.meatsci.2011.01.010

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Antibacterial and Antioxidant Activity of Extracts from Selected Probiotic Bacteria

Richard Nyanzi[1], Daniel S. S. Shuping[2], Piet J. Jooste[1] & Jacobus N. Eloff[2]

[1] Department of Biotechnology and Food Technology, Tshwane University of Technology, Pretoria, South Africa

[2] Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

Correspondence: Richard Nyanzi, Department of Biotechnology and Food Technology, Tshwane University of Technology, Pretoria, 175 Nelson Mandela Drive, Private Bag X680, Pretoria, 0001, South Africa. Tel: 27-12-382-6240. E-mail: nyanzir@tut.ac.za

Received: July 22, 2015 Accepted: August 27, 2015 Online Published: September 11, 2015

doi:10.5539/jfr.v4n5p122 URL: http://dx.doi.org/10.5539/jfr.v4n5p122

Abstract

Probiotic extracts can potentially be used as bio-preservatives and in reduction of oxidative stress. The study investigated the antibacterial and antioxidant activity of methanol extracts from freeze-dried cells of probiotic *Lactobacillus* strains identified using molecular techniques. The quantitative microplate method, which employed *p*-iodonitrotetrazolium (INT) and the method by Brand-Williams et al. (1995) were employed to investigate quantitatively the antibacterial and the antioxidant activity, respectively, of probiotic extracts. The MIC values extracts from most probiotic strains, tested against indicator bacterial pathogens, were in the range of 1.25 – 5 mg/mL while that of *Lb. casei* strain B and *Lc. lactis* subsp *lactis* strain X was at least 20 mg/mL after 24 h of incubation at 37°C. At the highest extract concentration of 20 mg/mL used in the study, *Lb. acidophilus*, *Lb. rhamnosus* and *Lb. casei* strains had 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities of 77.9 - 86.1%, 45.7 - 86.4% and 36.9 – 45.8% respectively. Quantitative antibacterial and antioxidant activities of methanol extracts from freeze-dried cells of probiotic *Lactobacillus* strains was determined for the first time.

Keywords: antibacterial, antioxidant, *Lactobacillus*, methanol extract, probiotic

1. Introduction

Probiotics are defined as 'live microorganisms of human intestinal origin, which, in adequate quantities, impart health benefits to the consumer beyond basic nutrition' (FAO/WHO, 2002). Several of the probiotic species belong to the *Lactobacillus* and *Bifidobacterium* genera and have been used extensively in foods, beverages and therapeutic formulations as probiotic strains due to their GRAS (generally recognized as safe) status (Franz et al., 2003; Nyanzi & Jooste, 2012). The potential health benefits of probiotics include improvement in gut health and lactose intolerance, prevention of diarrhoea, constipation alleviation, reduction in serum cholesterol, prevention of vaginitis and intestinal infections, management of diabetes mellitus, prevention of hepatic diseases and inflammatory bowel disease, risk reduction in colon tumours and cancer, oxidative stress reduction and host immune system modulation (Marchand & Vandenplas, 2000; Holzapfel & Schillinger, 2002; Blandino et al., 2003; Franz et al., 2003; Rousseau et al., 2005; Shen et al., 2011; Ejtahed et al., 2012; Li et al., 2012; Sharma et al., 2014; Nyanzi et al., 2014). A number of probiotic strains antagonize enteropathogens and the proposed mechanisms (although not always clear) include competition for nutrients and receptor sites, aggregation with bacterial pathogens and hydrogen peroxide production, production of bacteriocins (protein-like substances) and, stimulation of the immune system (Shalev et al., 1996; Atanassova et al., 2003; Strus et al., 2005; Guéniche et al., 2010; Bendali et al., 2011; Nyanzi et al., 2014).

Diarrhoea is a global public health challenge and annually as many as 2 million people, mainly under the age of 5 years, die in the developing world (Bendali et al., 2011). Enteropathogenic *E. coli* is the major cause of diarrhoea while *Salmonella enterica* Typhimurium is linked to human gastroenteritis (Bendali et al., 2011). Antibiotics can reduce infections, however, antibiotic resistance as in the case of methicillin-resistant *Staphylococcus aureus* implies that there is a need to have alternative pathogen inhibitors (Yasunaka et al., 2005).

Plant extracts have been shown to have antimicrobial activity, however, cytotoxicity can be a concern while, on the other hand, probiotics have not been shown to be toxic.

Antibacterial effects of lactic acid bacteria (LAB) have been reported (Ström et al., 2002). The antibacterial activity of the probiotic strains is largely attributed to low pH in their growth environments and the production of organic acids and bacteriocins (Atanassova et al., 2003; De Muynck et al., 2004; Strus et al., 2005). There are reports of the demonstrated antimicrobial activity of organic acids (Ström et al., 2002; De Muynck et al., 2004), probiotic culture supernatants and their ethyl acetate extracts (Lavermicocca et al., 2000). Besides antimicrobial activity, probiotics are also reported to have antioxidant and anti-ageing activities (Ejtahed et al., 2012; Li et al., 2012). The presence of excessive reactive oxygen species (ROS) such as hydroxyl radicals (OH), superoxide anions ($^{\cdot}O_2^-$) and hydrogen peroxide (H_2O_2), released during cellular metabolism, in the absence of adequate antioxidants can cause *in vivo* oxidative damage to biomolecules such as proteins, lipids and chromosomes (Choi et al., 2002; Kullisaar et al., 2002; Wang et al., 2009; Li et al., 2012). *Lactobacillus* strains with proven or demonstrated antioxidant activities *in vitro*, may be applied in the prevention or reduction of oxidative stress. However, information relating to the antibacterial and antioxidant activity of extracts from freeze-dried probiotic bacterial cells is scanty. Consequently the aim of the present study was to determine possible antibacterial and antioxidant activities of methanol extracts from freeze-dried preparations of selected *Lactobacillus* strains.

2. Materials and Methods

2.1 Probiotic Cultures

A range of *Lactobacillus* strains were isolated from probiotic dairy products, pharmaceutical probiotic supplements while others were obtained from supplier companies. Thirteen *Lactobacillus* strains examined in this study were selected from the original number of 32 isolates on the basis of their bile tolerance, acid tolerance and antibiotic resistance profiles (Nyanzi, 2013). Phenotypic identification of the probiotic isolates was done using API 50 CHL galleries as well as the API WEB (V3.2 and V5.1) software (API system, Biomerieux, France). The probiotic strains were preserved as frozen cultures according to the procedure described by Nyanzi (2007).

2.2 Confirmation of Identity of Presumptive Probiotic Isolates

The identities of the isolates were confirmed by employing molecular typing techniques (Nyanzi et al., 2013) and the MEGA 6.06 software (Tamura et al., 2013) for phylogenetic analysis using sequences of the 16S rRNA gene [amplified using 27F and 1492R universal primers (5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3' respectively; Guo et al., 2010)], and *rpoA* and *pheS* protein-coding genes [amplified using primer pairs, rpoA-21-F / rpoA-23-R (5'-ATGATYGARTTTGAAAAACC-3' / 5'-ACHGTRTTRATDCCDGCRCG-3') and pheS-21-F / pheS-23-R (5'-CAYCCNGCHCGYGAYATGC-3' / 5'-GGRTGRACCATVCCNGCHCC-3') respectively; Naser et al., 2005].

The PCR products were subjected to gel electrophoresis and sequenced in a Genetic analyser ABI PRISM^{TM} 3100 (Applied Biosystems, USA). The generated sequences were edited using softwares, Chromas Lite 2.0 and BioEdit v. 7.0.9 (http://en.bio-soft.net/format.html/BioEdit). The isolates were identified by comparing the sequences obtained to those in the nucleotide database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/blastn/) using the *blastn* search option.

2.3 Indicator Bacterial Pathogens

The indicator bacterial pathogens used were from the American Type Culture Collection (ATCC) and included the following: *Escherichia coli* ATCC 8739, *E. coli* ATCC 11775, *Staphylococcus aureus* ATCC 6538, *S. aureus* ATCC 12600 and *Salmonella typhimurium* ATCC 14028. The cultures were maintained in nutrient broth and were preserved as frozen cultures as described by Nyanzi (2007).

2.4 Probiotic Bacterial Cell Mass Growth and Extraction Procedures

The procedure followed was as described by Nyanzi et al. (2014). In brief, two litres of De Man, Rogosa and Sharpe broth (MRS broth), in a 2L Duran Schott bottle, were inoculated with 4% of an overnight probiotic broth culture and incubated in a water bath at 37°C for 18 to 20 h. The MRS broth culture was centrifuged (Sorvall RC 6 centrifuge, USA) at 7000 rpm at 4°C for 10 minutes (min) to separate the cell pellet from the supernatant. The cell pellet was washed twice with phosphate buffer saline (PBS) and then suspended in sterilized, distilled water. The suspension was frozen at -80°C for at least 4 h and then freeze-dried at -85°C for 24 h. The freeze-dried bacterial cell mass was then subjected to particle reduction using a mortar and pestle. The powdered bacterial cell mass was stored in sterile bottles ready for particle size analysis and extraction as described by Nyanzi et al. (2014).

The powdered cell pellet was extracted using methanol. Acetone was not used because in a previous study (Nyanzi et al., 2014), such extracts were found to be ineffective. The cell powder (2 g) and the methanol extractant (10 mL) were mixed, vigorously shaken and then sonicated (Bandelin electronic sonicator, Pro-Nr 780.06021340.008, Berlin, Germany) at 100% power (120/240 W, 1.2 A, 230 V, 35 KHz frequency) for 15 min prior to filtering using Whatman No. 1 filter paper. The filtrate was dried in a fume cupboard at room temperature and the dried extract was weighed before storage.

2.5 Determination of the Minimum Inhibitory Concentrations (MICs) of Extracts

The two-fold serial-dilution microtitre plate method described by Eloff (1998), which employs *p*-iodonitrotetrazolium (INT) violet as an indicator of microbial growth, was used to determine the MICs of the extracts obtained in section 2.4. The MIC value was recorded as the lowest concentration of extract that inhibits growth of test organisms (Shai et al., 2009). A 100 µL volume of sterile distilled water was pipetted into each of the wells of the microtitre plate.

2.5.1 Antibacterial Assay

One hundred microliters (100 µL) of the extracts (80 mg/mL) dissolved in 25% acetone in water was added to separate microtitre plate wells in row A in duplicate. After thorough mixing of the well contents in row A, 100 µL was withdrawn and added to the next well in row B and thoroughly mixed. The procedure was continued until all the wells were mixed with extract except for row H. Some of the columns were used for sterility control and growth control in which cases test bacteria were not added or extracts were replaced with a standard antibiotic agent (0.1 mg/mL gentamicin). Test bacterial pathogen cultures were prepared by adjusting the turbidity of the bacterial suspension to a McFarland standard 0.5 that was equivalent to a concentration of 1-5 x 10^7 cfu/mL and was further diluted (1:100) in nutrient broth to obtain a final concentration of 1-5 x 10^5 cfu/mL. A volume of 100 µL of the broth culture of each of the test pathogens was distributed into separate wells with the exception of the wells reserved for the sterility controls. The microtitre plates were then sealed in a plastic bag and incubated at 37°C for 16 -18 hours (hr) at 100% humidity. To demonstrate the presence or absence of growth, 40 µL of 0.2 mg/mL of INT violet, (dissolved in sterile distilled water) was added to each of the wells and the plates were incubated for a further 2 hours to promote visualization. Bacterial growth was shown by the development of a red colour arising from the formation of the red/purple formazan (Eloff, 1998).

2.5.2 Antioxidant Activity of Extracts

A solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Cat. No.: D913-2, Sigma-Aldrich, Germany) was prepared at a concentration of 3.7 mg DPPH/ 100 mL of absolute methanol (analytical grade). The initial concentration of probiotic extracts was 80 mg/mL in 25% acetone (Sigma-Aldrich). This concentration was subjected to a two-fold serial dilution using 25% acetone until a final concentration of 1.25 mg/mL was obtained. The initial concentration of ascorbic acid (control) was also two-fold serially diluted until a final concentration of 0.03125 mg/mL was obtained. The dilutions of the extracts and ascorbic acid were performed in a microplate deep well (Cat. No.: 219012, 96-well 1 mL round well, Porvair Sciences, UK). The antioxidant assay was performed in a 96 well microtitre plate with each well filled with 200 µL of DPPH solution. The extracts and ascorbic acid along with their sequential dilutions, in 50 µL volumes, were introduced to the microtitre plate using a multi-channel pipette. The assay was replicated 5 times and 25% acetone was used as the negative control. The method was based on the description by Shikanga et al. (2010). A spectrophotometric reading was taken in an ELX 800 Universal Microplate Reader (Serial No. 185050, BioTek Instruments Inc., USA). Corrected antiradical activity was calculated using the following formula:

Total DPPH antiradical activity (%) = [1-(A570 nm sample / A570 nm control)] X 100] (Ng et al., 2011).

3. Results and Discussion

3.1 Confirmation of Identity of Bacterial Isolates

The studied isolates were identified based on their 16S rDNA sequences. The identity of the study isolates was confirmed using *rpoA* and *pheS* gene sequences (Table 1). The 16S rRNA gene is the most frequently used but not very discriminative for sole use between closely related strains (Rajendhran & Gunasekaran, 2011), while the *rpoA* and *pheS* genes are reported to be more discriminative than the former (Naser et al., 2005; Naser et al., 2007). The measurable superiority of *rpoA* and *pheS* gene sequencing over the 16S rRNA gene sequencing in discriminating and identifying the LAB isolates was largely illustrated by Nyanzi et al. (2013). The strains studied were regarded as probiotic since most species or strains used as such belong to the genera *Lactobacillus* and *Bifidobacterium* (Blandino et al., 2003; Manning & Gibson, 2004; Commane et al., 2005; Picard et al., 2005) and are generally regarded as safe (Prado et al., 2008; Liu et al., 2009; Lannitti & Palmieri, 2010).

Table 1. Confirmation of the identity of the probiotic isolates used in the present study

Isolate code	16S rDNA sequence identification	*pheS* gene sequence identification	*rpoA* gene sequence identification
M	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
N	*Lb. casei*	*Lb. casei*	*Lb. casei*
O	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
P	*Lb. casei*	*Lb. casei*	*Lb. casei*
C	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
D	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
Y	*Lb. rhamnosus*	*Lb. rhamnosus*	*Lb. rhamnosus*
B	*Lb. casei*	*Lb. casei*	*Lb. casei*
X	*Lc. lactis* subsp. *lactis*	*Lc. lactis* subsp. *lactis*	*Lc. lactis* subsp. l*actis*
U	*Lb. acidophilus*	*Lb. acidophilus*	*Lb. acidophilus*
Z	*Lb. helveticus*	*Lb. helveticus*	*Lb. helveticus*
V	*Lb. acidophilus*	*Lb. acidophilus*	*Lb. acidophilus*
W	*Lb. acidophilus*	*Lb. acidophilus*	*Lb. acidophilus*

3.2 Particle Size of Extracted Powder from Freeze-Dried Cells of LAB

The particle size of powdered freeze-dried cells of the LAB strains was in the range 44.85 – 70.22 μM (Nyanzi, 2013). This meant that the differences were generally marginal and the powder samples would be expected to have relatively similar extractability. However, the yield of the extracts ranged between 4.14% and 42.08% (Nyanzi, 2013) and the differences may be attributed to species and strain differences of the probiotic organisms in Table 1.

3.3 The Minimum Inhibitory Concentrations (MIC) of Extracts

3.3.1 Antibacterial Activity of extracts from Freeze-Dried Probiotic Bacteria

The probiotic *Lactobacillus* methanol extracts had a dose related inhibition of the indicator bacterial pathogens *E. coli* ATCC 8739, *S. aureus* ATCC 6538 and *Salmonella typhimurium* ATCC 14028. Table 2 presents a summary of the MIC values of the extracts from selected strains of *Lactobacillus* species against the indicator pathogens after different periods of incubation at 37°C.

Table 2. The MICs of probiotic extracts of the *lactobacillus* test strains against the selected bacterial pathogens after different time intervals

Probiotic strain extract	MICs (mg/mL) against indicator organism								
	E. coli ATCC 8739			*S. aureus* ATCC 6538			*Sa. typhi* ATCC 14028		
	24 h*	48 h	60 h	24 h	48 h	60 h	24 h	48 h	60 h
U	5	5	7.5	2.5	5	10	5	5	5
V	5	5	10	2.5	5	10	5	5	10
W	10	10	20	2.5	10	10	5	10	20
Z	5	5	10	2.5	5	5	5	5	5
M	5	10	10	5	5	10	5	10	10
Y	2.5	5	5	2.5	5	5	2.5	5	10
O	5	5	10	5	5	10	5	10	10
D	2.5	5	10	2.5	5	5	2.5	5	5
C	5	5	5	5	5	5	5	10	10
X	20	>20	>20	10	20	>20	20	>20	>20
P	5	10	10	5	10	10	5	10	10
B	20	>20	>20	10	>20	>20	20	>20	>20
N	2.5	5	10	1.25	5	5	2.5	5	10

* h= hours.

The MIC value of extracts from *Lb. acidophilus* strain U and V, and *Lb. helveticus* strain Z was 2.5 mg/mL against *S. aureus* ATCC 6538 while against *E. coli* ATCC 8739 and *Salmonella typhimurium* ATCC 14028, it was 5 mg/mL after 24 h of incubation at 37°C. This implied that inhibition was not only specific to the probiotic strain, but also to the pathogen strain.

The MIC value of extracts from *Lb. rhamnosus* strain Y and D was 2.5 mg/mL while that of *Lb. rhamnosus* strains M, C and O was 5 mg/mL against indicator pathogens after 24 h of incubation at 37°C. *Lactobacillus casei* strain B and *Lactococcus lactis* subsp. *lactis* strain X had a MIC value of 20 mg/mL, or more, against indicator bacterial pathogens. However, *Lb. casei* strain N and P had values of 1.25 - 2.5 mg/mL and 5mg/mL as MIC values against the indicator pathogens after 24 h of incubation at 37°C.

Overall, extracts from *Lb. rhamnosus* strain Y and D and *Lb. casei* strain N had the highest antimicrobial activity since their MIC values, in the range 1.25 – 2.5 mg/mL after 24 h, were lower than extracts from the other *Lactobacillus* strains tested. Since extracts from *Lb. rhamnosus* strains (M, C and O) and *Lb. casei* strain P had an MIC of 5 mg/mL, they could also be considered along with extracts from strains Y, D and N for further investigation.

From the results in Table 2, it was clear that some extracts were bacteriostatic because the MICs increased after an extended incubation period. However, when 100 µL from row 'A' wells of the microtitre plate with a concentration of 20 mg/mL, was aseptically spread-plated on nutrient agar (Merck, Germany) plates, no growth was observed after incubating aerobically for 24 h at 37°C. In contrast contents from corresponding dilutions in rows 'F' and 'H' of the microtitre plate with concentrations of 0.625 mg/mL and 0 mg/mL respectively, led to confluent growth on the nutrient agar petri-plates. This indicates that at higher concentrations the extracts were bactericidal.

With the exception of recent results of Nyanzi et al. (2014), there has been hardly any report on determining the MICs of methanol extracts from probiotic cells. Other scientists have found evidence for the anti-pathogenic activity of probiotics using supernatants in agar disc diffusion methods (Voulgari et al., 2010; Bendali et al., 2011) or a spot-on lawn method (Bendali et al., 2011). These authors found that *S. aureus, L. monocytogenes* and other bacterial pathogens were inhibited by by-products of LAB and that *Pseudomonas aeruginosa*, *K. pneumoniae*, *E. faecalis*, *B. cereus* and *S. aureus* were inhibited by *Lb. paracasei* subsp. *paracasei* (Bendali et al., 2011). Using the spot-on lawn method, pathogen inhibition by LAB was attributed to bacteriocins (De Muynck et al., 2004) or to fermentation by-products such as organic acids, hydrogen peroxide and antibiotics (Ström et al., 2002; Atanassova et al., 2003; De Muynck et al., 2004).

A major problem with these methods is that agar diffusion and spot-on lawn methods do not provide results that can be compared between different laboratories. Furthermore, if plant extracts contain non-polar antimicrobial compounds, agar diffusion methods under estimate the activity because the non-polar compounds would not readily diffuse in an aqueous agar matrix (Eloff & McGaw, 2014). By determining the MICs, the potential that these preparations could be active *in vivo* can be evaluated.

There have been many viewpoints on what should be considered as an interesting MIC for plant extracts. These values varied from 8 mg/mL (Fabry et al., 1998) to 0.1 mg/mL (Eloff, 2004; Rios & Recio, 2005; Cos et al., 2006). The lowest MIC found in this study 1.25 mg/mL. In evaluating the probability of *in vivo* efficacy, the aim of using the extract should be kept in mind. For an oral dosage of a plant extract parental concentrations of higher than 0.1 mg/mL could be too high. In the case of probiotics, which are not known to be toxic, a concentration of probiotic extracts greater than 0.1 mg/mL may arguably not be considered to be too high.

Other mechanisms such as pathogen adhesion prevention against infections may also play a role (Johnson-Henry et al., 2007). Surface-layer protein (SLP) extracts from the paracrystalline layer on the outside of the cell wall of probiotic strain *Lb. helveticus* R0052, inhibited adhesion of *E. coli* 0157:H7 to epithelial cells (Johnson-Henry et al., 2007). Pathogen antagonism against adhesion to epithelial surfaces (Johnson-Henry et al., 2007) and inhibition of their growth, as observed in the present study, is indicative of potential benefits that can be derived from non-viable probiotic cell extracts. This implies that probiotic bacterial extracts may have the potential for application as biopreservatives. In the present study, lower extract concentrations of some LABs were bacteriostatic and higher extract concentrations were bactericidal.

3.3.2 Antioxidant Activity of Probiotic Cell Extracts

The 2,2-Diphenyl-picrylhydrazyl (DPPH) free radical reduction spectrophotometric assay has been used to determine the antioxidant activity of extracts and compounds from different sources (Armatu et al., 2010). The assay depends on the stability of DPPH free radicals, which get decolourised by an effective antioxidant (Armatu

et al., 2010). The antioxidant activity of methanol extracts of probiotics has been determined by reaction with 2,2-Diphenyl-picrylhydrazyl (DPPH), a stable free radical, in a solution of methanol (Brand-Williams et al., 1995).

Table 3 shows the antioxidant activity of the methanol extracts from freeze-dried probiotic bacteria at concentrations in the range of 1.25 – 20 mg/mL. The anti-radical activity was presented in the form of percentages (%) by which different concentrations of the probiotic extracts reduced the absorbance of control DPPH in methanol at a wave length of 570 nm. Different wavelengths have been used in the past, Brand-Williams et al. (1995) and Shikanga et al. (2010) used 515 nm and 492 nm respectively while Armatu et al. (2010) and Ng et al. (2011) used 517 nm in determining antiradical activities.

Among the *Lb. acidophilus* strains tested in the present study, extracts from strains U and V reduced the DPPH free radicals by 19% to 86.7% while extract from strain W, with a slightly weaker antiradical capacity, had a DPPH scavenging activity of 11.4% to 82%. Among the *Lb. rhamnosus* strains, the extract from strain Y (10.2% – 86.4%) had the highest free radical scavenging activity while the extract from strain M (4.9% - 45.7%) had the least DPPH absorbance reducing activity. *Lactobacillus rhamnosus* strain D (11.3% - 66.9%) was relatively moderate in its antiradical activity. The antioxidant activity of the extract from *Lc. lactis* subsp. *lactis* strain X in the range 15.6% to 81.2% was almost double the free-radical scavenging activity (2.6% to 45.8%) of the extracts from *Lb. casei* strains (P, B, N).

It is worth noting that while the extract from *Lb. rhamnosus* strain Y, which had the highest antibacterial activity, also had the highest antioxidant activity as already stated. On the other hand, the extract from *Lb. casei* strain N had a low antioxidant activity despite depicting the highest antibacterial activity (MIC value of 1.25 mg/mL against *S. aureus*).

Table 3. Antioxidant activity (%) of selected probiotic bacteria methanol extracts

	Concentration of extracts (mg/mL)						IC_{50} of extracts
Source of extracts	20	10	5	2.5	1.25	Linear equation	(mg/mL)
Lb. acidophilus group							
U	84.50 ± 1.13	82.8 7 ± 1.37	56.62 ± 3.62	33.11 ± 2.48	18.99 ± 3.56	Y = 9.9446X + 7.235	4.3
V	86.07 ± 0.7	80.11 ± 1.52	57.02 ± 0.95	33.88 ± 2.71	21.91 ± 0.63	Y = 9.3474X + 10.34	4.24
W	81.98 ± 1.77	52.20 ± 1.53	30.32 ± 2.21	17.13 ± 0.43	11.40 ± 1.42	Y = 5.0783X + 4.805	8.9
Z	77.93 ± 1.81	71.92 ± 1.26	55.08 ± 3.75	36.96 ± 1.42	24.11 ± 3.99	Y = 8.114X + 15.05	4.31
Lb. rhamnosus group							
M	45.71 ± 3.97	44.35 ± 7.32	26.80 ± 2.99	10.65 ± 3.14	4.91 ± 1.63	Y = 5.9263X -3.165	8.97
Y	86.38 ± 0.54	63.07 ± 0.81	39.23 ± 2.3	21.32 ± 2.66	10.23 ± 1.1	Y = -7.652X+ 98.725	6.37
O	73.07 ± 1.4	47.76 ± 1.99	29.11 ± 1.28	13.13 ± 0.76	7.80 ± 3.25	Y = -5.784X + 100.19	8.7
D	66.87 ± 1.67	55.87 ± 0.77	38.36 ± 0.79	19.57 ± 0.47	11.29 ± 1.82	Y = -7.2611X + 98.11	6.63
C	79.86 ± 1.07	62.85 ± 1.11	37.51 ± 1.82	21.26 ± 1.71	14.84 ± 2.24	Y = -2.663X + 70.995	7.88
Lb. casei group							
N	45.8 ± 1.19	39.76 ± 2.87	26.64 ± 2.24	11.94 ± 1.23	6.91 ± 2.33	Y = -5.350X + 100.44	9.43
P	45.24 ± 0.82	41.64 ± 2.33	29.86 ± 1.22	15.84 ± 1.12	12.68 ± 0.98	Y = -4.72X + 94.33	9.39
B	36.92 ± 0.44	22.27 ± 1.04	10.49 ± 1.42	5.77 ± 1.51	2.63 ± 0.64	Y = -2.066X + 99.73	24.07
X	81.22 ± 2.25	70.47 ± 0.86	48.42 ± 1.01	28.25 ± 1.69	15.62 ± 2.95	Y = 8.6497X + 5.535	5.14
Control (Ascorbic acid)	90.99 ± 1.14	82.8 ± 0.55	73.46 ± 0.94	69.22 ± 0.61	50.13 ± 1.52	Y = 62.515X + 40.913	0.15

To the best of our knowledge, this is the first time that methanol extracts from freeze-dried cells of selected *Lactobacillus* strains were shown to possess free radical DPPH scavenging activity. Cell-free supernatant extracts from *Lb. plantarum* C88 and *Lb. fermentum* had DPPH inhibition activities of 53.1% and 87.9% respectively (Wang et al., 2009; Li et al., 2012). In a study of *Bf. animalis* 01, Shen et al. (2011) observed that MRS broth, culture supernatant, intact cells and intracellular cell free extracts had a DPPH free radical scavenging activity of 6.6%, 73.1%, 11.1% and 27.7% respectively. In other studies, *Lactobacillus* strains such as *Lb. brevis* BJ20 (Lee et al., 2010; Li et al., 2012), *Lb. plantarum* 7FM10 (Kanno et al., 2012; Li et al., 2012) have also been shown to have DPPH anti-radical activity.

At the highest extract concentration of 20 mg/mL used in this study, *Lb. acidophilus*, *Lb. rhamnosus* and *Lb. casei* strains had DPPH scavenging activities of 77.9 - 86.1%, 45.71 - 86.4% and 36.92 – 45.80% respectively.

To compare the efficacy of different extracts, the concentration scavenging 50% of DPPH free radicals was calculated using a linear equation, derived for each extract, from a graph of percentage scavenging activity and probiotic extract concentration (IC_{50}). Extracts from *Lb. acidophilus* strains U, V and *Lb. helveticus* strain Z had the highest antioxidant activity since they each had the lowest IC_{50} of 4.24 - 4.31 mg/mL. All extracts from the *Lb. rhamnosus* strains studied had an IC_{50} in the range 6.37 – 8.97 mg/mL. Extracts from *Lb. casei* strains were the least active in reducing DPPH free radicals as they had higher IC_{50} values in the range 9.35 – 24.07 mg/mL. The antioxidant activity of extracts from non-probiotic *Lc. lactis* subsp. *lactis* strain X (5.14 mg/mL) was only second to that of *Lb. acidophilus* strains U and V (4.27 – 4.3 mg/mL). This augments the findings of Li *et al*. (2012) that *Lc. lactis* subsp *lactis* strain 12 had free-radical scavenging activity.

Oxidative damage and/or stress *in vivo* can negatively affect the health status of patients suffering from ailments that include cancer, allergies, cardiovascular diseases, atherosclerosis, inflammation, progression of HIV status to AIDS and senescence-related diseases such as rheumatoid arthritis, Parkinson's disease and Alzheimer's disease (Choi et al., 2002; Shen et al., 2011; Li et al., 2012). Unhindered oxidative stress may deteriorate type 2 diabetes and subsequently weaken the antioxidant defenses (Ejtahed et al., 2012). The present study showed that extracts from freeze-dried probiotic cells contain antioxidant components at varying levels. This suggests that the consumer of selected probiotic strains may benefit through oxidative stress reduction. This has been observed in reported nutritional intervention studies involving *Lb. acidophilus* LA5; *Bf. animalis* subsp. *lactis* Bb12; and *Lb. fermentum* ME-3 (Kullisaar et al., 2003; Ejtahed et al., 2012) and in vivo studies involving Lb. plantarum C88 (Li et al., 2012) and Lb. casei Zhang (Zhang et al., 2010). The antioxidant activity of LAB has been attributed to cell-surface proteins, exopolysaccharides and lipoteichoic acids which are capable of chelating iron metal and scavenging superoxide anions (Kullisaar et al., 2002; Li et al., 2012). These attributes were corroborated by Choi *et al*. (2006). The heat-killed cells (10^8 cfu/mL) and soluble polysaccharides (10 mg/mL) from *Lb. acidophilus* 606 exhibited a potent antioxidant activity of 52.06 ± 2.06% and 36.05 ± 3.65% respectively in terms of DPPH free radical scavenging ability (Choi et al., 2006). According to Choi et al. (2006), it was the soluble polysaccharide fraction, and not the enzymatic fraction, from *Lb. acidophilus* 606 that was responsible for the antioxidant activity. The findings of the present study, augmented by other workers' reports, imply that cells or components of certain *Lactobacillus* strains can be the source of effective antioxidant activity.

4. Conclusions

This study investigated possible antibacterial activity and antioxidant activity of methanol extracts from freeze-dried cells of probiotic *Lactobacillus* strains. The methanol extracts from certain *Lactobacillus* strains demonstrated measurable antibacterial activity. Extracts from *Lb. rhamnosus* strains, Y and D, and *Lb. casei* strain N had the highest antibacterial activity and their MIC values were in the range 1.25 – 2.5 mg/mL after 24 h of incubation at 37°C. Extracts from *Lactobacillus* strains M, C, O and P had a MIC value of 5 mg/mL, which, in a two-fold serial dilution, may arguably be relatively close to 2.5 mg/mL. Hence, they should also be considered for further investigation along with extracts from above strains Y, D and N.

This investigation illustrated that methanol extracts from freeze-dried probiotic cells of *Lactobacillus* strains tested had antioxidant components at varying levels. At the highest extract concentration of 20 mg/mL used in the study, *Lb. acidophilus*, *Lb. rhamnosus* and *Lb. casei* strains had DPPH scavenging activities of 77.9 - 86.1%, 45.7 - 86.4% and 36.9 – 45.8% respectively. This suggests that the consumer of selected probiotic strains may benefit through oxidative stress reduction. The extracts need to be investigated further for the constituent active compounds to be elucidated. Also, further investigation is required for possible use as biopreservatives in food and beverages in addition to the need for their non-cytotoxicity to be confirmed. The limitation to the present study was the need to have a considerably costly (but necessary) continuous centrifuge, which would facilitate faster collection of probiotic biomass.

Acknowledgements

The study was funded by Tshwane University of Technology and the Medical Research Council of South Africa. Dr Richard Nyanzi received a Postdoctoral Fellowship from the National Research Foundation (NRF) to work at Tshwane University of Technology.

References

Armatu, A., Colceru-Mihul, S., Bubueanu, C., Draghici, E., & Pirvu, L. (2010). Evaluation of antioxidant and free scavenging potential of some Lamiaceae species growing in Romania. *Romanian Biotechnological Letters,*

15(3), 5274-5280.

Atanassova, M., Choiset, Y., Dalgalarrondo, M., Chobert, J. M., Dousset, X., Ivanova, I., & Haertlé, T. (2003). Isolation and partial biochemical characterization of a proteinaceous anti-bacteria and anti-yeast compound produced by *Lactobacillus paracasei* subsp. *paracasei* strain M3. *International Journal of Food Microbiology, 87*, 63-73. http://dx.doi.org/10.1016/S0168-1605(03)00054-0

Bendali, F., Durand, A., Hebraud, M., & Sadoun, D. (2011). *Lactobacillus paracasei* subsp. *casei*: an Algerian isolate with antibacterial activity against enteric pathogens and probiotic fitness. *Journal of Food and Nutrition Research, 50*(3), 139-149.

Blandino, A., Al-Aseeri, M. E., Pandiella, S. S., Cantero, D., & Webb, C. (2003). Cereal-based fermented foods and beverages. *Food Research International, 36*, 527-543. http://dx.doi.org/10.1016/S0963-9969(03)00009-7

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-WIssenschaft und-Technologie-Food Science and Technology, 28*, 25-30. http://dx.doi.org/10.1016/S0023-6438(95)80008-5

Choi, C. W., Kim, S. C., Hwang, S. S., Choi, B. K., Ahn, H. J., Lee, M. Y., Park, S. H., & Kim, S. K. (2002). Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. *Plant Science, 163*, 1161-1168. http://dx.doi.org/10.1016/S0168-9452(02)00332-1

Choi, S. S., Kim, Y., Han, K. S., You, S., Oh, S., & Kim, S. H. (2006). Effects of *Lactobacillus* strains on cancer cell proliferation and oxidative stress in vitro. *Letters in Applied Microbiology, 42*, 452-458. http://dx.doi.org/10.1111/j.1472-765X.2006.01913.x

Commane, D., Hughes, R., Shortt, C., Rowland, J. (2005). The potential mechanisms involved in the anticarcinogenic action of probiotics. *Mutation Research, 591*, 276-289. http://dx.doi.org/10.1016/j.mrfmmm.2005.02.027

Cos, P., Vlietinck, A. J., Vanden Berghe, D., & Maes, L. (2006). Anti-infective potential of natural products: how to develop a stronger in vitro 'proof of concept'. *Journal of Ethnopharmacology, 106*, 290–302. http://dx.doi.org/10.1016/j.jep.2006.04.003

De Muynck, C., Leroy, A. I. J., De Maeseneire, S., Arnaut, F., Soetaert, W., & Vandamme, E. J. (2004). Potential of selected lactic acid bacteria to produce food compatible antifungal metabolites. *Microbiological Research, 159*, 339-346. http://dx.doi.org/10.1016/j.micres.2004.07.002

Ejtahed, H. S., Mohtadi-Nia, J., Homayouni-Rad, A., Niafar, M., Asghari-Jafarabadi, M., & Mofid, V. (2012). Probiotic yogurt improves antioxidant status in type 2 diabetic patients. *Nutrition, 28*, 539-543. http://dx.doi.org/10.1016/j.nut.2011.08.013

Eloff, J. N., & McGaw L. J. (2014). Using African plant biodiversity to combat microbial infections pp 163-173. In GURIB-FAKIM A (Ed) Novel Plant Bioresources: Applications in Food Medicine and Cosmetics. John Wiley. http://dx.doi.org/10.1002/9781118460566.ch12

Eloff, J. N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica, 64*, 711-713. http://dx.doi.org/10.1055/s-2006-957563

Eloff, J. N. (2000). A proposal on expressing the antibacterial activity of extracts – a small first step in applying scientific knowledge to rural primary health care in South Africa. *South African Journal of Science, 96*, 116-118.

Eloff, J. N. (2004). Quantifying the bioactivity of plant extracts during screening and bioassay-guided fractionation. *Phytomedicine, 11*(4), 370-371. http://dx.doi.org/10.1078/0944711041495218

Fabry, W., Okemo, P. O., & Ansorg, R. (1998). Antibacterial activity of East African medicinal plants. *Fitoterapia, 60*, 79-84. http://dx.doi.org/10.1016/s0378-8741(97)00128-1

FAO/WHO. (2002): Guidelines for the evaluation of probiotics in food. Food Agriculture Organisation of the United Nations and World Health Organisation Working group Report (pp. 1-11) (London, Ontario, Canada. Retrived from ftp://ftp.fao.org/es/esn/food/wgreport2.pdf

Franz, C. M. A. P., Stiles, M. E., Schleifer, K. H., & Holzapfel, W. H. (2003). Enterococci in foods - a conundrum for food safety. *International Journal of Food Microbiology, 88*, 105-122. http://dx.doi.org/10.1016/S0168-1605(03)00174-0

Guo, X. H., Kim, J. M., Nam, H. M., Park, S. Y., & Kim, J. M. (2010). Screening lactic acid bacteria form swine origins for multistrain probiotics based on in vitro functional properties. *Anaerobe, 16*, 321-326. http://dx.doi.org/10.1016/j.anaerobe.2010.03.006

Holzapfel, W. H., Schillinger, U. (2002). Introduction to pre- and probiotics. *Food Research International, 35*, 109-116. http://dx.doi.org/10.1016/S0963-9969(01)00171-5

Johnson-Henry, K., Hagen, K. E., Gordonpour, M., Tompkins, T. A., & Sherman, P. (2007). Surface-layer protein extracts from *Lactobacillus helveticus* inhibit enterohaemorrhagic *Escherichia coli* 0157:H7 adhesion to epithelial cells. *Cellular Microbiology, 9*(2), 356-367. http://dx.doi.org/10.1111/j.1462-5822.2006.00791.x

Kanno, T., Kuda, T., An, C., Takahashi, H., & Kimura, B. (2012). Radical scavenging capacities of Saba-narezushi, a Japanese fermented chub mackerel, and its lactic acid. *LWT-Food Science and Technology, 47*, 25-30. http://dx.doi.org/10.1016/j.lwt.2012.01.007

Kullisaar, T., Songisepp, E., Mikelsaar, M., Zilmer, K., Vihalemm, T., & Zilmer, M. (2003). Antioxidative probiotic fermented goat's milk decreases oxidative stress-mediated atherogenecity in human subjects. *British Journal of Nutrition, 90*, 449-456. http://dx.doi.org/10.1079/BJN2003896

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C., & Kilk, A. (2002). Two antioxidative lactobacilli strains as promising probiotics. *International Journal of Food Microbiology, 72*(3), 215-224. http://dx.doi.org/10.1016/S0168-1605(01)00674-2

Lannitti, T., & Palmieri, B. (2010). Therapeutic use of probiotic formulations in clinical practice. *Clinical Nutrition, 29*, 701-725. http://dx.doi.org/10.1016/j.clnu.2010.05.004

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., & Gobbetti, M. (2000). Purification and characterisation of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Applied and Environmental Microbiology, 66*(9), 4084-4090. http://dx.doi.org/10.1128/AEM.66.9.4084-4090.2000

Lee, B. J., Kim, J. S, Mi Kang, Y., Lim, J. H., Kim, Y. M., Lee, M. S., … Je, J. Y. (2010). Antioxidant activity and γ-aminobutyric acid (GABA) content in sea tangle fermented by *Lactobacillus brevis* BJ20 isolated from traditional fermented foods. *Food Chemistry, 122*, 271-276. http://dx.doi.org/10.1016/j.foodchem.2010.02.071

Li, S., Zhao, Y., Zhang, L., Zhang, X., Huang, L., Li, D., … Wang, Q. (2012). Antioxidant activity of Lactobacillus plantarum strains isolated from traditional Chinese fermented foods. *Food Chemistry, 135*, 1914-1919. http://dx.doi.org/10.1016/j.foodchem.2012.06.048

Liu, C., Zhang, Z. Y., Dong, K., Yuan, J. P., & Guo, X. K. (2009). Antibiotic resistance of probiotic strains of lactic acid bacteria isolated from marketed foods and drugs. *Biomedical and Environmental Science, 22*, 401-412. http://dx.doi.org/10.1016/S0895-3988(10)60018-9

Manning, T. S., & Gibson, G. R. (2004). Prebiotics. *Best Practice & Research in Clinical Gastroenterology, 18*(2), 287-298. http://dx.doi.org/10.1016/j.bpg.2003.10.008

Marchand, J., & Vandenplas, Y. (2000). Microorganisms administered in the benefit of the host: myths and facts. *European Journal of Gastroenterology & Hepatology, 12*(10), 1077-1088. http://dx.doi.org/10.1097/00042737-200012100-00003

Naser, S. M., Dawyndt, P., Hoste, B., Gervers, D., Vandemeulebroecke, K., Cleenwerck, I., … Swings, J. (2007). Identification of lactobacilli by pheS and rpoA gene sequence analyses. *International Journal of Systematic and Evolutionary Microbiology, 57*, 2777-2789. http://dx.doi.org/10.1099/ijs.0.64711-0

Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawnyndt, P., Vancanneyt, M., & Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of Enterococcus species based on rpoA and pheS genes. *Microbiology, 151*, 2141-2150. http://dx.doi.org/10.1099/mic.0.27840-0

Ng, C. C., Wang, C. Y., Wang, Y. P., Tzeng, W. S., & Shyu, Y. T. (2011). Lactic acid bacteria fermentation on the production of functional antioxidant herbal Anoectochilus formosanus Hayata. *Journal of Bioscience and Bioengineering, 111*(3), 289-293. http://dx.doi.org/10.1016/j.jbiosc.2010.11.011

Nyanzi, R. (2007). Enhancing the functional quality of mageu. MTech Disssertation, Tshwane University of Technology, South Africa.

Nyanzi, R. (2013). Identification and properties of potential probiotic bacteria for application in mageu. DTech thesis, Tshwane University of Technology, Pretoria, South Africa.

Nyanzi, R., & Jooste, P. J. (2012). Cereal-based functional foods. In: Rigobelo, E. C. (Ed). Probiotics/ Book2. InTech, ISBN: 978-953-51-0776-7. http://dx.doi.org/10.5772/50120

Nyanzi, R., Awouafack, M. D., Steenkamp, P., Jooste, P. J., & Eloff, J. N. (2014). Antibacterial activity of cell extracts from 13 probiotic *Lactobacillus* strains and characterization of lactic acid and a novel fatty acid derivative from one strain. *Food Chemistry, 164*, 470-475. http://dx.doi.org/10.1016/j.foodchem.2014.05.067

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F., & Matuchansky, C. (2005). Bifidobacteria as probiotic agents – physiological effects and clinical benefits. *Alimentary Pharmacology & Therapeutics, 22*, 495-512. http://dx.doi.org/10.1111/j.1365-2036.2005.02615.x

Prado, F. C., Parada, J. L., Pandey, A., & Soccol, C. R. (2008). Trends in non-diary probiotic beverages. *Food Research International, 41*, 111-123. http://dx.doi.org/10.1016/j.foodres.2007.10.010

Rajendhran J., & Gunasekaran, P. (2011). Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. *Microbiological Research, 166*, 99-110. http://dx.doi.org/10.1016/j.micres.2010.02.003

Rios, J. L., & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology, 100*, 80-84. http://dx.doi.org/10.1016/j.jep.2005.04.025

Rousseau, V., Lepargneur, J. P., Roques, C., Remaud-Simeon, M., & Paul, F. (2005). Prebiotic effects of oligosaccharides on selected vaginal lactobacilli and pathogenic microorganisms. *Anaerobe, 11*, 145-153. http://dx.doi.org/10.1016/j.anaerobe.2004.12.002

Shai, L. J., McGaw, L. J., & Eloff, J. N. (2009). Extracts of the leaves and twigs of the threatened tree Curtisia dentata (Cornaceae) are more active against Candida albicans and other microorganisms than the stem bark extract. *South African Journal of Botany, 75*, 363-366. http://dx.doi.org/10.1016/j.sajb.2008.11.008

Shalev, E., Battino, S., Weiner, E., Colodner, R., & Keness, Y. (1996). Ingestion of yoghurt containing *Lactobacillus acidophilus* compared with pasturised yoghurt as prophylaxis for recurrent candidal vaginitis and bacterial vaginosis. *Archives of Family Medicine, 5*, 593-596. http://dx.doi.org/10.1001/archfami.5.10.593

Sharma, P., Tomar, S. K., Goswami, P., Sangwan, V., & Singh, R. (2014). Antibiotic resistance among commercially available probiotics. *Food Research International, 57*, 176-195. http://dx.doi.org/10.1016/j.foodres.2014.01.025

Shen, Q., Shang, N., & Li, P. (2011). In vitro and in vivo antioxidant activity of *Bifidobacterium animalis* 01 isolated from centenarians. *Current Microbiology, 62*, 1097-1103. http://dx.doi.org/10.1007/s00284-010-9827-7

Shikanga, E. A., Combrick, S., & Regnier, T. (2010). South African Lippia herbal infusions: Total phenolic content, antioxidant and antibacterial activities. *South African Journal of Botany, 76*, 567-571. http://dx.doi.org/10.1016/j.sajb.2010.04.010

Ström, K., Sjögren, J., Broberg, A., & Schnürer, J. (2002). *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Applied and Environmental Microbiology, 68*, 4322-4327. http://dx.doi.org/10.1128/AEM.68.9.4322-4327.2002

Strus, M., Kucharska, A., Kukla, G., Brzychczy-Wloch, M., Maresz, K., & Heczko, P. B. (2005). The in votro activity of vaginal Lactobacillus with probiotic properties against Candida. *Infectious Diseases Obstetrics and Gynecology, 13*(2), 69-75. http://dx.doi.org/10.1080/10647440400028136

Tamura K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution, 30*, 2725-2729. http://dx.doi.org/10.1093/molbev/mst197

Voulgari, K., Hatzikamari, M., Delepoglou, A., Georgakopoulous, P., Litopoulou-Tzanetaki, E., & Tzanetakis, N. (2010). Antifungal activity of non-starter lactic acid bacteria isolates from dairy products. *Food Control, 21*, 136-142. http://dx.doi.org/10.1016/j.foodcont.2009.04.007

Wang, A. N., Yi, X. W., Yu, H. F., Dong, B., Qiao, & S. Y. (2009). Free radical scavenging activity of Lactobacillus fermentum in vitro and its antioxidative effect on growing-finishing pigs. *Journal of Applied Microbiology, 107*, 1140-1148. http://dx.doi.org/10.1111/j.1365-2672.2009.04294.x

Yasunaka, K., Abe, F., Nagayama, A., Okabe, H., Lozada-Pérez, L., López-Villafranco, E., … Reyes-Chilpa, R. (2005). Antibacterial activity of crude extracts from Mexican medicinal plants and purified coumarins and xanthones. *Journal of Ethnopharmacology, 97*, 293-299. http://dx.doi.org/10.1016/j.jep.2004.11.014

Zhang, Y., Du, R., Wang, L., & Zhang, H. (2010). The antioxidant effects of probiotic Lactobacillus casei Zhang on the hyperlipidemic rats. *European Food Research and Technology, 231*, 151-158. http://dx.doi.org/10.1007/s00217-010-1255-1

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Quantitative Evaluation of the Effects of Moisture Distribution on Enzyme-Induced Acylation of Trehalose in Reduced-Moisture Organic Media

Takashi Kuroiwa[1, 2], Kazuyuki Kimura[1], Yoshihiro Aoki[2], Marcos A. Neves[1], Seigo Sato[1], Sukekuni Mukataka[1], Akihiko Kanazawa[2], & Sosaku Ichikawa[1]

[1] Faculty of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan

[2] Department of Chemistry and Energy Engineering, Faculty of Engineering, Tokyo City University, Tamazutsumi 1-28-1, Setagaya, Tokyo 158-8557, Japan

Correspondence: Sosaku Ichikawa, Faculty of Life and Environmental Sciences, University of Tsukuba, Tennodai 1-1-1, Tsukuba, Ibaraki 305-8572, Japan. Tel: 81-29-853-4627. Fax: 81-29-853-4605. E-mail: ichikawa.sosaku.fn@u.tsukuba.ac.jp

Received: May 18, 2015 Accepted: August 19, 2015 Online Published: September 11, 2015

doi:10.5539/jfrv4n5p133 URL: http://dx.doi.org/10.5539/jfrv4n5p133

Abstract

Enzymatic condensation of trehalose and myristic acid in organic media (2-methyl-2-butanol and acetone) with reduced moisture content was evaluated. Monomyristoyl trehalose was synthesized using immobilized lipase B from *Candida antarctica* (Novozym® 435). The product yield was significantly affected by process parameters, such as the initial moisture content in organic media, as well as in immobilized enzymes, and the added concentration of molecular sieves. Up to 25% yield of monomyristoyl trehalose could be attained, depending on the process. The experimental setup used in this study consisted of a multiphase component, i.e., Novozym® 435, an organic solvent, and molecular sieves. Moisture adsorbed either onto immobilized enzymes, or molecular sieves, and free moisture content in the organic solvent were characterized using individual experiments. The relationship between process parameters and the quantitative moisture distribution in the system was also investigated. The results presented in this paper indicates that a process design considering moisture distribution in the reaction system is important for understanding the effect of moisture on the reaction as well as for optimizing the process parameters.

Keywords: lipase, trehalose ester, multiphase reaction, moisture content, organic media, moisture adsorption isotherm

1. Introduction

Sugar-fatty acid esters, consisting of hydrophilic groups of mono-, di- or tri-saccharide and hydrophobic alkyl chain groups from fatty acids, have wide application fields such as food, cosmetic, pharmaceutical and chemical industries. Among them, trehalose-fatty acid esters have various potential industrial applications including foods, cosmetics, or medical supplies because of its surface activities (Chen, Kimura, & Adachi, 2005; Piao, Takase, & Adachi, 2007; Raku, Kitagawa, Shimakawa, & Tokiwa, 2003), physiological and biological activities (Okabe et al., 1999; Khan, Stocker, & Timmer, 2012; Hsieh et al., 2015) and biodegradability (Raku et al., 2003). For their intended uses, it is desirable to synthesize such esters enzymatically because they should be highly biocompatible. The lipase- catalyzed reactions to synthesize sugar-fatty acid esters by condensation with sugars and fatty acids in organic media with reduced moisture content have been widely studied (Plou et al., 2002; Adachi & Kobayashi, 2005; Kobayashi, 2011).

Water is a product from the condensation reaction and thus the moisture content in the reaction system affects the thermodynamic reaction equilibrium (Kobayashi & Adachi, 2004). As reported in the literature (Rupley, Gratton, & Careri, 1983; Klibanov, 2001), the small amount of water bound to enzyme molecules is called "essential water", which is critical for catalytic activity in organic media. Enzymatic reactions in organic media are often carried out in a multiphase system consisting of solid enzymes including those in immobilized forms with solid supports, organic solvent and desiccants such as molecular sieves. In such experiments, moisture has to be

distributed among each component in the experimental setup. Therefore, in order to elucidate the effects of moisture on the enzymatic reaction in the multiphase system, consideration of the quantitative moisture distribution among the enzymes, solvent and desiccant should be investigated.

In this study, we synthesized a trehalose-fatty acid ester in two organic media, 2-methyl-2-butanol and acetone with reduced moisture content, using immobilized lipase fraction B from *C. antarctica* (Novozym® 435). 2-Methyl-2-butanol is water-miscible, relatively hydrophobic solvent (log*P* = 1.3), while acetone is water-miscible, relatively hydrophilic solvent (log*P* = -0.23), where log*P* is octanol-water partition coefficient (Janssen et al., 1992). Both solvents have been widely used for lipase-catalyzed synthesis of sugar-based surfactants because they can partly dissolve a variety of sugars and fatty acids. Novozym® 435 has also been widely investigated as a catalyst for synthesis of sugar esters from di- and tri-saccharides and fatty acids (Chen et al., 2005; Piao et al., 2007; Woudenberg, van Rantwijk, & Sheldon, 1996; Ferrer, Cruces, Plou, Bernabe, & Ballesteros, 2000; Zhang et al., 2003). The authors investigated the effects of moisture in the experimental setup on the synthetic reaction of a trehalose-fatty acid ester using trehalose and myristic acid as substrates. The effects of moisture content in the organic media, and that of Novozym® 435, as well as the concentration of molecular sieves are reported here. Then, a comprehensive method for evaluating the effects of moisture on the yield of synthetic product is presented based on experimental results in relation to the quantitative moisture distribution amongst each component in the experimental setup. The results of this study should be applicable for various reaction systems containing multi-phase components for synthesis of sugar-based surfactants.

2. Materials and Methods

2.1 Materials

Novozym® 435 (a product of Novo Nordisk A/S, Bagsvaerd, Denmark) was obtained from Sigma Co. (St. Louis, MO). This was an immobilized lipase from *C. antarctica* fraction B (CALB), and a porous acrylic resin with a diameter of 0.3–0.9 mm was used as support material for the enzyme. Trehalose dihydrate, myristic acid, 2-methyl-2-butanol, acetone, methanol of HPLC analytical grade, and molecular sieves 3A were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). 2-Methyl-2-butanol and acetone were dried with 200 g/L of molecular sieves 3A for at least a week before use.

2.2 Enzymatic Acylation of Trehalose in Presence of Myristic Acid

Trehalose dihydrate (50 μmol), myristic acid (50 μmol), immobilized enzyme (100 mg), and molecular sieves 3A (0 – 400 mg) were placed in a 30-mL Erlenmeyer flask. Five milliliters of 2-methyl-2-butanol with moisture content of 0.058 – 46 g/L or acetone with moisture content of 0.040 – 2.5 g/L was added to the flask. The moisture contents of both solvents were adjusted by adding little moisture levels to the dried solvents and measured by Karl Fischer titration, using a Coulometer KF 737 (Metrohm, Switzerland) before use. The flask was sealed tightly with a screw cap and kept on incubation bath at 40 ˚C. After incubation for a given time (1-96 h), the solvent was evaporated from the reaction mixture, 15 mL of chloroform/methanol (2:1, v:v) were added to the solid residue, followed by removal of immobilized enzyme and molecular sieves from the mixture by filtration. After solvent evaporation, the solid residue was dried under reduced pressure and used for product analysis.

2.3 Purification of Reaction Products and Structural Analysis by Fourier-transform Infrared (FT-IR) Spectroscopy

The synthetic product in the enzymatic reaction mixture was purified by a column chromatography using a glass column containing octadecyl silica (Wakosil, Wako Pure Chemical Industries, Co., Ltd, Osaka, Japan). The mobile phase used consisted of methanol/water (85:15, v:v). Purified product was detected by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). Silica gel 60 plate (Merck) was used for TLC analysis. The samples containing the synthetic products were spotted onto the TLC plate and then developed by using a mixture of chloroform/methanol (75:25, v:v). After drying the plate, the spots of hexose-containing compounds were visualized in purple color by heating at 110 ˚C with 20 wt% sulfuric acid containing 2 mg/mL of orcinol. HPLC analysis was also carried out to determine the purity of the reaction products. The operation conditions of HPLC were as described below. FT-IR spectra of myristic acid, trehalose, and the purified product were obtained by FT-IR spectrophotometer (FT/IR 5300, JASCO, Tokyo, Japan) using the KBr tablet method. Myristic acid and trehalose were used for analysis as they were purchased.

2.4 Determination of Product Yield

The yield of reaction product was determined using a HPLC system equipped with a COSMOSIL 5C18-MS-II column (ϕ 4.6 x 250 mm; Nacalai Tesque Inc., Kyoto, Japan) and a refractive index detector (Model RI-2031,

JASCO Co., Tokyo). Methanol/water (85:15, v: v) was used as the mobile phase at a flow rate of 1.0 mL/min. The column temperature was kept at 40 ˚C during analysis. Monomyristoyl trehalose was chemically synthesized according to a procedure previously reported (Ikemoto & Minamino, 1993) with a slight modification and used as a standard compound after purification. The synthetic yield was determined as the molar yield of monomyristoyl trehalose based on the amount of trehalose dihydrate used initially.

2.5 Isoterms of Moisture Adsorption onto Novozym® 435 and Molecular Sieves in Organic Solvents

Moisture adsorption equilibria on solid components in the experimental setup were investigated, basically by a procedure described previously (Kuroiwa et al., 2007). A specified amount of immobilized enzyme or molecular sieves 3A and 2-methyl-2-butanol or acetone with certain moisture content were incubated in a screw-capped glass vial at 40 ˚C. After a 100-h incubation period, the moisture content in the liquid phase was measured by the Karl Fischer titration. The amount of moisture adsorbed onto solids were calculated using equation (1), based on the moisture mass balance:

$$q_e = q_i + V(C_i - C_e)/m \quad (1)$$

where q_e and q_i are amounts of moisture adsorbed onto the solid components at the initial and equilibrium states, respectively, [g/g], C_i and C_e indicated the concentrations of free moisture in solvent at the initial and equilibrium states, respectively, [g/L], V is volume of solvent [L], and m is amount of solid component [g].

3. Results and Discussion

3.1 Synthesis of Monomyristoyl Trehalose by Immobilized Enzyme

We conducted the acylation of trehalose in presence of myristic acid using a commercial immobilized lipase, Novozym® 435, in organic media with reduced moisture content. Figure 1 shows the FTIR spectra of myristic acid, trehalose, and the purified synthetic product with scanning range from 4,000 to 1,000 cm^{-1}. The absorption band attributed to carboxyl groups (1,701 cm^{-1}) of myristic acid disappeared, and a new absorption band (1,734 cm^{-1}) assigned to ester bond appeared in the FTIR spectra of the purified product. The bands of hydroxyl groups and alkyl ether bonds (around 3,390 and 1,150-1,000 cm^{-1}) of trehalose moieties were also detected in the purified product. Furthermore, only one component was detected in the purified product by TLC and HPLC analyses, and its R_f value around 0.3 in TLC and retention time of 5.1 min in HPLC were identical to those of a standard monomyristoyl trehalose synthesized chemically. These results confirmed that monomyristoyl trehalose was successfully obtained by lipase-catalyzed condensation between trehalose and myristic acid. In the following studies, we determined the synthetic yield of the products from the area underneath the peak with a retention time of 5.1 min in HPLC analysis.

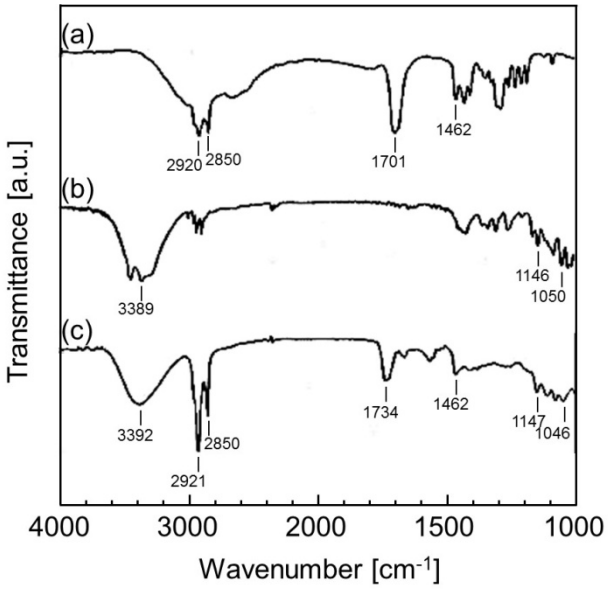


Figure 1. FT-IR spectra of (a) myristic acid, (b) trehalose and (c) purified synthetic product. The absorption band attributed to carboxyl groups (1,701 cm^{-1}), esters (1,734 cm^{-1}), methylene chains (2850 and 2920 cm^{-1}), methyl groups (1462 cm^{-1}), hydroxyl groups and alkyl ethers (around 3,390 and 1,150-1,000 cm^{-1})

Figure 2 shows the time courses of the synthetic yield of the monomyristoyl trehalose in 2-methyl-2-butanol with different initial moisture content. In both cases, the yield was increased as the reaction proceeded. However, the yields reached at the later stage of reaction (after 70 h) were considerably different each other. Therefore, the effects of operational factors on the yield of monomyristoyl trehalose in two different organic solvents, 2-methyl-2-butanol and acetone were further investigated.

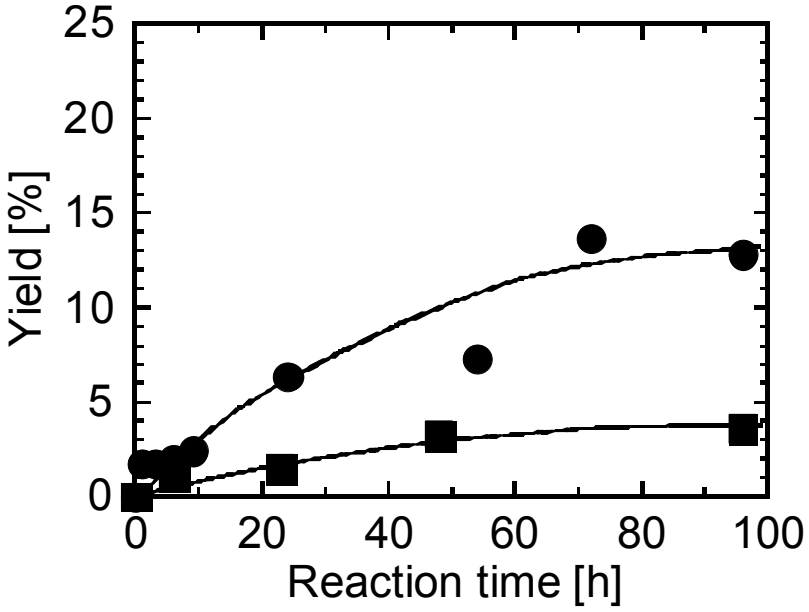


Figure 2. Time courses of the yield of monomyristoyl trehalose in 2-methyl-2-butanol with different initial moisture content of 0.42 g/L (●), 3.2 g/L (■). Amounts of added molecular sieves 3A and the immobilized enzyme moisture content were 40 g/L and 0.045 g-water/g-Novozym® 435, respectively

3.2 Effect of Process Parameters on the Synthetic Yield

Previous studies have reported that the moisture content of the solvent, and that of the solid catalyst, besides the amount of molecular sieves added to the system may affect enzyme-induced acylation of sugars and sugar moieties of natural compounds (Yahya, Anderson, & Moo-Young, 1998; Chamouleau, Coulon, Girardin, & Ghoul, 2001; Gayot, Santarelli, & Coulon, 2003; Zhang, Adachi, Watanabe, Kobayashi, & Matsuno, 2003). As shown in the previous section, reduced moisture content in the system also affected the synthesis of monomyristoyl trehalose using immobilized enzymes. Therefore, we studied the effect of process parameters associated to the moisture distribution in the system, on the product yield; namely, the moisture content of the organic solvent, and that of the solid enzymes immobilized on the solid support particles, as well as the amount of added molecular sieve were investigated.

Figure 3 shows the effect of the initial moisture content in two different solvents on the yield of monomyristoyl trehalose after 72 h of reaction. In both systems, the yield increased with the decrease of the initial moisture content. Low yields at high moisture levels can be explained by the reaction equilibrium, because water is a product in condensation of trehalose with myristic acid as well as monomyristoyl trehalose, and increased moisture leads to shift the reaction equilibrium toward hydrolysis. In the case of the 2-methyl-2-butanol system, the maximum yield appeared at about 0.5 g/L of moisture, and a lower yield was obtained in the region below the peak. However, a different water dependency was observed when immobilized enzymes with different moisture levels were used (Figure 2a, squares). These results indicate that the presence of moisture not only in the solvent but also in other solid components (immobilized enzymes and molecular sieves) affects the product yield, and the effect of moisture on different components are dependent on each other.

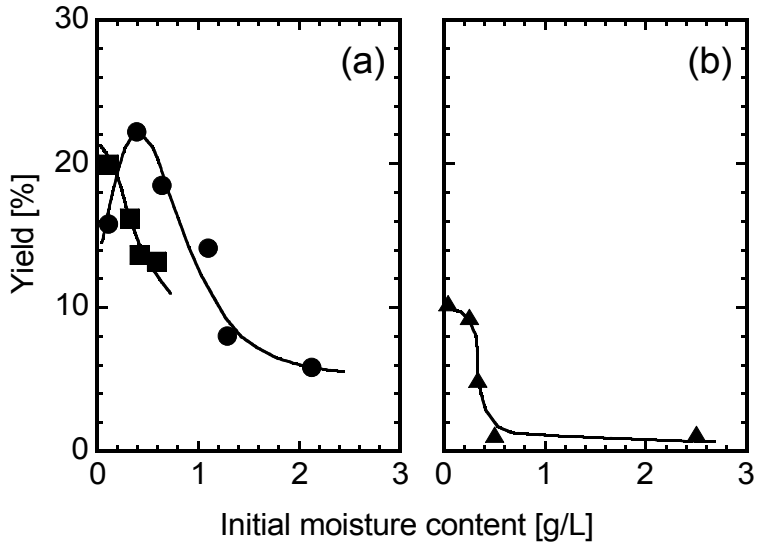


Figure 3. Effect of initial moisture content in organic solvent on the yield of monomyristoyl trehalose after 72 h reaction. Solvents used were: (a) 2-methyl-2-butanol containing 40 g/L of molecular sieves 3A; (b) acetone containing 20 g/L of molecular sieves 3A. Moisture contents of immobilized enzymes were 0.036 (●), 0.045 (■) and 0.028 (▲) g-water/g-Novozym® 435

Molecular sieves are often used for the enzymatic synthesis in organic solvent to keep the moisture content in the medium low, in order to suppress the undesirable reaction with water, i.e. hydrolysis of the products. Figure 4 shows the effect of addition of molecular sieve 3A to the reaction system on the product yield. The addition of the molecular sieve apparently affected the yield of the product at 72 h, as well as the initial moisture content in the solvents. The maximum yield was also obtained in 2-metyl-2-butanol system using the immobilized enzymes with a moisture content of 0.045 g-water/g-solid. Similar result is depicted in Figure 3a: the maximum yield was obtained at a certain initial moisture level in the solvent. However, these results suggest that the effects of above process parameters cannot be discussed separately; that is, a more comprehensive approach is needed to quantify moisture effect on the product yield. Therefore, the authors attempted to estimate the moisture distribution in the experimental setup.

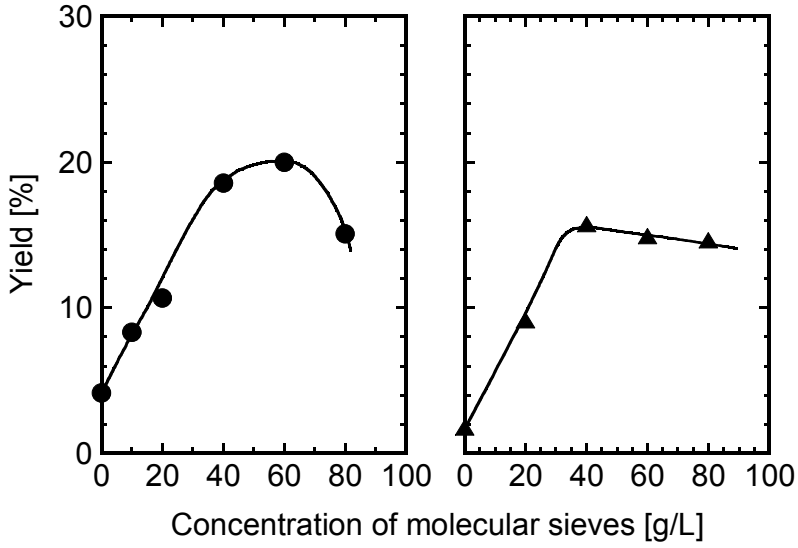


Figure 4. Effect of molecular sieves addition on the yield of monomyristoyl trehalose after 72 h reaction. Solvents used were (a) 2-methyl-2-butanol with initial moisture content of 0.11 g/L, and (b) acetone with initial moisture content of 0.044 g/L. As for the immobilized enzyme, 0.036 g-water/g- Novozym® 435 were used

3.3 Moisture Distribution in the System

The experimental setup in this study was a multicomponent system consisting of liquid and solid phases. Water in such a system has to be distributed among the individual components (Figure 5). Thus, in order to understand the effect of moisture on the product yield, it is necessary to quantify the distribution of moisture in the experimental setup.

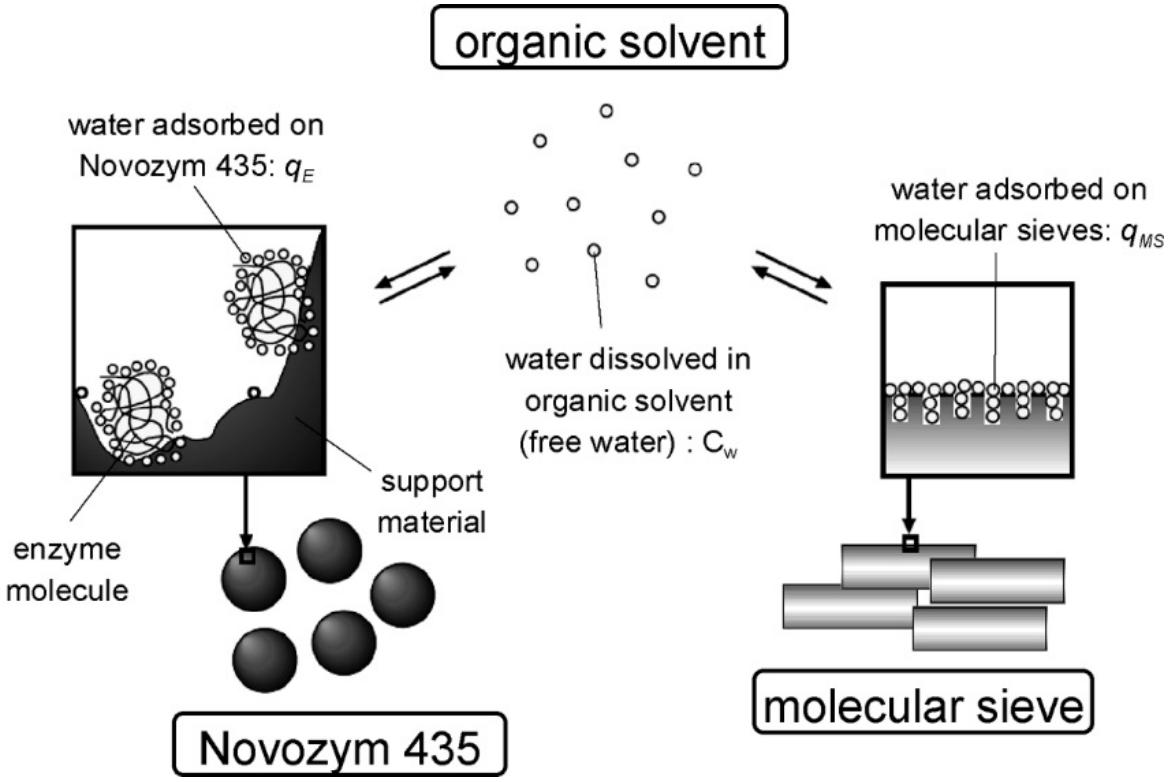


Figure 5. Schematic illustration of moisture distribution during immobilized lipase-induced reaction in a reduced-moisture organic solvent. Water in a multiphase system consisting of liquids containing solid components has to be distributed among the individual components such as a solvent, immobilized enzyme molecules, and molecular sieves

The water mass balance is given by the following equation:

$$V C_i + m_E q_{E,i} + m_{MS} q_{MS,i} + V C_r = V C_e + m_E q_{E,e} + m_{MS} q_{MS,e} \quad (2)$$

where C_r: is concentration of free water in solvent produced by synthetic reaction [g/L], m_E and m_{MS} are amounts of immobilized enzyme and molecular sieve 3A, respectively, [g], $q_{E,i}$ and $q_{E,e}$ are amounts of moisture adsorbed onto immobilized enzyme at the initial and equilibrium states, respectively, [g/g], and $q_{MS,e}$ and $q_{MS,i}$ are amounts of moisture adsorbed onto molecular sieves 3A at the initial and equilibrium states, respectively, [g/g].

In this study, the value of $q_{MS,i}$ can be set to zero because the molecular sieves were sufficiently dried before use. Therefore, Equation 2 can be rearranged as follows:

$$C_i + C_r = C_e + (m_E/V)(q_{E,e} - q_{E,i}) + (m_{MS}/V)q_{MS,e} \quad (3)$$

As shown later, the amount of moisture adsorbed onto the immobilized enzymes and molecular sieves at equilibrium, $q_{E,e}$ and $q_{MS,e}$, can be determined from moisture adsorption equilibria based on separate experimental results for each component. Thus, we can use Equation 3 to calculate the moisture distribution in the system at the equilibrium state — that is, moisture adsorbed onto the immobilized enzymes, moisture adsorbed onto the molecular sieves, and the moisture dissolved in the organic solvent.

Isotherms for moisture adsorption onto immobilized enzymes (Figure 6a) and onto molecular sieves 3A (Figure 6b) could be expressed by a Langmuir-type equation (Equation 4). The adsorption parameters q_s and K determined from Figure 5 are listed in Table 1.

$$q_e = q_s C_e/(K + C_e) \quad (4)$$

where q_s is saturated amount of moisture adsorbed onto the solid component [g/g] and K is adsorption equilibrium constant [g/L].

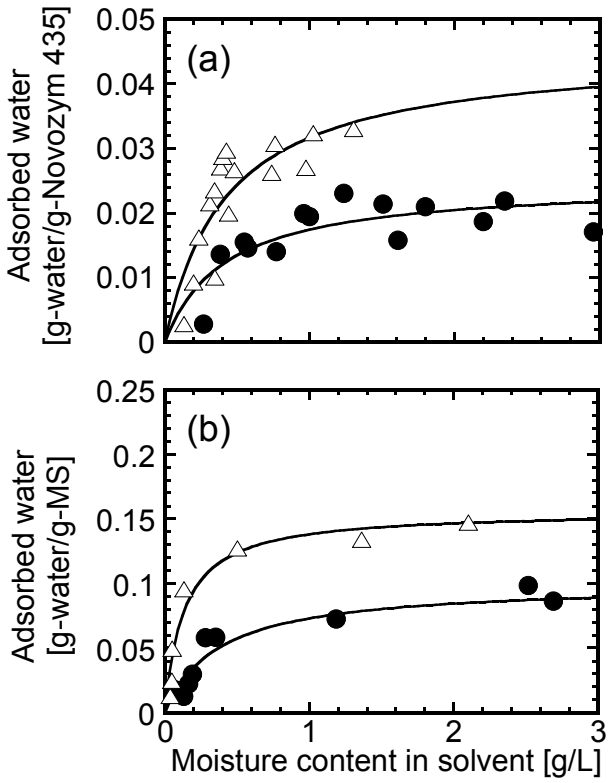


Figure 6. Isotherms for moisture adsorption onto (a) Immobilized enzyme and (b) molecular sieves 3A in 2-methyl-2-butanol (●) and acetone (△, data from Kuroiwa et al., 2007) at 40˚C

Table 1. Moisture adsorption parameters for Novozym 435 and Molecular sieve 3A in two different solvents

Solvent	Novozym 435		Molecular sieve 3A	
	q_{max} [g-water/g-solid]	K [g/L]	q_{max} [g-water/g-solid]	K [g/L]
2-Methyl-2-butanol	0.025	0.42	0.10	0.37
Acetone	0.045	0.42	0.16	0.13

3.4 Effect of Moisture Distribution on the Product Yield

The relationships between the yield of monomyristoyl trehalose and the initial (at 0 h reaction) and final (after 72 h reaction) moisture in 2-methyl-2-butanol and acetone were shown in Figure 7. The final moisture content in each solvent was calculated using Equation 3 with the initial process parameters for all experiments. As shown in Figures 7 (a) and (b), clear relationships between the yield and the initial moisture in both solvents could not be found, because the yield was affected by not only the initial moisture in the solvent but also the water content of immobilized enzymes and the concentration of added molecular sieves. On the other hand, the product yield was clearly correlated with the final moisture in each solvents (Figures 7(c) and (d)), which were calculated according to Equations 3 and 4; the resulting data are presented in Table 1. These results suggest that the effect of moisture in the experimental setup could be evaluated comprehensively by quantitative prediction of the state of moisture distribution to each component.

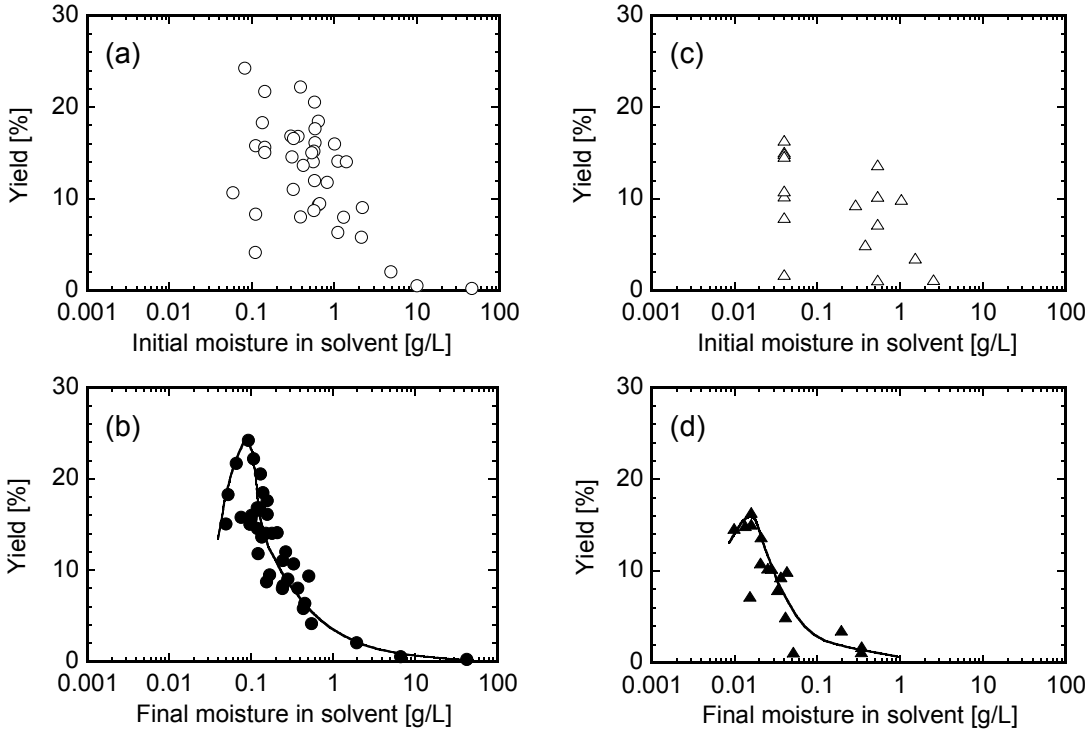


Figure 7. Relationships between free moisture at (a) (b) the initial (0 h) or (c) (d) final (after 72 h reaction) stages and the yield of monomyristoyl trehalose in (a) (c) 2-methyl-2-butanol, and (b) (d) acetone media. Values of final moisture in both solvents were calculated with equation (3) from each experimental condition

For both solvents, the product yield decreased with increasing free moisture when the final moisture in the solvent was high (> 0.1 g/L for 2-methyl-2-butanol, and > 0.02 g/L for acetone). Since water is a product of the condensation reaction, an increase in free moisture lowers the equilibrium concentrations of the condensed products. Therefore, the low yield obtained at high moisture content can be attributed to a shift in the reaction equilibrium towards hydrolysis, due to the existence of a large amount of free water molecules.

However, the product yield did not increase with the moisture decrease when it was very low (< 0.1 g/L for 2-methyl-2-butanol, and < 0.02 g/L for acetone); especially in the case of 2-methyl-2-butanol system, the yield decreased simultaneously with moisture. For synthetic reactions induced by immobilized preparations of CALBs, the initial catalytic activity and the conversion decreased at very low water content in previous studies (Kuroiwa et al., 2007; Gayot et al., 2003; Pepin & Lortie, 2001). These results cannot be explained in terms of reaction equilibrium. According to the literature, water molecules binding to enzyme molecules are critical for enzymatic activity in organic media; enzyme molecules have to be hydrated by a sufficient amount of water to maintain their active conformation and molecular flexibility (Rupley et al., 1983; Klibanov, 2001; Serdakowski & Dordick, 2007). The amount of such "essential" water for catalytic activity is around 0.4 g-water/g-enzyme, although the actual value depends on the enzyme (Rupley et al., 1983; Klibanov, 2001). The amount of such water approximately corresponds to the order of monolayer of water molecules on the surface of enzyme molecules, and in this state, several hundreds of water molecules bind to one enzyme molecule as previously calculated (Rupley et al., 1983). Based on these considerations, the conditions of low-moisture side from the peak of the product yield would correspond to the state that, at most, only several tens of water molecules are bound to one enzyme molecule with the assumption that Novozym® 435 contains 5 - 10% of protein (Gayot et al., 2003; Secundo, Carrea, Soregaroli, Varinelli, & Morrone, 2001). Therefore, the authors believe that the low product yield at low moisture content was due to the low catalytic activity of enzymes hydrated insufficiently.

4. Conclusions

The lipase-induced acylation of trehalose with a myristic acid was investigated, focusing on the effect of moisture in the experimental setup on the synthesis and yield of a trehalose-fatty acid ester. The acylation between trehalose and myristic acid by Novozym® 435 (a commercial lipolytic immobilized enzyme from *C.*

antarctica) in 2-methyl-2-butanol and acetone, so that synthesizing monomyristoyl trehalose. The yield of the reaction product was significantly affected by the moisture content of the solvent, the moisture content of the catalyst, and by the concentration of added molecular sieves. The effects of the operating conditions are discussed in relation to the quantitative distribution of moisture in the experimental setup, that is, moisture adsorbed either onto immobilized enzymes, or molecular sieves, and moisture content in the organic solvents. The changes in the yield of the synthetic product would be attributed to both the thermodynamic reaction equilibrium and the enzymatic activity in reduced-moisture organic solvents. In the present paper, we demonstrated that quantitative analysis of moisture in the system, as well as controlling process parameters are essential to elucidate the behavior of lipase-induced acylation in organic media with low moisture content. Actually, characteristics of the experimental setup, such as immobilized lipase, organic solvent and desiccant, are expected to play major roles on the enzymatic acylation. The technique and experimental results reported here may serve as basis for elucidating such multiphase systems, foreseeing the design of optimized processes for non-aqueous enzymatic catalysis. These considerations also should effective for designing continuous production process, such as packed-bed enzyme reactors feeding a non-aqueous substrate solution continuously.

Acknowledgements

This work was partly supported by research grants from the Cosmetology Research Foundation, and the Hokuto Foundation for Bioscience, both in Japan. We thank Dr. T. Iwamura of Tokyo City University, Japan, for his expert advices on FT-IR analysis of the products.

References

Adachi, S., & Kobayashi, T. (2005). Synthesis of esters by immobilized-lipase-catalyzed condensation reaction of sugars and fatty acids in water-miscible organic solvent. *Journal of Bioscience and Bioengineering, 99*, 87-94. http://dx.doi.org/10.1263/jbb.99.87

Chamouleau, F., Coulon, D., Girardin, M., & Ghoul, M. (2001). Influence of water activity and water content on sugar esters lipase catalyzed synthesis in organic media. *Journal of Molecular Catalysis B: Enzymatic, 11*, 949-954. http://dx.doi.org/10.1016/S1381-1177(00)00166-1

Chen, J., Kimura, Y., & Adachi, S. (2005). Synthesis of linoleoyl disaccharides through lipase-catalyzed condensation and their surface activities. *Journal of Bioscience and Bioengineering, 100*, 274-279. http://dx.doi.org/10.1263/jbb.100.274

Ferrer, M., Cruces, M. A., Plou, F. J., Bernabe, M., & Ballesteros, A. (2000). A simple procedure for the regioselective synthesis of fatty acid esters of maltose, leucrose, maltotriose and n-dodecyl maltosides. *Tetrahedron, 56*, 4053-4061. http://dx.doi.org/10.1016/S0040-4020(00)00319-7

Gayot, S., Santarelli, X., & Coulon, D. (2003). Modification of flavonoid using lipase in non-conventional media: effect of the water content. *Journal of Biotechnology, 101*, 29-36. http://dx.doi.org/10.1016/S0168-1656(02)00286-9

Hsieh, S. W., Lee, M. R., Tsai, C. W., Lai, L. Y., Yeha, T., Hsieh, C. W., … Chang, S. W. (2015). Enzymatic synthesis, purification andidentification of bioactive trehalose esterderivatives for health applications. *Food and Bioproduct Processing, 95*, 163-172. http://dx.doi.org/10.1016/j.fbp.2015.05.003

Ikemoto, T., & Minamino, H. (1993). Surfactants. Japanese Patent Disclosure, H05-168893.

Janssen, A. E. M., Hadini, M., Wessels Boer, N., Walinga, R., Van der Padt, A., Van Sonsbeek, H. M., & Van't Riet, K. (1992). The effect of organic solvents on enzymatic esterification of polyols. In *Biocatalysis in Non-Conventional Media* (*Progress in Biotechnology* 8, edited by Tramper et al., pp. 155-161), Elsevier Science Ltd.

Kahn, A. A., Stocker, B. L., & Timmer, S. M. S. (2012). Trehalose glycolipids—synthesis and biological activities. *Carbohydrate Research, 356*, 25-36. http://dx.doi.org/10.1016/j.carres.2012.03.010

Klibanov, A. M. (2001). Improving enzymes by using them in organic solvents. *Nature, 409*, 241-246. http://dx.doi.org/10.1038/35051719

Kobayashi, T. (2011). Lipase-catalyzed synthesis of sugar esters in non-aqueous media. *Biotechnology Letters, 33*, 1911-1919. http://dx.doi.org/10.1007/s10529-011-0663-z

Kobayashi, T., & Adachi, S. (2004). Reaction equilibrium for lipase-catalyzed condensation in organic solvent systems. *Biotechnology Letters, 26*, 1461-1468. http://dx.doi.org/10.1023/B:BILE.0000044481.31933.5d

Kuroiwa, T., Kimura, K., Ichikawa, S., Nakajima, M., Sato, S., & Mukataka, S. (2007). Lipase-catalysed

synthesis of fatty acid modified chitobiose in a low-water organic solvent: effect of water distribution in a multiphase reaction system. *Food and Bioproduct Processing, 85*, 98-103. http://dx.doi.org/10.1205/fbp06049

Okabe, S., Suganuma, M., Tada, Y., Ochiai, Y., Sueoka, E., Kohya, H., … Fujiki, H. (1999). Disaccharide esters screened for inhibition of tumor necrosis factor-α release are new anti-cancer agents. *Japanese Journal of Cancer Research, 90*, 669-676. http://dx.doi.org/10.1111/j.1349-7006.1999.tb00799.x

Pepin, P., & Lortie, R. (2001). Influence of water activity on the enantio selective esterification of (R, S)-ibuprofen by crosslinked crystals of *Candida antarctica* lipase B in organic solvent media. *Biotechnology and Bioengineering, 75*, 559-562. http://dx.doi.org/10.1002/bit.10051

Piao, J., Takase, K., & Adachi, S. (2007). Enzymatic synthesis of myristoyl disaccharides and their surface activity. *Journal of the Science of Food and Agriculture, 87*, 1743-1747. http://dx.doi.org/10.1002/jsfa.2909

Plou, F. J., Cruces, M. A., Ferrer, M., Fuentes, G., Pastor, E., Bernabe, M., … Ballesteros A. (2002). Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent. *Journal of Biotechnology, 96*, 55-66. http://dx.doi.org/10.1016/S0168-1656(02)00037-8

Raku, T., Kitagawa, M., Shimakawa, H., & Tokiwa, Y. (2003). Enzymatic synthesis of trehalose esters having lipophilicity. *Journal of Biotechnology, 100*, 203-208. http://dx.doi.org/10.1016/S0168-1656(02)00224-9

Rupley, J. A., Gratton, E., & Careri, G. (1983). Water and globular proteins. *Trends in Biochemcal Sciences, 8*, 18-22. http://dx.doi.org/10.1016/0968-0004(83)90063-4

Secundo, F., Carrea, G., Soregaroli, C., Varinelli, D., & Morrone, R. (2001). Activity of different *Candida antarctica* lipase B formulations in organic solvents. *Biotechnology and Bioengineering, 73*, 157-163. http://dx.doi.org/10.1002/bit.1047

Serdakowski A. L., & Dordick, J. S. (2007). Enzyme activation for organic solvents made easy. *Trends in Biotechnology, 26*, 48-54. http://dx.doi.org/10.1016/j.tibtech.2007.10.007

Woudenberg, M., van Rantwijk, F., & Sheldon, R. A. (1996). Regioselective acylation of disaccharides in tert-butyl alcohol catalyzed by *Candida antarctica* lipase. *Biotechnology and Bioengineering, 49*, 328-333. http://dx.doi.org/10.1002/(SICI)1097-0290(19960205)49:3<328::AID-BIT11>3.0.CO;2-A

Yahya, A. R. M., Anderson, W. A., & Moo-Young, M. (1998). Ester synthesis in lipase-catalyzed reactions. *Enzyme and Microbial Technology, 23*, 438-450. http://dx.doi.org/10.1016/S0141-0229(98)00065-9

Zhang, X., Adachi, S., Watanabe, Y., Kobayashi, T., & Matsuno, R. (2003). Prediction of the equilibrium conversion for the synthesis of acyl hexose through lipase-catalyzed condensation in water miscible solvent in the presence of molecular sieve. *Biotechnology Progress, 19*, 293-297. http://dx.doi.org/10.1021/bp0202822

Zhang, X., Kobayashi, T., Watanabe, Y., Fujii, T., Adachi, S., Nakanishi, K., & Matsuno, R. (2003). Lipase-catalyzed synthesis of monolauroyl maltose through condensation of maltose and lauric acid. *Food Science and Technology Research, 9*, 110-113. http://dx.doi.org/10.3136/fstr.9.110

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Functional, Physical and Sensory Properties of Pulse Ingredients Incorporated into Orange and Apple Juice Beverages

Fatemeh Zare[1,2], Valérie Orsat[1] & Joyce I. Boye[2]

[1] Bioresource Engineering Department, McGill University (Macdonald Campus), 21111 Lakeshore Road, Ste. Anne de Bellevue, QC, H9X 3V9, Canada

[2] Agriculture and Agri-Food Canada, 3600 Casavant Boul. West, Saint-Hyacinthe, QC, J2S 8E3, Canada

Correspondence: Joyce I. Boye, Agriculture and Agri-Food Canada, 3600 Casavant Boul. West, Saint-Hyacinthe, QC J2S 8E3, Canada. Tel: 1-(450)-768-3232. Fax: 1-(450)-773-8461. E-mail: joyce.boye@agr.gc.ca

Received: February 27, 2015 Accepted: August 23, 2015 Online Published: September 14, 2015

doi:10.5539/jfr.v4n5p143 URL: http://dx.doi.org/10.5539/jfr.v4n5p143

Abstract

The objective of this study was to explore the use of pulse ingredients in the development of orange juice and apple juice supplemented beverages. Commercially available pulse ingredients including pea protein (PP), chickpea flour (CPF), lentil flour (LF) and pea fibre (PF) were selected and characterized with respect to specific functional properties (water holding capacity, fat absorption capacity, protein solubility, emulsifying and foaming properties). Apple juice was supplemented with 1-4% pulse ingredients, whereas a supplementation level of 1-2% was used for orange juice. The physical and sensory properties of the supplemented beverages were measured after production and during 3 weeks of refrigerated storage. Sensory attributes for both orange and apple juice supplemented with 1% and 2% pulse ingredients were similar to their respective controls (with and without pectin added). In terms of turbidity, supplementation increased the turbidity of apple juice and orange juice beverages at all levels, in comparison with control and pectin-added control samples. Supplemented samples, showed less satisfactory results in terms of cloud stability and color especially for orange juice beverages in comparison with their respective controls. Overall, while there are some hurdles to be overcome, the results suggest that when used at the 1-2% levels, PP, PF, CPF and LF could serve as potential value-added ingredients for beverage supplementation based on their physical and sensory properties. Further studies are, however, required in this promising area to improve the stability of the final production especially during storage.

Key words: pulse, functional properties, supplementation, apple juice, orange juice

1. Introduction

Pulses are the dry seeds of low fat legumes including bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), lentil (*Lens culinaris*), chickpea (*Cicer arietinum)* and lupin (*Lupinus perennis*). They are nutritional and contain high amounts of complex carbohydrates (e.g., resistant starch and oligosaccharides), protein, vitamins and minerals (e.g., folate and iron) as well as antioxidants, and only very small amounts of unsaturated fats (Ofuya & Akhidue, 2005; Curran, 2012).

Various research studies have suggested that regular dietary intake of pulses may reduce the risk of developing chronic diseases such as obesity, diabetes, heart disease and cancer (Hu, 2003; Jacobs & Gallaher, 2004; Kelly, Frost, Whittaker & Summerbell, 2004; Williams, O'Shea, & Gafenauer, 2004; Schatzkin & Mouw, 2007; Curran, 2012). Furthermore, regular consumption of pulses may assist with weight management by increasing the feeling of satiety and also controlling blood sugar and appetite due to their low glycemic index (Koh-Banerjee et al., 2004; Curran, 2012).

In addition to their health and nutritional benefits, the functional properties of pulse ingredients could play an important role in food systems. Techno-functional properties of interest in food formulations include solubility, water binding, fat binding, emulsification, foaming, gelation, thickening and flavour binding capacity. These physico-chemical properties play an important role during food processing, storage, preparation and consumption (Kinsella, 1976). Amino acid composition, structure and conformation and interactions between proteins and other food components (e.g., salts, fats, carbohydrates and phenolics) as well as pH, temperature

and other process specifications all affect to some extent the quality and functionality of food ingredients (Boye, Zare, & Pletch, 2010).

Beverage supplementation with nutraceutical components and traditional nutritional ingredients has been shown to improve the nutritional and rheological quality of beverages (Renuka, Kulkarni, Vijayanand, & Prapulla, 2009). Several studies have reported beverage supplementation with different food ingredients such as fiber (Dahl, Whiting, Isaac, Weeks, & Arnold, 2005; Beristain et al., 2006), whey and whey protein (Vojnovic, Ritz, & Vahcic, 1993; Kazmierski, Agboola, & Corredigi, 2003; Pescuma, Hébert, Mozzi, & Valdez, 2010), soy flour and soy protein (Jasentuliyana, Toma, Klavons, & Medora, 1998; Kent & Harper, 2003; Tiziani, & Vodovotz, 2005), peanut (Deshpande, Chinnan, & McWatters, 2008), fructooligosaccharide (Renuka et al., 2009), β-Glucan (Temelli, Bansema, & Stobbe, 2004; Din, Anjum, Zahoor, & Nawaz, 2009) and more. So far, however, only a few studies have considered beverage supplementation using pulses (Luz-Fernandez de Tonella, & Berry, 1987) and pulse ingredients (Jackman & Yada, 1989).

In spite of their high nutritional value, pulses do not represent a significant share of the western diet. Food supplementation with pulse ingredients could offer a promising opportunity to improve the nutritional properties of formulated food products. To ensure market acceptability, however, supplemented products made with pulse ingredients need to be comparable to non-supplemented products in terms of quality, shelf life and consumer acceptability.

In this study, therefore, beverage (i.e., apple juice and orange juice) supplementation with pulse ingredients was considered as a potential avenue to increase pulse utilisation and consumption. Commercially available pulse ingredients, including pea protein, chickpea flour, lentil flour and pea fiber, were selected and their functional properties were studied. Subsequently the physical and sensory properties of beverages supplemented with the pulse ingredients (in the presence and absence of pectin which was used as a stabilizer) were studied and compared with soy supplemented beverage as well as non-supplemented control beverages (i.e., apple and orange juice with and without pectin).

2. Materials and Methods

2.1 Materials

Pulse ingredients used in this study were as follows: chickpea flour from Diefenbaker Seeds Company (Elbow, SK, Canada), lentil flour from K2 Milling Company (Tottenham, ON, Canada), pea fiber from Best Cooking Pulses Inc (Rowatt, SK, Canada) and pea protein from Nutri-Pea Company (Portage La Prairie, MB, Canada). Soy protein concentrate (71.6% protein content) was from Oleanergie F2001 Company (St. Hyacinthe, QC, Canada). Unfiltered and unpasteurized apple juice prepared from the McIntosh variety was obtained from Quinn farm (Ile Perrot; QC, Canada) and fresh oranges (Navel Orange variety) were purchased from the retail market. Low-methoxy pectin was purchased from TIC Gum Company (PA, USA).

2.2 Functional Properties of Pulse Ingredients

Proximate analysis of the pulse ingredients including protein, moisture, fat and ash measurements were done using standard AOAC methods (AOAC, 1990). pH was measured using a pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada). Functional properties studied included water holding capacity using AACC method 88-04 (AACC, 1983); fat absorption capacity according to the method described by Lin, Humbert, & Sosulski (1974); protein solubility was measured based on the amount of protein in solution at specified pH values as measured using UV-visible light according to the Bradford method (1976); emulsifying properties were determined with the method described by Pearce & Kinsella (1978); and foaming capacity was studied using the method described by Waniska & Kinsella (1979).

2.3 Sample Preparation and Supplementation

Apple juice was stored in the refrigerator at 4 °C before use. Oranges were washed with tap water and the juice extracted with a household juice extractor model E415 (Presse-Agrumes, France) and stored in a refrigerator prior to use. Figure 1 presents a schematic diagram of the processes used for juice supplementation. With consideration of the supplementation range used for commercial fruit juices, which is mostly 0.5-3% of thickening agents such as pectin, this study considered a range of 1-4% for apple juice and orange juice supplementation. Apple juice was supplemented with 1-4% pulse ingredients, whereas a supplementation level of 1-2% was used for orange juice. For comparison, apple juice and orange juice were also supplemented with 2% soy protein concentrate. Furthermore, as control samples, non-supplemented apple and orange juices (with and without 2% pectin) were prepared. All samples were stored for 3 weeks and they were analyzed to determine

their physico-chemical (i.e., pH, turbidity, loss of cloud stability and color) and sensory properties (i.e., flavour, mouthfeel and overall acceptance).

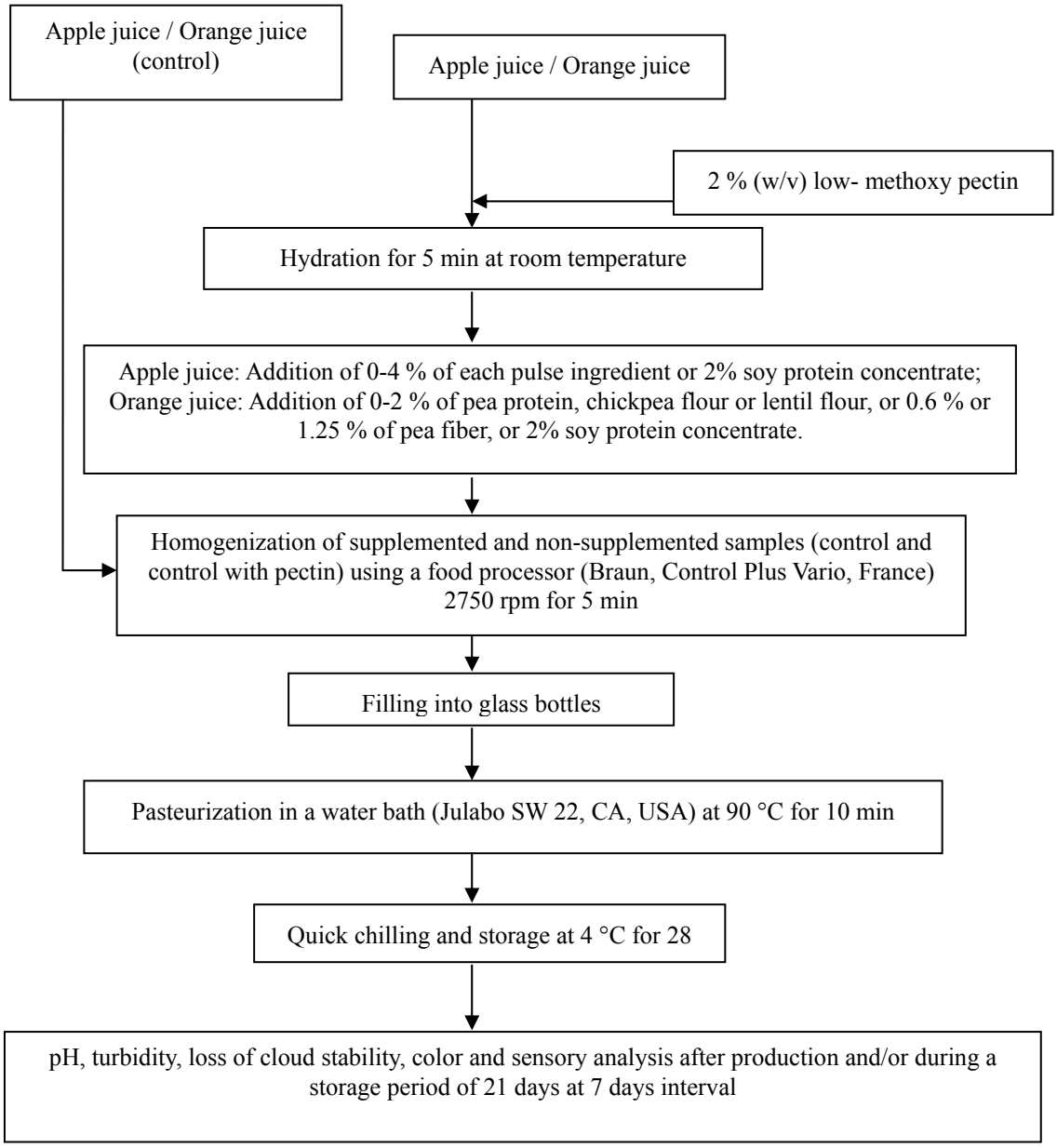


Figure 1. Schematic processes used for supplementation of apple juice and orange juice with pulse ingredients

2.4 Beverage Characterization

Turbidity of the beverages was measured according to the method described by Stähle-Hamatschek & Gierschner (1989); with some modifications. Briefly, the transparency of 100 mL of sample was measured (Ts) using a UV-Visible spectrophotometer (Cary 300 Bi, Varian, Canada) at 695 nm. Samples were then centrifuged at 20 °C for 20 min at 2015 g (4200 rpm) using a SARSTEDT centrifuge (AG & Co., Germany). Subsequently, the transparency of the supernatant was measured (Tc) and the percentage of turbidity (% T) was calculated as (Tc/Ts)*100. Cloud stability in fortified juices was measured by the method described by Kazmierski et al. (2003). Transparency of the supernatant of the centrifuged samples (20 °C /15 min/1028 g (3000 rpm), SARSTEDT centrifuge, AG & Co., Germany) was measured at 659 nm using a UV-Visible spectrophotometer

(Cary 300 Bi, Varian, Canada). An increase in transparency was considered as an indication of loss of cloud stability. This measurement was carried out at 7-day intervals during the storage period. The color of the beverages was also measured using a Labscan II colorimeter (Hunter Associate Laboratory, Inc., Restone, VA). Beverage pH was measured with an Accumet pH meter (Accumet AP61, Fisher Scientific Inc, ON, Canada).

2.5 Sensory Analyses of Beverages

Sensory analyses (flavour, mouthfeel and overall acceptance) of the supplemented and control samples were evaluated after production by 25 untrained panelists, adult males and females; using the nine point hedonic scale method. Each panelist was provided with a maximum of 3 samples at a time and they were asked to score samples from extremely like (1) to extremely dislike (9). The sensory room was equipped with red light to blind the panelists to the color of the beverages.

2.6 Statistical Analysis

Excel 2007 was used for the calculation of means and standard deviations. Statistical analysis was conducted using ANOVA analysis (SAS 9.1, SAS Institute Inc. NC, US). Comparisons were made using the Student–Newman-Keuls test and the two sample t-test for comparison of two means. All experiments were done in three separate independent trials.

3. Results and Discussion

3.1 Proximate analysis of pulse ingredients

Proximate composition of the pulse ingredients are summarized in Table 1. As expected, the pea protein concentrate showed the highest protein content (79.97% w/w) whereas pea fibre contained the lowest (7.21% w/w). Fat content of the pulse ingredients ranged between 0.06% (w/w) for lentil flour to 7.39% (w/w) for chickpea flour. Moisture content varied from 3.18% to 9.99% for the different pulse ingredients.

Table 1. Proximate analysis of pulse ingredients

Sample	Protein % (w/w)	Moisture% (w/w)	Fat % (w/w)	Ash% (w/w)
	Average ± SD	Average ± SD	Average ± SD	Average ± SD
Pea protein	79.97 ± 0.13 a	3.18 ± 0.07 b	0.53 ± 0.86 b	4.79 ± 0.42 a
Chick pea flour	23.52 ± 0.09 b	9.99 ± 0.01 a	7.39 ± 12.77 a	3.16 ± 0.36 b
Lentil flour	24.83 ± 0.12 b	9.45 ± 0.14 a	0.06 ± 0.10 b	2.68 ± 0.27 b
Pea fiber	7.21 ± 0.17 c	5.29 ± 0.04 b	0.38 ± 0.14 b	1.95 ± 0.29 c

Means with the same letters are not significantly different, for a given column (P<0.05).

3.2 Functional Properties of Pulse Ingredients

Results of the functional properties of the samples studied are summarised in Table 2. The water holding capacity (WHC) of pulse ingredients ranged from 0.8 to 3.1 (mL/g). WHC of pea protein concentrate was 3.13 mL/g and was the highest among the pulse ingredients (Table 2). This value is similar to the WHC reported for soy protein concentrate (3.9 mL/g) and soy protein isolates (4.3 mL/g) (L'Hocine, Boye, & Arcand, 2006). Not surprisingly, pea fibre had the next highest WHC (2.73 mL/g). This value is close to the value reported by Wang & Toews (2011). Chickpea flour and lentil flour exhibited very low WHC of 0.83 and 0.88 mL/g, respectively; which are comparable with the WHC of sunflower flour (107.01 % or 1.07 mL/g; Lin et al., 1974). WHC is affected by percentage of protein, cultivar and processing treatments (Kaur & Singh, 2007) as well as the number of hydrophilic sites on the protein molecules (Lin et al., 1974) and fiber content (Heller & Hackler, 1977). The higher protein content in pea protein in comparison with the other pulse ingredients may explain its higher water holding capacity. Pea fiber's second ranking for WHC is likely due to its high capacity of fiber to absorb water molecules (Heller & Hackler, 1977).

Solubility profiles of pulse ingredients at pH ranging from 1-11 are presented in Figure 2. The isoelectric point of legume proteins is generally between pH 4 and pH 6 (Fernandez-Quintela, Macarulla, Del Barrio, & Martõanez, 1997). Thus, for most pulse proteins, solubility is highest at low acidic and high alkaline pH values. In this study, the isoelectric point of pea protein and lentil flour ranged between pH 3.5 to 4.5 (region of lowest solubility), whereas that of chickpea flour was between pH 2.5 to 4.5. Interestingly, the solubility of both the

chickpea flour and lentil flour (at the region of highest solubility) was higher (60 – 80%) than for pea protein concentrate (20-25%). Processing treatments used for the production of pulse ingredients can affect their functionality (Obatolu, Fasoyiro, & Ogunsunmi, 2007). In particular, production methods such as precooking and drum-drying or spray-drying can reduce the nitrogen solubility of pulse flours and ingredients (Carcea-Bencini, 1986). Processing treatments used for preparing the pulse ingredients are proprietary; nevertheless, the lower solubility of pea protein concentrate, in spite of its higher protein content, suggests protein denaturation and changes to the molecular structure (L'hocine et al., 2006).

Fat absorption capacities (FAC) of all samples ranged from 76 % to 116 % (w/w). The lowest FAC was found for lentil flour (76%, w/w) and highest for pea fibre (116% w/w). FAC of pea protein and chickpea flour were 79% and 87% (w/w) respectively. All the FACs measured in this study were lower than for soy protein concentrate (SPC) or soy protein isolate (SPI) (FAC= 218-251 % reported by L'hocine et al., 2006), but they are comparable with FACs of soy flour, SPC and SPI ranging from 84.4 % to 154.5 % (w/w), reported by Lin et al., 1974. Fernandez-Quintela et al. (1997) also reported higher FAC of 160 % and 120 % (w/w) for faba bean isolate and pea protein isolate, respectively. The fat absorption mechanism is attributed to either oil entrapment and/or absorption and/or the lipophilic properties of the proteins contained in the pulses. The size of each particle offers different surface area to absorb the oil (Wang &Toews, 2011). The particle size of the pulse ingredients was not measured however as part of this study. Future studies focusing on the impact of particle size will therefore need to be undertaken. Non-polar sides of protein chains could also bind the hydrocarbon chains of fat molecules (Lin et al., 1974; Kinsella, 1976). In general, the difference of FAC of pulse ingredients could be attributed to a variety of factors including potential differences in protein structure. Specifically, the high FAC of pea fibre compared to the other samples could be due to the superior ability of fiber to physically entrap or bind with fat molecules (Kinsella, 1976).

Emulsifying activity index (EAI) of the pulse ingredients ranged between 11-14 m^2/g, whereas the emulsifying stability index (ESI) varied between 26-33 min (Table 2). Due to the nature of the sample, it was impossible to determine the emulsifying properties of pea fibre. Of the other samples, lentil flour and chickpea flour had the lowest emulsifying properties, whereas pea protein had the highest emulsifying properties. Barac et al. (2010), studied the EAI and ESI of pea protein isolates at different pH and they reported that the EAI of neutral pea protein (pH=7) ranged between 25 -115 m^2/g and their ESI ranged between 20-80 min; the ESI values are in the same range as that reported in this study. Proteins can form a thin layer or film around oil droplets in a food system to make an emulsion. EAI may be defined as the amount of oil that can be emulsified per unit of protein whereas ESI shows the ability of the emulsion to oppose changes to the structure of the emulsion over a period of time (Pearce & Kinsella, 1978; Boye et al., 2010). Emulsifying properties are affected by hydrophobicity and hydrophilicity properties of proteins and amino acids that are contained in the structure of proteins. The total protein content can also affect the EAI and ESI (Paredes-Lopez, Ordorica-Falomir, & Olivares-Vázquez, 1991). Thus, the higher emulsifying property of pea protein in comparison with the other samples is likely due to its higher protein content.

Foaming expansion of our pulse ingredients ranged between 400-1500 %. The highest value was found for chickpea flour and the lowest for lentil flour. The foaming expansion of pea protein and lentil flour however, was not significantly different ($P<0.05$). In comparison with soy protein isolates which gave foaming expansion (FE) values ranging from 400-550 % (L'hocine et al., 2006), pulse ingredients such as chickpea flour may have better foaming properties. Foaming capacity (FC) or foaming expansion (FE) is expressed as the volume (%) of foam increase due to whipping whereas foam stability (FS) is defined as the change in the volume of foam over a time period (Boye et al., 2010). Foaming properties (FC or FE) are related to protein content, protein structure and processing treatments (Paredes-Lopez et al., 1991; Obatolu, et al., 2007). Obatolu et al., (2007), reported greater foaming capacities in raw yam bean in comparison with boiled yam bean, which suggested that a thermal processing treatment may lower the FC. The differences in foaming properties of the pulse ingredients could thus be due to differences in protein content as well as processing treatments which could in some instances result in protein denaturation.

Table 2. Functional properties of pulse ingredients

Sample	WHC (mL/g)	FAC % (w/w)	Emulsifying Properties		Foam Expansion (FE %)
			EAI (m^2/g)	ESI (min)	
	Average ± SD	Average ± SD	Average ± SD	Average ± SD	Average ± SD
Pea protein	3.13 ± 0.02 a	79.70 ± 4.85 c	13.37 ± 0.00 a	32.75 ± 0.30 a	514.97 ± 49.50 b
Chickpea flour	0.83 ± 0.01 d	87.69 ± 5.18 b	11.93 ± 0.01 a	25.79 ± 4.46 a	1348.20 ± 114.94 a
Lentil flour	0.88 ± 0.01 c	76.70 ± 2.71 c	12.98 ± 0.02 a	26.11 ± 4.18 a	478.26 ± 7.62 b
Pea fiber	2.73 ± 0.00 b	116.28 ± 3.67 a	ND	ND	ND

Means with the same letters are not significantly different (P<0.05); ND: not defined.

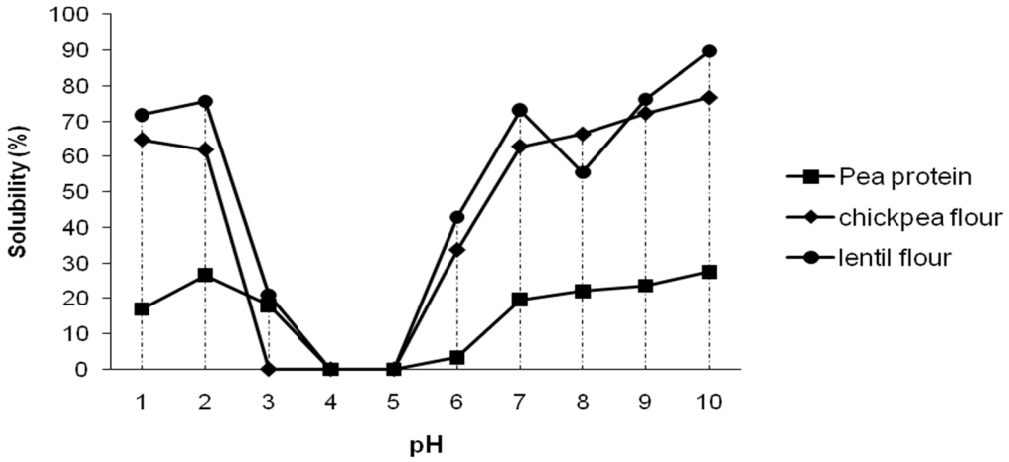


Figure 2. Solubility profile of pulse ingredients

3.3 Physico-chemical Properties of Beverages Supplemented with Pulse Ingredients

3.3.1 pH

Figure 3 (a, b), presents data on the pH values of apple and orange juice supplemented with the pulse ingredients. pH of apple juice beverages ranged from 3.50 to 4.21 and the pH of orange juice beverages ranged from 3.75 to 4.02. Pulse supplementation significantly increased the pH in all apple juice beverages compared to both control samples (i.e., non-supplemented juice with and without pectin added) (P<0.05). This could be due to the fact that pulse ingredients, by themselves are less acidic than fruit juices and so they add lower acidity and therefore lead to a higher pH of the matrices. Also, the higher protein content (pea protein) of the pulses resulted in a higher pH value, which could be due to a buffering capacity of the pulse protein. Supplementation with 2% soy protein concentrate also significantly increased the pH in comparison with the control samples. SPC supplemented samples were comparable with pea protein supplemented apple juice at the same level of supplementation (P<0.05). For orange juice beverages, pH significantly increased as a result of supplementation in all samples in comparison with control samples (P<0.05), except for the pea fiber supplemented beverages and 1% chickpea flour and 1% lentil flour supplemented samples. A comparison of the two control samples (with and without pectin) for both apple and orange juices showed that addition of pectin did not alter the pH of the juices (P<0.05).

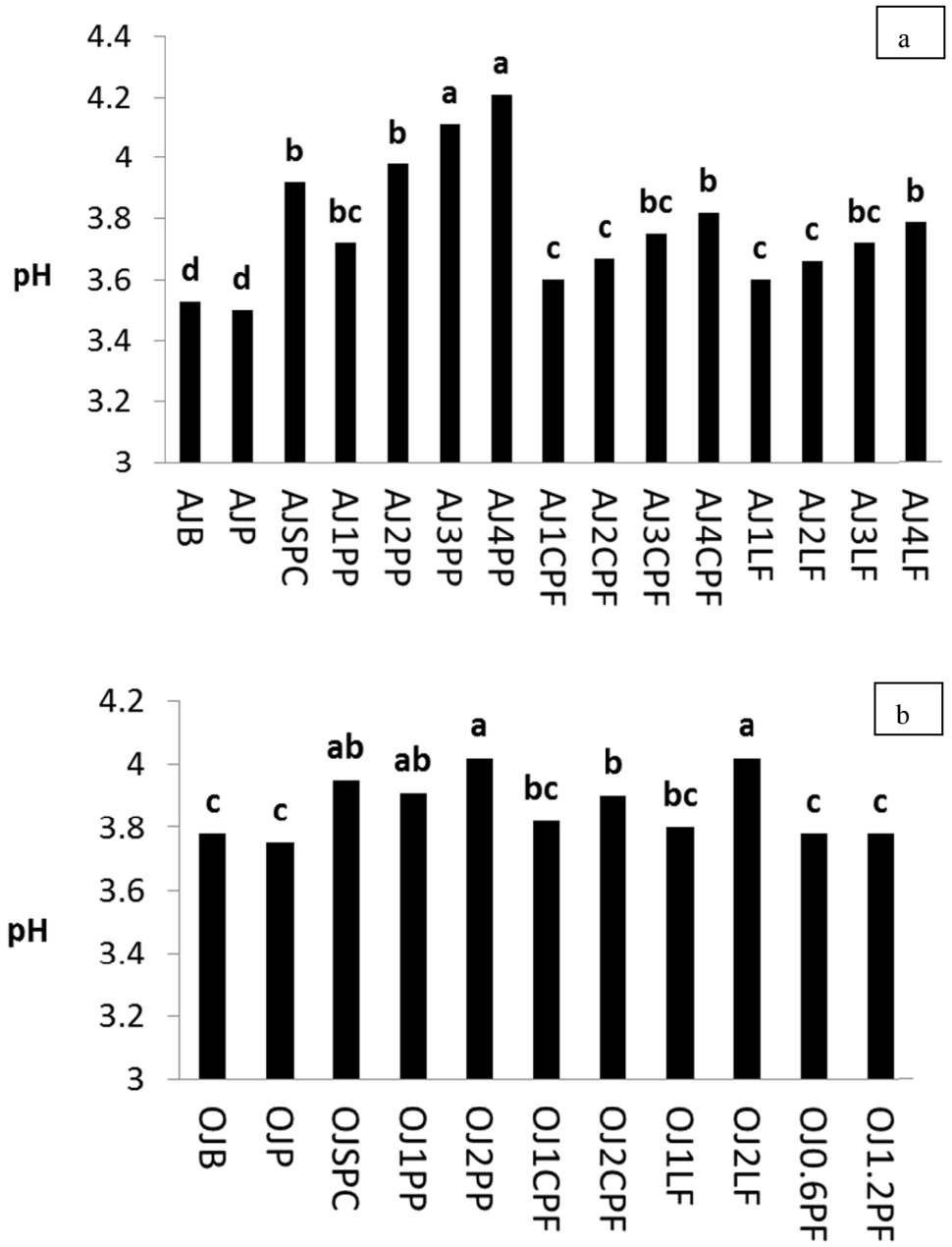


Figure 3. a): pH of supplemented and non-supplemented apple juice with 1-4% of pulse ingredients, b): pH of supplemented and non-supplemented orange juice with 1-2% of pulse ingredients; (AJ: apple juice, OJ: orange juice, B: blank, P: pectin, SPC: soy protein concentrate, PP: pea protein, CPF: chickpea flour, LF: lentil flour, 0.6PF: 0.6 % pea fiber, 1.2PF: 1.25% pea fiber; means with difference letters are significantly different *(P<0.05)*)

3.3.2 Turbidity

Figure 4 (a,b), present data on turbidity of apple and orange juice supplemented with pulse ingredients. For both apple juice and orange juice beverages, supplementation significantly increased turbidity of all samples in comparison with both control samples (i.e., non-supplemented juices with and without pectin added) and it was also concentration dependent for both beverages (P<0.05). Considering the protein solubility of pulse ingredients which is generally lowest at pH 3-6 (Figure 2), it is reasonable to expect that they would have lower solubility in both apple and orange juice; this may explain why the turbidity increased as a result of supplementation and pulse concentrations. The effect of soy protein concentrate and pea protein in increasing the turbidity was greater than for lentil flour and chickpea flour for both apple juice and orange juice supplemented beverages (P<0.05).

Addition of pectin did not alter the turbidity of both apple juice and orange juice in comparison with the control sample with no pectin.

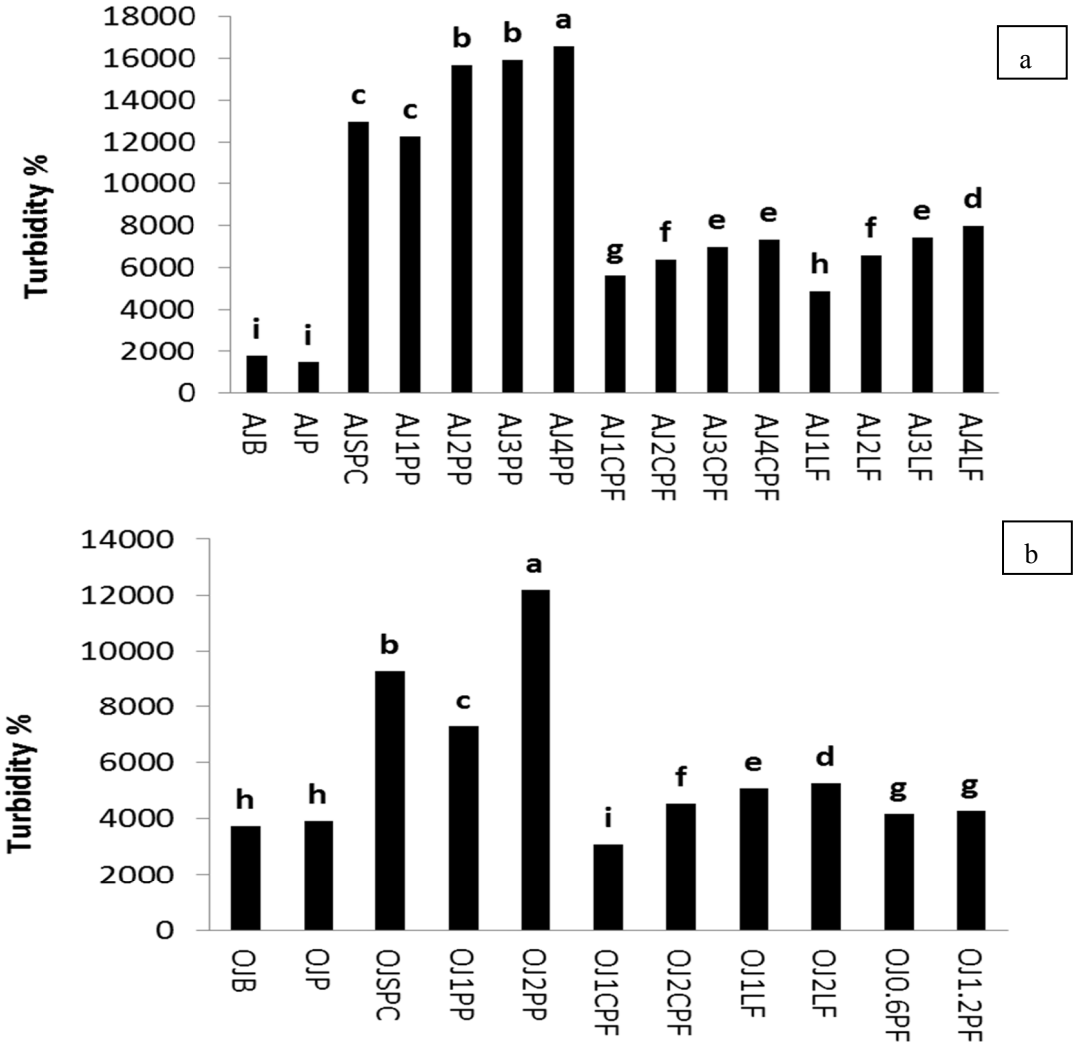


Figure 4. a): turbidity of supplemented and non-supplemented apple juice with 1-4% of pulse ingredients, b): turbidity of supplemented and non-supplemented orange juice with 1-2% of pulse ingredients; (AJ: apple juice, OJ: orange juice, B: blank, P: pectin, SPC: soy protein concentrate, PP: pea protein, CPF: chickpea flour, LF: lentil flour, 0.6PF: 0.6 % pea fiber, 1.2PF: 1.25% pea fiber; means with difference letters are significantly different *(P<0.05)*)

3.3.3 Loss of Cloud Stability (L.C.S)

Loss of cloud stability (L.C.S) in supplemented apple juice and orange juice after 1 week and during 3 weeks refrigerated storage are presented in Figures 5 (a,b). Our results showed that cloud stability of supplemented samples decreased during storage, and loss of cloud stability is negatively related to the level of supplementation. Cloud stability of the apple juice supplemented beverages was higher than for the orange juice supplemented beverages. Supplementation with soy protein concentrate also, resulted in greater cloud stability in comparison with all other apple juice and orange juice supplemented samples. Addition of pectin helped to maintain the cloud stability of both supplemented beverages in comparison with blank samples.

Cloudiness of fruit juice occurs due to the presence of pulp particles and pectin naturally present in the apple and orange juices (Kazmierski et al., 2003). As a result of gravity, settling of the pulp in both apple and orange juices is expected to increase during storage. Kazmierski et al. (2003) reported that the most important cause of cloud de-stabilization and clarification of fresh juice is the activity of the enzyme pectin methyl esterase (PME). It is,

therefore, important to inactivate the PME enzyme by heating to minimize its activity on the juice pectin. This was not an issue in the present study as the pasteurization process of the juices would have inactivated the PME enzyme. Kazmierski et al. (2003) further indicated that the introduction of proteins, the electrical charge of the juice particles, pH and temperature may also affect cloud stability due to possible interactions between pulse proteins and juice components. In our study, supplementation may have affected the cloud stability of the beverages due to the changes in protein content, electrical charge of juice particles and also the pH (as presented in section 3.3.1).

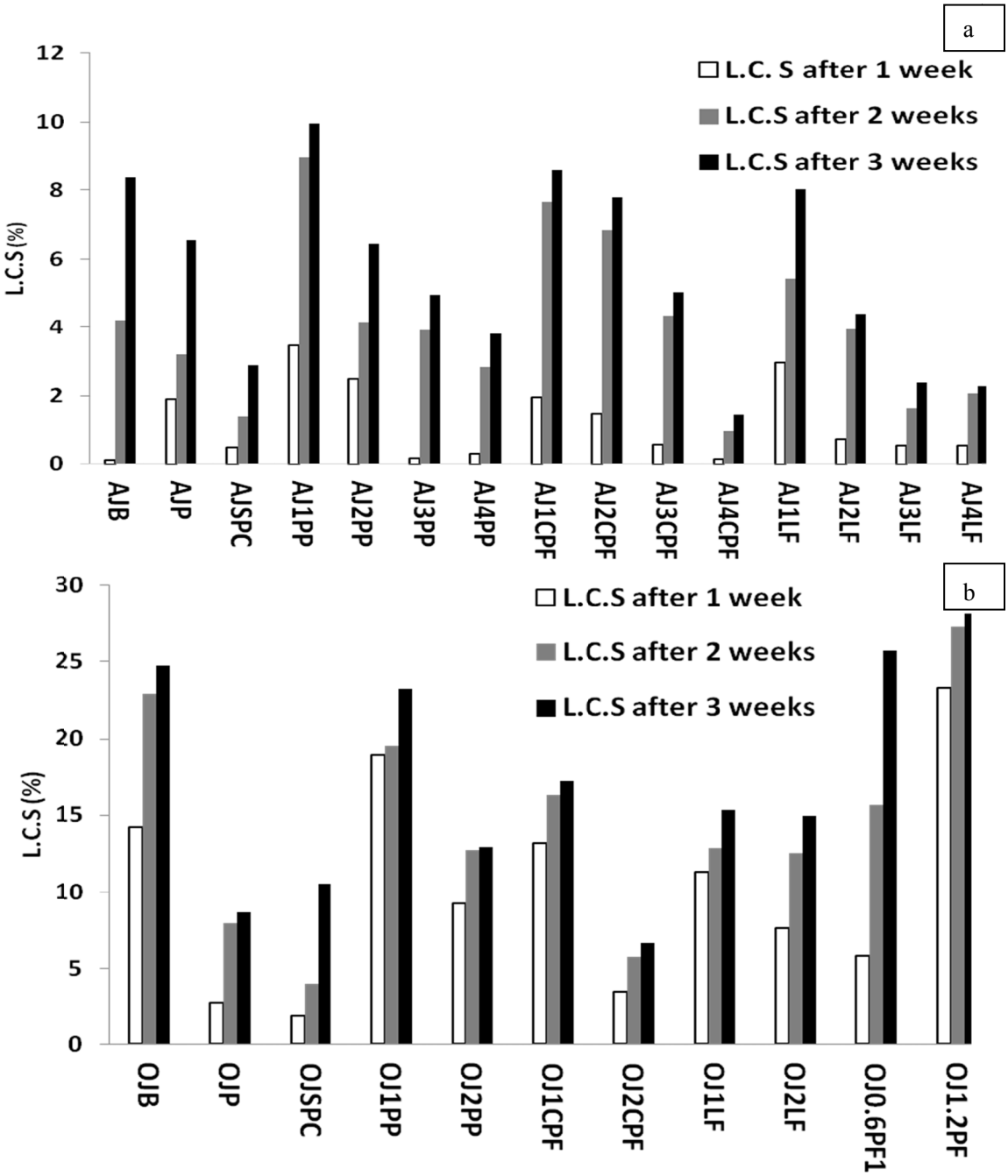


Figure 5. a): loss of cloud stability (L.C.S) of supplemented apple juice and control sample after 1 week and 3 weeks storage, b): loss of cloud stability of supplemented orange juice and control sample after 1 week and 3 weeks storage (AJ: apple juice, OJ: orange juice, B: blank, P: pectin, SPC: soy protein concentrate, PP: pea protein, CPF: chickpea flour, LF: lentil flour, 0.6PF: 0.6 % pea fiber, 1.2PF: 1.25% pea fiber)

3.3.4 Color

Color measurements obtained for the supplemented apple juice, orange juice and control samples are presented in Table 3. In apple juice the "L" factor (representing lightness, 0 = black to 100 = white) remained in the same range of 22.3- 42.3 in all supplemented samples as well as the control. The "a" value (negative values indicate green whereas positive values indicate red) increased in all samples supplemented with chickpea flour and pea protein but it was almost equal to the control sample for apple juice supplemented with the lentil flour. The "b" value (negative values indicate blue and positive values indicate yellow) dramatically increased in all samples. Results for ΔE (color difference between supplemented apple juices and control samples) showed that addition of the pulse ingredients changed the color of the juices. Addition of soy protein concentrate also changed the colour of the apple juice significantly except when it was compared with samples supplemented with 3% and 4% chickpea flour. Pea protein had the largest effect when compared to chickpea and lentil flours, however, there were no significant differences between 1 to 3% pea protein supplemented samples (P<0.05). There was also, no notable color difference between 2% chickpea flour and 2% lentil flour supplemented apple juice sample compared to the control sample with pectin.

Table 3. Color parameters (L), (a), (b) and color difference (ΔE) in supplemented apple and orange juice and control samples

Sample	L (Average ± SD)	a (Average ± SD)	b (Average ± SD)	ΔE (Average ± SD)
AJ control	31.40 ± 2.64	3.98 ± 0.22	0.13 ± 2.71	----------
AJ control+ pectin only	22.31 ± 1.56	3.19 ± 0.10	11.76 ± 3.65	14.81 ± 3.8 f
AJ + 2%SPC	31.36 ± 0.48	3.36 ± 0.04	21.44 ± 1.37	21.32 ± 1.36 cd
AJ + 1%PP	26.17 ± 0.06	5.16 ± 0.01	32.66 ± 0.06	32.97 ± 0.07 b
AJ + 2 %PP	34.16 ± 0.15	6.12 ± 0.03	32.62 ± 0.01	32.68 ± 0.02 b
AJ + 3 %PP	35.97 ± 0.05	6.33 ± 0.01	32.40 ± 0.08	32.68 ± 0.08 b
AJ + 4 %PP	37.89 ± 0.01	7.60 ± 0.02	36.43 ± 0.16	37.05 ± 0.16 a
AJ + 1 %CPF	30.8 ± 0.21	4.29 ± 0.04	26.55 ± 0.49	11.40 ± 0.16 g
AJ + 2 %CPF	34.68 ± 0.14	5.16 ± 0.07	31.18 ± 0.30	16.29 ± 0.03 ef
AJ + 3 %CPF	37.07 ± 0.00	6.89 ± 0.00	25.25 ± 0.00	20.46 ± 3.38 d
AJ + 4 %CPF	42.38 ± 0.07	4.99 ± 0.05	24.73 ± 0.17	22.92 ± 0.06 c
AJ + 1 %LF	28.03 ± 0.05	2.87 ± 0.02	21.97 ± 0.08	9.36 ± 0.07 h
AJ + 2 %LF	34.84 ± 0.01	2.86 ± 0.03	21.48 ± 0.09	15.94 ± 0.03 ef
AJ + 3 %LF	37.05 ± 0.13	3.13 ± 0.06	21.18 ± 0.12	18.13 ± 0.16 e
AJ + 4 %LF	42.27 ± 0.12	3.68 ± 0.02	23.61 ± 0.08	22.88 ± 0.13 c
OJ control	36.35 ± 0.30	5.64 ± 0.20	48.90 ± 1.54	----------
OJ control+ pectin only	35.33 ± 0.21	7.1 ± 0.27	55.42 ± 0.93	6.76 ± 0.998 d
OJ + SPC	44.02 ± 1.48	7.49 ± 0.71	47.83 ± 6.36	9.32 ± 2.80 cd
OJ + 1 %PP	43.03 ± 0.81	8.17 ± 0.35	55.38 ± 3.79	10.05 ± 1.75 c
OJ + 2 %PP	46.49 ± 0.17	9.42 ± 0.05	57.24 ± 0.34	13.66 ± 0.12 b
OJ + 1 %CPF	48.39 ± 0.07	5.93 ± 0.09	43.0 ± 0.65	13.43 ± 0.34 b
OJ + 2 %CPF	47.96 ± 0.03	7.05 ± 0.05	46.22 ± 0.66	12.01 ± 0.13 bc
OJ + 1 %LF	38.24 ± 0.08	7.60 ± 0.24	57.54 ± 0.14	9.06 ± 0.17 cd
OJ + 2 %LF	43.52 ± 0.97	8.69 ± 1.58	65.28 ± 3.03	18.24 ± 2.59 a
OJ + 0.6% PF	26.26 ± 0.07	16.30 ± 0.06	45.09 ± 0.126	15.17 ± 0.04 b
OJ + 1.25% PF	28.42 ± 0.61	17.46 ± 0.20	48.78 ± 1.07	14.26 ± 0.47 b

Means with the same letter are not significantly different (P<0.05); AJ: apple juice; OJ: orange juice; SPC: soy protein concentrate; PP: pea protein; CPF: chickpea flour; LF: lentil flour; PF: pea fiber

For supplemented orange juice the results indicated that supplementation slightly affected "b" and "L" compared to the control sample (non-supplemented without pectin) (i.e., "b" was 42.9 - 57.23 in comparison to 48.9 for the non-supplemented control sample and "L" was 26.25 - 48.93 in comparison with 36.35 for the non-supplemented control sample). "a" increased for all samples supplemented with pea fiber (16.3 and 17.3) but it remained in the same range (between 5.6-8.6) for all the other supplemented and control samples. ΔE, which represents the color difference between the supplemented orange juice and control samples, varied between 6.7 and 18.25. ΔE values were generally higher for samples supplemented with higher amounts of the pulse ingredients, but there was no significant difference between ΔE of 1% and 2% chickpea flour, 2% pea protein and 0.6% and 1.2% pea fiber samples. Besides, 1% lentil flour and 2% soy protein concentrate supplemented orange juice showed no significant color difference when compared with the control sample containing pectin (P<0.05). The smallest color difference was observed between the two control samples for both orange and apple juices, which indicated that pectin did not affect the color of both juices (P<0.05).

3.4 Sensory Properties of Beverages

Results of the sensory evaluation for flavour, mouthfeel and overall acceptance of the supplemented apple juice and orange juice samples are presented in Table 4. Samples were ranked from extremely like (1) to extremely dislike (9). Overall, the results showed that apple juice supplemented with 1% of all the pulse ingredients and also 2% soy protein concentrate were acceptable in terms of flavour in comparison with both control samples (P<0.05). For mouthfeel and overall acceptance, all supplemented samples were ranked significantly higher (i.e., less acceptable) in comparison with both controls. The control apple juice containing pectin was found to be as good as the control sample without pectin in terms of flavour, mouthfeel and overall acceptance. For orange juice, in terms of flavour, mouthfeel and overall acceptance, there were no significant differences found between all the supplemented beverages and the non-supplemented control sample, except for the 2% soy protein concentrate and 2% pea protein supplemented samples (P<0.05). It is also notable that apple juice or orange juice supplementation with 1% of each of the pulse ingredients resulted in overall acceptance scores of 5 or lower (i.e., neither like nor dislike or better scores). This may suggest that a 1% supplementation level could be a promising target for the creation of innovative products using pulse ingredients. Future studies and further formulation development work could therefore target this supplementation level.

Table 4. Sensory evaluation scores (ranged from extremely like = 1 to extremely dislike = 9) of supplemented apple and orange juice and control samples

Sample	Flavour	Mouthfeel	Overall acceptance
	Average ± SD	Average ± SD	Average ± SD
AJ control	3.64 ± 1.46 b	3.44 ± 1.26 b	3.40 ± 1.22 b
AJ control+ pectin only	3.48 ± 1.66 b	3.44 ± 1.15 b	3.44 ± 1.32 b
AJ + 2%SPC	4.68 ± 1.93 ab	5.16 ± 1.77 a	5.24 ± 1.69 a
AJ +1 %PP	4.64 ± 2.03 ab	5.00 ± 2.08 a	4.96 ± 2.07 a
AJ + 2 %PP	5.32 ± 1.93 a	5.28 ± 2.05 a	5.40 ± 1.80 a
AJ + 1 %CPF	4.92 ± 1.95 ab	5.20 ± 2.0 a	4.92 ± 1.77 a
AJ + 2 %CPF	5.48 ± 1.75 a	5.44 ± 1.29 a	5.48 ± 1.35 a
AJ + 1 %LF	4.32 ± 1.34 ab	4.68 ± 2.13 a	4.56 ± 1.52 a
AJ + 2 %LF	5.6 ± 2.08 a	5.72 ± 1.81 a	5.88 ± 1.92 a
OJ control	4.56 ± 1.91 cd	4.24 ± 2.00 c	4.68 ± 2.05 b
OJ control+ pectin only	4.88 ± 2.38 bcd	5.04 ± 2.14 bc	4.92 ± 2.28 ab
OJ + %SPC	6.24 ± 1.98 ab	6.28 ± 1.79 a	6.44 ± 1.73 a
OJ + 1 %PP	5.96 ± 1.88 abc	5.48 ± 1.80 abc	6.16 ± 1.97 ab
OJ + 2 %PP	6.4 ± 2.04 a	6.12 ± 2.18 ab	6.4 ± 2.10 a
OJ + 1 %CPF	4.48 ± 1.87 cd	4.96 ± 1.79 abc	5.00 ± 1.95 ab
OJ + 2 %CPF	5.48 ± 1.73 abcd	5.52 ± 1.32 abc	5.56 ± 1.41 ab
OJ + 1 %LF	4.32 ± 1.46 d	4.64 ± 1.70 bc	4.68 ± 1.77 b
OJ + 2 %LF	4.56 ± 1.35 cd	5.52 ± 1.19 abc	5.28 ± 1.36 ab
OJ + 0.6% PF	4.84 ± 1.31 bcd	4.32 ± 1.46 c	4.60 ± 1.25 b
OJ +1.25% PF	5.8 ± 1.63 abcd	5.36 ± 1.57 abc	5.72 ± 1.56 ab

Means with the same letter are not significantly different (P<0.05); AJ: apple juice; OJ: orange juice; SPC: soy protein concentrate; PP: pea protein; CPF: chickpea flour; LF: lentil flour; PF: pea fiber.

4. Conclusion

Functional properties of food ingredients in beverage applications are affected by a variety of factors including protein content, pH, ionic strength and temperature. This research illustrated differences in the functional properties of the different pulse ingredients studied. The physical analysis on the beverage systems showed that supplementation at all levels and in both orange and apple juice matrices increased pH and turbidity. Also, apple juice and orange juice supplementation with pulse ingredients decreased cloud stability, however higher level of supplementation resulted in a lower loss of cloud stability over storage time. Color of apple juice and orange juice was significantly affected by pulse supplementation and they were altered towards red and yellow hues respectively. In terms of sensory attributes (flavour, mouthfeel and overall acceptance), 1% or 2% of all pulse ingredients in apple juice and orange juice supplementation gave relatively acceptable products in comparison with the control samples. This result was highlighted for chickpea flour and pea fiber supplemented orange juice.

Considering the growing interest in healthier food products and the importance of continued innovation in different product streams, including the development of heathier beverages, there is good potential to examine the use of pulse ingredients in the formulation of pulse supplemented beverages. Further research on the sensory properties, storage stability and marketability of such pulse supplemented beverages would be useful.

Acknowledgement

The study was supported with funds from Pulse Canada and Agriculture and Agri-Food Canada.

References

AACC. (1983). *American Association of Cereal Chemists Methods 1983* (8th ed.). Washington DC.

AOAC. (1990). *Official Methods of Analysis* (15th ed.). Association of Official Analytical Chemists, Washington DC.

Barac, M., Cabrilo, S., Pesic, M., Stanojevic, S., Zilic, S., Macej, O., & Ristic, N. (2010). Profile and functional properties of seed proteins from six pea (*Pisum sativum*) genotypes. *International Journal of Molecular Sciences, 11*, 4973-4990. http://dx.doi.org/10.3390/ijms11124973

Beristain, C. I., Cruz-Sosa, F., Lobato-Calleros, C., Pedroza-Islas, R., Rodriguez-Huezo, M. E., & Verde-Calvo, J. R. (2006). Application of soluble dietary fibers in beverages. *Revista Mexican De Ingenieria Quimica, 5*(1), 81-95.

Boye, I. J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*, 414-431. http://dx.doi.org/10.1016/j.foodres.2009.09.003

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of micrograin quantities of protein using the method of protein-dye binding. *Analytical Biochemistry, 7*(72), 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3

Carcea-Bencini, M. (1986). Functional properties of drum dried chickpea (*Cicer arietinum* L.) flour. *Journal of Food Science, 51*, 1518-1526. http://dx.doi.org/10.1111/j.1365-2621.1986.tb13849.x

Curran, L. (2012). The nutritional value and health benefits of pulses in relation to obesity, diabetes, heart disease and cancer. *British Journal of Nutrition, 108*, S1-S2. http://dx.doi.org/10.1017/S0007114512003534

Dahl, W. J., Whiting, S. J., Isaac, T. M., Weeks, Sh., J., & Arnold, Ch. J. (2005). Effects of thickened beverages fortified with inulin on beverage acceptance, gastrointestinal function, and bone resorption in institutionalized adults. *Nutrition, 28*, 308-311. http://dx.doi.org/10.1016/j.nut.2004.06.025

Deshpande, R. P., Chinnan, M. S., & McWatters, K. H. (2008). Optimization of a chocolate-flavored, peanut–soy beverage using response surface methodology (RSM) as applied to consumer acceptability data. *Food Science and Technology*, 1485-1492. http://dx.doi.org/10.1016/j.lwt.2007.08.013

Din, A., Anjum, F. M., Zahoor, T., & Nawaz, H. (2009). Extraction and utilization of barley β-glucan for the preparation of functional beverage. *International Journal of Agriculture and Biology, 11*, 737-740.

Fernandez-Quintela, A., Macarulla, M. T., Del Barrio, A. S., & Martõanez, J. A. (1997). Composition and functional properties of protein isolates obtained from commercial legumes grown in northern Spain. *Plant Foods for Human Nutrition, 51*, 331-342. http://dx.doi.org/10.1023/A:1007936930354

Hu, F. B. (2003). Plant-based foods and prevention of cardiovascular disease: an overview. *American Journal of Clinical Nutrition, 78,* 544-551.

Jackman, R. L., & Yada, R.Y. (1989). Functional Properties of Whey-Pea Protein Composite Blends in a Model System. *Journal of Food Science, 54*(5), 1287-1292. http://dx.doi.org/10.1111/j.1365-2621.1989.tb05975.x

Jacobs, D. R., & Gallaher, D. D. (2004). Whole grain intake and cardiovascular disease: A review: *Current Atherosclerosis Reports, 6*, 415-423. http://dx.doi.org/10.1007/s11883-004-0081-y

Jasentuliyana, N., Toma, R. B., Klavons, J. A., & Medora, N. (1998). Beverage cloud stability with isolated soy protein. *Journal of the Science of Food and Agriculture, 78*, 389-394. http://dx.doi.org/10.1002/(SICI)1097-0010(199811)78:3<389::AID-JSFA130>3.0.CO;2-Z

Kazmireski, M., Agboola, S., & Corresigi, M. (2003). Optimizing stability of orange juice fortified with whey protein at low pH values. *Journal of Food Quality, 26*, 337-352. http://dx.doi.org/10.1111/j.1745-4557.2003.tb00249.x

Kelly S., Frost, G., Whittaker, V., & Summerbell, C. (2004). Low glycaemic index diets for coronary heart disease. *Cochrane Database of Systematic Reviews, 18*(4), CD004467. http://dx.doi.org/10.1002/14651858.cd004467.pub2

Kent, K. D., & Harper, W. J. (2003). Effect of whey protein addition and pasteurization method on the characteristics of tomato juice. *Journal of Milchwissenschaft, 58*, 400-403.

Kinsella, J. E. (1976). Functional properties of proteins in foods. *Critical Reviews in Food Science and Nutrition*, *1*(3), 219-280. http://dx.doi.org/10.1080/10408397609527208

Koh-Banerjee, P., Franz, M., Sampson, L., Liu, S., Jacobs, D. R., Spiegelman, D., Willett, W., & Rimm, E. (2004). Changes in whole-grain, bran and cereal fibre consumption in relation to 8-y weight gain among men. *American Journal of Clinical Nutrition, 80*, 1237-1245.

L'hocine, L., Boye, I. J., & Arcand, Y. (2006). Composition and functional properties of soy protein isolates prepared using alternative defatting and extraction procedures. *Journal of Food Science, 71*(3), C137-C145. http://dx.doi.org/10.1111/j.1365-2621.2006.tb15609.x

Lin, M. Y., Humbert E. S., & Sosulski, F. W. (1974). Certain Functional properties of Sunflour meal products. *Journal of Food Science, 39*(2), 368-370. http://dx.doi.org/10.1111/j.1365-2621.1974.tb02896.x

Luz-Fernandez de Tonella, M., & Berry, J. W. (1987). Characteristics of a Chocolate Beverage from Germinated Chickpeas. *Journal of Food Science, 52*(3), 726-728. http://dx.doi.org/10.1111/j.1365-2621.1987.tb06712.x

Obatolu, V. A., Fasoyiro, S. B., & Ogunsunmi, L. (2007). Processing and functional properties of yam beans (Sphenostylis Stenocarpa). *Journal of Food Processing and Preservation, 31*, 240-249. http://dx.doi.org/10.1111/j.1745-4549.2007.00112.x

Ofuya, Z. M., & Akuidue, V. (2005). The Role of Pulses in Human Nutrition: *A Review. Journal of Applied Science and Environmental management, 9*(3), 99-104.

Paredes-López, O., Ordorica-Falomir, C., & Olivares-Vázquez, M. R. (1991), Chickpea protein isolates: physicochemical, functional and nutritional characterization, *Journal of Food Science, 56*, 726. http://dx.doi.org/10.1111/j.1365-2621.1991.tb05367.x

Pearce, K. W., & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26*(3), 716-723. http://dx.doi.org/10.1021/jf60217a041

Pescuma, M., Hébert, E. M., Mozzi, F., & Valdez, G. F. (2010). Functional fermented whey-based beverage using lactic acid bacteria. *International Journal of Food Microbiology, 30*(141), 73-81. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.04.011

Renuka, B., Kulkarni, S. G., Vijayanand, P., & Prapulla, S. G. (2009). Fructooligosaccharide fortification of selected fruit juice beverages: Effect on the quality characteristics. *Food Science and Technology, 42*, 1031-1033. http://dx.doi.org/10.1016/j.lwt.2008.11.004

Schatzkin, A. & Mouw, T. (2007). Dietary Fiber and Whole-Grain Consumption in Relation to Colorectal Cancer in the NIH-AARP Diet and Health Study. *American Journal of Clinical Nutrition, 85*(5), 1353-1360.

Stähle-Hamatschek, S., & Gierschner, K. (1989).Trubzusammensetzung und ihr Einfluß auf die Trübungsstabilität in naturtrüben Apfelsäften, *Flüssiges Obst, 56,* 543-558.

Temelli, F., Bansema, C., & Stobbe, S. (2004). Development of an orange-flavored barley β-glucan beverage. *Cereal Chemistry, 81*, 499-503. http://dx.doi.org/10.1094/CCHEM.2004.81.4.499

Tiziani, S., & Vodovotz, Y. (2005). Rheological Characterization of a Novel Functional Food: Tomato Juice with Soy Germ. *Journal of Agricultural and Food Chemistry, 53*, 7267-7273. http://dx.doi.org/10.1021/jf0511087

Vojnovic, V., Ritz, M., & Vahcic, N. (1993). Sensory evaluation of whey based fruit beverages. *Die Nahrung, 37*, 246-251. http://dx.doi.org/10.1002/food.19930370309

Wang, N., & Toews, R. (2011). Certain physichochemical and functional properties of fiber fractions from pulses. *Food Research International, 44*, 2515-2523. http://dx.doi.org/10.1016/j.foodres.2011.03.012

Waniska, R. D., & Kinsella, J. E. (1979). Foaming properties of proteins: evaluation of a column aeration apparatus using ovalbumin. *Journal of Food Science, 44*, 1398-1402. http://dx.doi.org/10.1111/j.1365-2621.1979.tb06447.x

Williams, P. G., Gafenauer, S. J., & O'Shea, J. E. (2004). Cereal grains, legumes, and weight management: a comprehensive review of the scientific evidence. *Nutrition Reviews, 66*(4), 171-182. http://dx.doi.org/10.1111/j.1753-4887.2008.00022.x

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Quality of Minimally Processed Products Marketed in Cuiabá, Mato Grosso, Brazil

Fabíola Gonçalves da COSTA[1], Adelino Cunha Neto[2], Luiz José Rodrigues[3] & Eduardo Eustáquio de Souza Figueiredo[3]

[1] Graduated in Food Science and Technology, School Nutrition, Federal University of Mato Grosso, Cuiabá, MT, Brazil

[2] Doctorate student in Veterinary Hygiene and Technological Processing of Animal Products, School Nutrition, Federal University of Mato Grosso, Cuiabá, MT, Brazil

[3] Professor of School Nutrition, Federal University of Mato Grosso, Cuiabá, MT, Brazil

Correspondence: Eduardo Eustáquio de Souza Figueiredo, Professor of School Nutrition, Federal University of Mato Grosso, Cuiabá, MT, Brazil. Tel: 55-65-3615-8589. E-mail: figueiredoeduardo@hotmail.com

Received: May 26, 2015 Accepted: August 26, 2015 Online Published: September 14, 2015

doi:10.5539/jfr.v4n5p157 URL: http://dx.doi.org/10.5539/jfr.v4n5p157

Abstract

Consumers have been increasingly seeking healthier foods without sacrificing sensory satisfaction and convenience, which are highly acclaimed attributes in modern times. Minimally processed products can meet these demands. The present study evaluated the microbiological, microscopic and physicochemical quality of minimally processed fruits and vegetables marketed in Cuiabá, Brazil. A total of 36 samples, consisting of sliced melon, fruit salad in pieces, grated carrot, diced melon, sliced papaya and pineapple rounds were subjected to microbiological, microscopic and physicochemical analyses. *Salmonella* spp. was absent in all samples, while 27.8% (10/36) of the samples showed coliform counts at 45 °C over 2.0 and 2.7 log $CFU.g^{-1}$ in fruit and vegetables, respectively. Light dirt and foreign matter were present in 55.5% of the samples (20/36), including wood fragments and insect/animal excrements (rat hair) in 13.9% (5/36) of the samples. The pH and soluble solids (°Brix) results ranged from 3.84 to 6.66 and from 8.19 to 10.24, respectively. The products were in different stages of maturation and 27.8% (10/36) of the sliced papaya and grated carrots were in unsatisfactory sanitary conditions under the current Brazilian legislation.

Keywords: microbiological, microscopic, physicochemical analyses; fruits; vegetables; minimal processing

1. Introduction

According to the IFPA (2013) (International Association of Minimally Processed Products), minimally processed products are defined as any fruit or vegetable, or even a combination of both, which has been physically altered from its original form, while maintaining freshness. In general, the minimally processed vegetable sector faces major challenges, such as maintaining fresh products without loss of sensory and nutritional quality, in addition to guaranteeing that the products will not result in potential health risks to consumers (Huxsoll & Bolin, 1989**)**.

According to organizations such as Food Standards Agency – FSA(United Kingdom), and Centers for Disease Control and Prevention – CDC (United State America), the frequency of foodborne illness outbreaks associated with the consumption of fresh products, especially minimally processed ones, has increased notably (CDC, 2009; Paula, Vilas Boas, Rodrigues, Carvalho, & Picolli, 2009; FSA, 2007). The Food and Drug Administration (FDA) states that, of 72 cases of outbreaks related to fresh products, 25% (18 cases) are associated to minimally processed products (FDA, 2008).

The presence of injured cells and loss of its components (nutrients and enzymes) during processing operations provide optimum conditions for microorganism growth. The microbial type and species, as well as levels, in minimally processed food products vary according to the fruit or vegetable, cultivation practices, sanitary conditions during handling and processing, and storage temperature, among other factors (Smanioto, Pirolo, Simionato, & Arruda, 2009).

Microorganism indicators are an important verification tool of food sanitary hygienic conditions. This group consists mainly of coliform and bacteria from the *Salmonella* genus, which is a group of gram-negative, facultative anaerobic bacteria. Coliforms are an indicator of fecal contamination. They are lactose fermenters and, therefore, produce CO_2, used in the identification of these bacteria (Menezes & Moreira, 2012). Pathogens can originate from this microflora and lead to potential food safety problems. Neglecting to maintain appropriate utensils and handler hygiene conditions can contribute to the transmission of pathogenic and spoilage microorganisms (Xisto, Vilas Boas, & Nunes, 2012). Minimally processed vegetables evaluated in southeastern Brazil presented the high coliform bacteria counts in 50% of the samples, revealing neglect regarding hygiene precautions during the processing of these products (Albuquerque, Santos, & Rall, 2013; Pereira & Hoffmann, 2011; Pires, Donadone, Chaud, & Pereira, 2011; Silva & Rall, 2011; Furlaneto, Santini, & Velasco, 2005).

The aim of this study was to evaluate the quality of minimally processed products during different periods which were marketed in two supermarkets at the city of Cuiabá, Mato Grosso, Brazil.

2. Material and Methods

2.1 Sampling

A total of 36 fresh-cut fruit and vegetable samples were collected in refrigerated counters of two large supermarkets (A and B) located at Cuiabá, Brazil. The samples were composed of fruit salad, melon slices and grated carrot, collected in supermarket, A as well as cut melon, papaya slices and pineapple slices, collected at supermarket B, with all samplings conducted weekly from June 2013 until August of the same year. Samples were represented by two packets of minimally processed vegetables during the period, weighing approximately 300 grams. Six samples consisted of fruit salad, melon slices and grated carrot from supermarket A, and six samples consisted of diced melon, sliced papaya and pineapple rounds from supermarket B. All samples weighed approximately 300 grams, collected weekly from June until August 2013. The samples were placed in isothermal bags and sent to the Nutrition School laboratories at the Federal University of Mato Grosso for analysis. The applied parameters were the maximum permissible microbiologic and microscopic limits contained in Brazilian resolutions RDC No. 12 from January 2, 2001 (BRASIL 2001) and RDC No. 175 from July 8, 2003 (BRASIL 2003), which follow the World Health Organization (WHO) guidelines.

2.2 Microbiological Analyses

Twenty-five grams (25 g) of each sample were diluted in 225 mL of sterile peptone saline solution 0.1% (w/v) in plastic 720 mL *Stomacher* bags (Hexis Scientific S/A, Brazil) and processed in *Stomacher* sample homogenizers (Marconi, Brazil) for 60 seconds. All analyses were performed according to the International Commission on Microbiological Specification for Foods (ICMSF, 1983) and American Public Health Association (APHA, 2001).

Coliforms were determined by analysis of multiple sets of three laurel sulfate tryptose broth tubes (Himedia®, Mumbai, India), inoculated with 1 mL aliquots of the diluted samples at 10, 100 and 1000 times, and incubated at 35 °C for 24-48 hours during the presumptive phase. The confirmation analysis of coliforms was conducted from positive fermentation tubes and gas production at 35 °C and 45 °C in brilliant green bile broth 2% and *Escherichia coli* broth (Himedia®, Mumbai, India), incubated for 24-48 hours. The results were expressed in log $MPN.g^{-1}$ (APHA, 2001; ICMSF 1983).

The *Salmonella* spp. investigation consisted of three phases: pre-enrichment, selective enrichment and differential isolation. The pre-enrichment was conducted in buffered peptone water (Himedia®, Mumbai, India), which included 25 grams of each sample, incubated at 35 °C for 24 hours. Aliquots of 0.1 to 1 mL of the homogenate were transferred to 10 mL Rappaport-Vassiliadis broth (RV) and tetrathionate broth (TT) (Himedia®, Mumbai, India) and incubated at 42 °C and 35 °C, respectively, for 24 hours. Subsequently, typical *Salmonella* colonies were seeded in rambach (Merck, Germany) and brilliant green agar (Himedia®, Mumbai, India) by groove, incubated at 42 °C and 35 °C for 24 hours and then subjected to biochemical tests for identification (APHA, 2001; ICMSF 1983).

2.3 Physicochemical Analyses

After grinding and homogenizing the tissues in a 1:5 ratio (20 g of diluted pulp in 100 mL of distilled water), titratable acidity (TA), pH and soluble solids (SS) assays were performed. The determination of titratable acidity (% correspondent to citric acid) was performed by titration with 0.1 N NaOH solution using phenolphthalein as an indicator (AOAC, 2000). The pH was determined using a T-1000 Model pH meter (Tecna, Brazil) (AOAC, 2000). The SS were determined by refractometry, using a digital ATAGO PR-100 refractometer (Atago, Ribeirão Preto, Brazil) with automatic temperature compensation at 25 °C (AOAC, 2000).

2.4 Microscopical Analyses

Microscopical analyses were performed by the light debris floating method using a Wildman trap. Sample aliquots of 20 grams were introduced into the Wildman trap and homogenized with distilled water and soybean oil, and the filth contained in the oil phase was captured on a filter paper, dried through a vacuum pump (Tecnal, model TE058, São Paulo, Brazil) and observed by use of a stereoscopic microscope (Olympus, model SZST, Japan). The detected structures were confirmed by observation on an optical microscope (Olympus, model CX40RF100, Japan) (Beux, 1992).

2.5 Statistical Analyses and Experimental Design

The experiment was conducted in a randomized block design (RBD) with three blocks that comprised the manufacturing periods of the minimally processed products (1st, 2nd and 3rd manufacturing dates), performed weekly. The statistical analyses of the physicochemical assays were performed using the Statistical Programme Sisvar 4.3 (Ferreira, 2000). After the analysis of variance, the significance level applying an F test was observed. The treatment means, when significant, were compared by the Scott-Knott test at 5% probability. The results of the microbiological and microscopic analyses were obtained from indicative samples consisting of two portions of fruit and/or vegetables and are expressed as frequency and/or log of Colony Forming Unit (CFU), following Brazilian legislations (BRAZIL, 2003; BRAZIL, 2001) and recommendations from the World Health Organization. The results of the microbiological and microscopic analyses were performed with a 2 fruit or vegetable portion pool, being considered indicative tests. These procedures were performed for all collecting periods.

3. Results and Discussion

No specific legislation for minimally processed exists in Brazil. Thus, the microbiological standards set out in Resolution RDC No. 12 from 2001 of the Brazilian National Health Surveillance Agency (BRASIL, 2001) are the norm for fruit and/or vegetables "fresh, prepared (peeled, selected or cracked), sanitized, chilled or frozen for direct consumption". The RDC 12/2001 establishes the absence of *Salmonella* spp. in 25 g of fruit or vegetable and a maximum thermotolerant fecal coliform count at 45 °C or *Escherichia coli* of 5×10^2 CFU.g^{-1} (log 2.7 CFU.g^{-1}) for fruit and 1×10^2 CFU.g^{-1} (log 2.0 CFU g^{-1}) for vegetables.

The 36 fruit and vegetable samples evaluated in this study were in accordance to the recommended limits dictated by Brazilian legislation (BRASIL, 2001), such as the absence of *Salmonella* in 25 g of product. However, 27.8% of the samples, represented by five pools of two sample portions (Table 1), totalizing 10 of the 36 samples, were in unsatisfactory sanitary conditions due to the presence of coliforms at 45 °C, with counts above log 2.7 CFU.g^{-1}, detected in sliced papaya (supermarket B) from the 1st and 3rd collection period, and above log 2.0 CFU.g^{-1} detected in grated carrots (supermarket A) during all three collection periods, with coliforms also detected at 35 °C.

The absence of *Salmonella* spp. associated with a percentage of 71.2% of negative samples for coliforms at 35 °C and 45 °C may indicate that good manufacturing practices were implemented, such as the use of detergents to carefully wash the fruits and vegetables exteriors, as well as environment and utensil sanitizing on the processing site (Shale, Mukamugema, Lues, Venter, & De Smidt, 2012). The absence of *Salmonella* in fresh-cut fruits and vegetables was also observed in Campinas-SP, by Santos et al. (2010), Bauru-SP (Smanioto, Pirolo, Simionato, & Arruda, 2009), in a salad mix at Camburiú and Florianópolis-SC (Marmentini, Felipe, Lemos, Pedrozo, & Bender, 2011) and also in fruit salad evaluated in Fortaleza-CE (Pinheiro, Abreu, Maia, Sousa, Figueiredo, Rocha, & Costa, 2011).

The presence of *Salmonella* spp. in minimally processed vegetables was detected in 25% of fruit samples (Pinheiro, Figueiredo, Figueiredo, Maia, & Sousa, 2005) and in 66.6% of vegetables and tubers (Bruno, Queiroz, Andrade, Vasconcelos, & Borges, 2005) sold in supermarkets in Fortaleza-CE, and in 25% of fruit from Catanduva-SP (Virgolin, Geromel, Manfrin, & Fazio, 2013). The presence of bacteria from this genus reflects poor sanitary conditions and health risks, such as the possibility of infections, that the products may present, due to cross-contamination and inefficient sanitizing techniques (Menezes & Moreira, 2012).

Among the tested samples, only 27.8% (10/36) presented coliform counts of 2.7 and 2.0 log CFU.g^{-1} at 35 °C and 45 °C in fruits and vegetables, respectively (Table 1). The percentage of samples with high coliform results in the present study was lower than in other studies conducted in Brazil as reported. The occurrence of high coliform counts at 35 °C and 45 °C classifies these products as presenting unsatisfactory sanitary conditions (Franco & Landgraf, 2005), since the occurrence of this bacteria group can indicate the possible presence of pathogenic microorganisms due to possible direct or indirect contact with feces (Oliveira, Costa, & Maia, 2006).

Table 1. Coliforms at 35 °C and 45 ºC (log MPN.g^{-1}) in minimally processed vegetables sampled in different manufacturing periods

Supermarket	Product	log MPN.g^{-1} of coliforms per period			
		Coliforms	1st	2nd	3rd
A	Fruit salad	35 °C	2.66	1.97	1.54
		45 °C	2.66	1.97	1.54
A	Diced melon	35 °C	1.36	0.48	1.88
		45 °C	0.56	0.48	1.18
A	Grated carrots	35 °C	3.04	3.04	3.04
		45 °C	3.04	3.04	3.04
B	Pineapple rounds	35 °C	0.96	0.48	0.48
		45 °C	0.48	0.48	0.48
B	Sliced papaya	35 °C	3.04	1.63	3.04
		45 °C	3.04	1.63	3.04
B	Sliced melon	35 °C	1.97	0.48	2.18
		45 °C	1.18	0.48	1.43

Table 2. Mean titratable acidity (TA), pH and soluble solids (SS) values in minimally processed vegetables sampled in three different manufacturing periods

Product/Supermarket	Period	TA (% citric acid)	pH	SS (ºBrix)
Fruit salad/A	1rst	0.35^{a}	4.65^{b}	8.14^{a}
	2nd	0.35^{a}	4.46^{a}	8.15^{a}
	3rd	0.39^{b}	4.69^{b}	10.00^{b}
Diced melon/A	1rst	0.16^{b}	6.44^{a}	8.24^{b}
	2nd	0.13^{a}	6.66^{b}	8.25^{b}
	3rd	0.15^{b}	6.40^{a}	6.00^{a}
Grated carrots/A	1rst	0.17^{a}	6.44^{b}	4.47^{b}
	2nd	0.19^{a}	6.36^{a}	4.49^{b}
	3rd	0.28^{b}	6.41^{b}	4.19^{a}
Pineapple rounds/B	1rst	0.54^{a}	4.01^{b}	10.23^{b}
	2nd	0.52^{a}	3.84^{a}	10.24^{b}
	3rd	0.59^{b}	3.98^{b}	8.09^{a}
Sliced papaya/B	1rst	0.12^{b}	5.27^{b}	8.34^{a}
	2nd	0.10^{a}	5.18^{a}	8.28^{a}
	3rd	0.12^{b}	5.28^{b}	9.87^{b}
Sliced melon/B	1rst	0.12^{b}	6.41^{b}	8.31^{b}
	2nd	0.10^{a}	6.22^{a}	8.27^{b}
	3rd	0.12^{b}	6.39^{b}	8.19^{a}

* Means followed by the same letter represent statistical similarities between the manufacturing periods at 5% probability according to the Scott-Knott test.

The different processing stages of minimally processed products may be responsible for spoilage and contamination by pathogenic microorganisms (Coelho, Scalcon, Guaitanele, & Haida, 2008). According to

Pinheiro et al. (2005), microorganism growth in minimally processed vegetables is influenced by multiple factors, depending on the type of plant (pH, water activity and nutrients), origin, processing steps (washing, sanitizing, peeling, cutting, packaging storage temperature), sanitary conditions of the handler, equipment and utensils, as well as the environment. In the present study, pH, titratable acidity and soluble solids were evaluated (Table 2). Among these parameters, the pH showed the greatest influence on microbial growth, with values ranging from 3.84 in pineapple rounds to 6.66 in diced melon.

The papaya and carrot samples with high coliform counts at 35 °C and 45 °C presented a mean pH of 5.28 and 6.40 (Table 2), respectively. Fruits have low acidity content due to their naturally occurring organic acids (ICMSF, 1980), while greens and vegetables usually present a pH ranging from 5 to 7 (ICMSF, 1985).

According to Franco and Landgraf (2005), the pH of carrot ranges from 4.9 to 6.0. The pH tending to neutrality detected in carrots and papaya are within the minimum and optimum pH values, in which some coliforms, as *Escherichia coli* (4.3 to 8.0) and *Enterobacteriaceae*, such as *Erwinia carotovora* (4.9 to 7.1), *Proteus vulgaris* (4.4 to 7.0), *Serratia marcescens* (4.6 to 7.0) and *Salmonella* spp. (4.5 to 7.5), *S. typhi* (4.0 to 7.2) and *S. choleraesus* (5.0 to 7.6) can grow (Franco & Landgraf, 2005). Among these, *E. coli* and the genus *Salmonella* can represent consumer health risks.

The results displayed in Table 3 show that 55.5% (20/36) of the vegetable samples contained light dirt and foreign materials. However, 13.9% (5/36) showed filth such as insect or animal droppings (rat hair) and wood fragments (Table 3 and Figure 1), respectively, in sliced papaya and grated carrot. These samples were characterized as unfit for human consumption, since, according to the RDC No. 175 of July 8, 2003, insects, in any stage of development, living or dead, whole or in part; insect or animal excrement; rigid, sharp and/or cutting objects are substances harmful to human health (BRASIL, 2003).

Table 3. Results of the microscopic analyses on minimally processed products sold in two supermarkets at Cuiabá, Brazil

Foreign matter	Incidence	Product
Fiber fragments	2	Pineapple
	3	Grated carrot
	3	Sliced papaya
	2	Melon
	3	Sliced melon
Insect/animal excrements	1	Sliced papaya
Wood fragments	1	Grated carrot

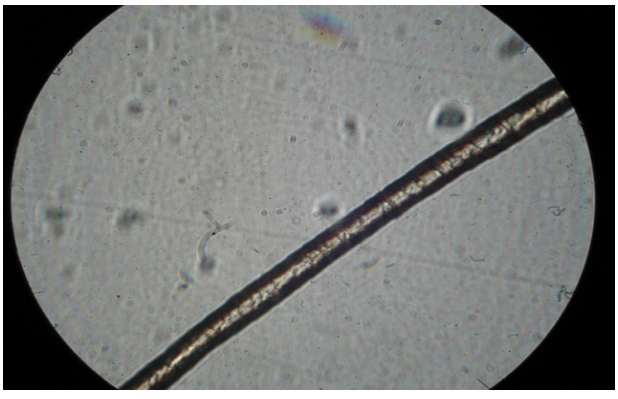


Figure 1. Rat hair found in papaya slices

In the present study, samples considered unsuitable due to the presence of light dirt also showed unsatisfactory sanitary conditions, due to the presence of coliforms at 45 °C. The situation described herein confirms the

statement by Pineli and Araújo (2006) that the contamination of minimally processed vegetables by human pathogens may occur directly or indirectly by different factors during production, acquisition and processing, including by animal or insect excrements.

4. Conclusions

The minimally processed vegetables sold in supermarkets in the city of Cuiabá, sampled from June to August 2013, were in different maturation stages, with 71.2% (26/36) of the samples presenting good quality and being fit for consumption, while 27.8 % (10/36), composed of a pool of sliced papaya and grated carrots, were in unsatisfactory sanitary conditions and unfit for human consumption according to the current Brazilian legislation microbiological and microscopic standards.

Acknowledgements

The author's would like to thank funding agency "*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES – Brasil"* for financial support.

References

AOAC. (2000). *Association of Official Analytical Chemists.* Official methods of analysis (16th ed.). Washington: Association of Official Analytical Chemists.

APHA. (2001). *American Public Health Association. Compendium of methods of the microbiological examination of foods* (p. 676). Washington, D.C.

Albuquerque, M. M., Santos, R. B. F., & Rall, V. L. M. (2013). Minimally processed vegetables in the city of Botucatu: Quality sanitary hygienic and research staphylococcal enterotoxin .*Higiene Alimentar, 27*, 166-170.

Beux, M. R. (1992). Notions of food microscopy: Research foreign matter and identification of histological elements. *Revista do CEPPA/Centro de Pesquisas e Processamento de Alimentos*. Centro Politécnico – UFPR, Paraná, Brazil.

BRASIL. (2003). Ministry of Health. National Health Surveillance Agency. resolution – RDC n° 175, of July 8, 2003. *"Macroscopic Materials Assessment Technical Regulation and Microscopic Harmful to Human Health in Packaged Food"*. Retrieved from http://www.anvisa.gov.br/legis/resolucoes/

BRASIL. (2001). Ministry of Health. National Health Surveillance Agency. Resolution – RDC n° 12, of January 2, 2001. *Technical Regulation On Microbiological Standards for Food.* Retrieved from http://www.anvisa.gov.br/legis/resolucoes/

Bruno, L. M., Queiroz, A. A. M., Andrade, A. P. C., Vasconcelos, N. M. & Borges, M. F. (2005). Microbiological evaluation of vegetables and fruits minimally processed marketed in Fortaleza (CE). *Revista da CEPPA/Centro de Pesquisas e Processamento de Alimentos, 23*, 75-84. http://dx.doi.org/10.5380/cep.v23i1.1272

CDC. (2008-2009). Center for Disease Control and Prevention. *Investigation update: outbreak of Salmonella Typhimurium infections*. Retrieved from http://www.cdc.gov/print.do?url=http%3A//www.cdc.gov/salmonella/typhimurium/

Coelho, S. R. M., Scalcon, F. R., Guaitanele, J., & Haida, K. S. (2008). Quality of minimally processed cassava roots produced in the western region of Paraná. *Higiene alimentar, 22*, 90-93.

Ferreira, D. F. (2000). *System Variance Analysis for Balanced Data.* Lavras: UFLA. (SISVAR 4. 1. computer Package).

Franco, B. D. G. M., & Landgraf, M. (2005). *Food Microbiology* (p. 182). São Paulo: Atheneu.

FDA. (2008). United State Food and Drugs Administration/Center for Food Safety and Nutrition Applied. Guidance for Industry – Guide to minimize microbial safety hazards of fresh-cut vegetables. Silver Spring. Retrieved from http://www.cfsan.fda.gov/guidance.html

FSA. (2007). Food Standards Agency, *Morrison's recalls leaf lettuce*. London. Retrieved from http://www.food.gov.uk/news/newsarchive/2007/apr/morrisalad.

Furlaneto, L., Santini, M. S., & Velasco, F. A. S. (2005). Microbiological analysis of minimally processed vegetables and greenery. *Higiene Alimentar, 19*, 68-71.

Huxsoll, C. C., & Bolin, H. R. (1989). Processing and distribution alternatives for minimally processed fruits and vegetables. *Food Technology, 43*, 124-128.

ICMSF. (1985). International Comission on Microbiological Specfications for Foods. *Ecologia microbiana de los alimentos. Volume II, Productos alimentícios*(p. 989). Editor Acribia, Zaragoza, Espanha.

ICMSF. (1980). International Comission on Microbiological Specfications for Foods. *Ecologia microbiana de los alimentos. Volume I, Factores que afectan a La supervivencia de los microorganimos em los alimentos* (p. 332). Editor Acribia, Zaragoza, Espanha.

ICMSF. (1983). International Comission on Microbiological Specifications for Foods (). *Microorganisms in foods. I. Their significance and methods of enumerations* (3rd ed., p. 431). Toronto: University of Toronto Press.

IFPA. (2013). International Fresh-Cut Produce Association. *Fresh production*. Retrieved from http://www.fresh-cuts.org

Marmentini, G. F., Felipe, M. R., Lemos, M. P., Pedrozo, P., & Bender, T. (2011). Microbiological analysis of the mix of fresh-cut salads, marketed in the resort of municipalities Camburiú and Florianopolis , SC. *Higiene Alimentar, 25*, 121-125.

Menezes, L. M. & Moreira, V. S. (2012). Microbiological analysis of minimally processed pumpkin (*Cucurbita moschata* Duch) in Itapetinga-BA *UNOPAR, Científica Ciências Biológicas e da Saúde, 14*, 159-163. Retrieved from http://www.pgss.com.br/revistacientifica/index.php/biologicas/article/view/416/403

Oliveira, A. M. C., Costa, J. M. C. & Maia, G. A. (2006). Hygienic-sanitary quality of fresh-cut "pearl" pineapple. *Revista Brasileira de Promoção da Saúde*. 19, 19-24. Retrieved from http://www.redalyc.org/articulo.oa?id=40819105/.1806-1222/19/1/

Paula, N. R. F., Vilas Boas, E. V. B., Rodrigues, L. J., Carvalho, R. A. & Picolli, R. H. (2009). Quality of fresh-cut produce commercialized on supermarket shelves in the cities of Lavras-MG, Brasília-DF, and São Paulo-SP. *Ciência e Agrotecnologia, 33*, 219-227. Retrieved from http://dx.doi.org/10.1590/S1413-70542009000100031. http://www.scielo.br/1413-7054/33/1

Pereira, A. P. M., & Hoffmann, F. L. (2011). Microbiological quality of minimally processed vegetables. *Higiene Alimentar, 25*, 60-63.

Pineli, L. L. O., & Araújo, W. M. C. (2006). Production, quality and health safety of fresh-cut vegetables. *Higiene Alimentar, 20*, 55-60.

Pinheiro, A. M., Abreu, C. R. A., Maia, G. A., Sousa, P. H. M., Figueiredo, E. A. T., Rocha, E. M. F. F. & Costa, J. M. C. (2011) Evaluation of quality characteristics, bioactive compounds and microbiological quality of tropical ready-to-eat fruit salad. *Alimentos e Nutrição, 22*, 435-440. Retrieved from http://serv-bib.fcfar.unesp.br/seer/index.php/alimentos/article/view/1494/1139.0103-4235/22/3/

Pinheiro, N. M. S., Figueiredo, E. A. T., Figueiredo, R. W., Maia, G. A., & Sousa, P. H. M. (2005). Evaluation of microbiological quality of fruits minimally processed commercialized in supermarkets in Fortaleza. *Revista Brasileira de Fruticultura, 27*, 153-156. Retrieved from http://www.scielo.br/pdf/rbf/v27n1/24589.pdf

Pires, K. R., Donadone, V. S., Chaud, D. M. A., & Pereira, I. R. O. (2011). Microbiological quality of minimally processed vegetables, marketed in São Paulo. *Higiene Alimentar, 25*, 100-104.

Santos, T. B. A., Silva, N., Junqueira, V. C. A., & Pereira, J. L. (2010). Indicator microorganisms in minimally processed fruits and vegetables. *Brazilian Journal of Food Technology, 13*, 141-146. http://dx.doi.org/10.4260/BJFT2010130200019.

Shale, K., Mukamugema, J., Lues, R. J., Venter, P., & De Smidt, O. (2012). Microbiota associated with commercially produced traditional banana beer in Rwanda. *Scientific Research and Essays, 7*(47), http://dx.doi.org/10.5897/SRE12.252.

Silva, N. C. C., & Rall, V. M. (2011). Sanitary hygienic quality of fresh-cut products. *Higiene Alimentar, 25*, 121-124.

Smanioto, T. F., Pirolo, N. J., Simionato, E. M. R. S., & Arruda, S. C. (2009). Microbiological quality of minimally processed fruits and vegetables. *Revista do Instituto Adolfo Lutz, 68*, 150-154. http://revistas.bvs-vet.org.br/rialutz/article/view/7122/7347.0073-9855/68/1

Virgolin, L. B., Geromel, M. R., Manfrin, S. C. P., & Fazio, M. L. S. (2013). Sanitary hygienic quality of minimally processed vegetables, marketed in the region of Catanduva, SP. *Higiene Alimentar, 27*, 130-134.

Xisto, A. L. R. P., Vilas Boas, E. V. B., & Nunes, E. E. (2012). Maintenance of the microbiological quality of minimally processed watermelon. *Journal of Biotechnology and Biodiversity, 3*, 15-20. Retrieved from http://revista.uft.edu.br/index.php/JBB/article/view/193/131./2170-4804/3/2

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Sensory Evaluation of *Moringa*- Probiotic Yogurt Containing Banana, Sweet Potato or Avocado

Megan Kuikman[1] & Colleen P. O'Connor[1]

[1] Division of Food and Nutritional Sciences, Brescia University College, London, Canada

Correspondence: Colleen P. O'Connor, Brescia University College, Western University, London, Ontario, N6G 1H2, Canada. Tel: 1-519-432-8353 x28249. E-mail: colleen.oconnor@uwo.ca

Received: May 5, 2015 Accepted: August 25, 2015 Online Published: September 21, 2015

doi:10.5539/jfr.v4n5p165 URL: http://dx.doi.org/10.5539/jfr.v4n5p165

Abstract

This study evaluated the effects of adding selected fruits and vegetables local to Mwanza, Tanzania on the sensory qualities of probiotic yogurt supplemented with *Moringa oleifera,* a local tree with a high micronutrient and protein content. A total of five samples were evaluated: 1) Probiotic yogurt (control), 2) *Moringa* probiotic yogurt, 3) *Moringa*-banana probiotic yogurt, 4) *Moringa*-sweet potato probiotic yogurt, and 5) *Moringa*-avocado probiotic yogurt. Consumers (n= 37) rated the five different samples on a 9-point hedonic scale for four sensory characteristics (flavour, appearance, texture and overall quality). The control sample and the *Moringa*-banana sample had significantly higher ratings ($p<0.05$) than the *Moringa* sample for appearance, flavour, texture and overall quality. The *Moringa*-banana sample was not found to be significantly different than the control sample for all sensory characteristics ($p>0.05$). Overall, the addition of banana to *Moringa* probiotic yogurt resulted in a product with comparable sensory qualities to probiotic yogurt alone.

Keywords: *Lactobacillus rhamnosus* GR-1, *Moringa oleifera,* probiotic, consumer test, yogurt, Tanzania

1. Introduction

"Yogurt is the food produced by culturing one or more optional dairy ingredients …. with a characterizing bacterial culture that contains the lactic acid-producing bacteria, Lactobacillus bulgaricus and Streptococcus thermophilus" (Food and Drug Administrations [FDA], 2014). These lactic-acid bacteria convert lactose into lactic acid by first splitting lactose into glucose and galactose and then converting glucose into lactic acid, resulting in a decreased pH while the galactose tends to accumulate in the fermenting milk (Lourens-Hattingh & Viljoen, 2001). Yogurt temperature is then reduced to stop bacterial growth (Clark, Costello, Drake, & Bodyfelt, 2009). Many compounds are present in yogurt but acetylaldehyde is mainly responsible for the typical yogurt aroma (Hadman, Kunsman, & Deanne, 1971).

Probiotics, which can be defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Reid, 2010), grow well in milk, making yogurt an excellent carrier (Reid, 2010). The conventional yogurt starter cultures do not have the ability to survive passage through the human gastrointestinal tract (Gilliland, 1979), while probiotics do have the ability to alter the composition of the microbiota (Conly & Johnston, 2004). Probiotic yogurt has been shown to be comparable to standard yogurt for appearance, flavour, texture and overall quality (Hekmat & Reid, 2006).

Western Heads East (WHE) is Western University's community response to the HIV/AIDS crisis in Africa. Eastern and Southern Africa represent only 5% of the world's population, yet half of the world's population living with the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) reside in these areas (UNICEF, 2013). Additionally, the HIV/AIDS epidemic is occurring in populations where malnutrition is endemic (World Health Organization [WHO], 2003). WHE began a probiotic micro-enterprise initiative in Eastern Africa by introducing community probiotic yogurt kitchens where local women make yogurt containing the probiotic strain *Lactobacillus rhamnosus* GR-1 and sell it in the community. Numerous health benefits for those living with HIV have been associated with the consumption of probiotics, including an increase in CD4 count (Irvine et al., 2010) and resolved diarrhea (Anukam, Osazuwa, Osadolor, Bruce, & Reid, 2008). This venture not only supplies a healthy product to the community, but also empowers the women involved by providing an income source.

Moringa oleifera is a drought-tolerant tree that is cultivated in East Africa and has an outstanding nutrient content (Moringanews network, 2007). On a gram per gram comparison, *Moringa* leaves contain at least twice the protein of milk, are richer in iron than beef and lentils, are richer in calcium than milk and are as rich in vitamin A as carrots (Moringanews network, 2007). Since externally fortifying the probiotic-yogurt with micronutrients is unsustainable due to the high costs of importation, fortifying the probiotic-yogurt with *Moringa* is a locally available and sustainable alternative (Van Tienen et al., 2011).

Table 1. Nutrient content of 17.09g of *Moringa* vs. Recommended Dietary Allowance (RDA)

Nutrient	17.09g *Moringa*	RDA	% RDA/17.09g of *Moringa*
Proteins	4956 mg	0.8g/kg body weight/day	N/A
Vitamin A	2669 IU ≈ 133.5 µg	Males- 900 µg/day	Males- 15%
		Females- 700 µg/day	Females- 19%
Calcium	329 mg	1000 mg/day	33%
Magnesium	72 mg	Males- 420 mg/day	Males- 17%
		Females- 320 mg/day	Females- 23%
Phosphorus	46 mg	700 mg/day	6.6%
Potassium	237 mg	4700 mg/day*	5.0%
Copper	0.17 mg	0.9 mg/day	19%
Iron	4.8 mg	Males- 8 mg/day	Males- 60%
		Females- 18 mg/day	Females- 27%
Zinc	0.43 mg	Males- 11 mg/day	Males- 3.9%
		Females- 8 mg/day	Females- 5.4%
Manganese	1. 43 mg	Males- 2.3mg/day*	Males- 62%
		Females- 1.8mg/day*	Females- 79%

*Adequate intake (AI) because no RDA available.

Moringa's addition to probiotic yogurt may have growth-enhancing effects on *Lactobacillus rhamnosus* GR-1 when the probiotic yogurt is stored at 4°C (Van Tienen et al., 2011). A similar study also found that *Moringa* may have growth enhancing properties on *Lactobacillus rhamnosus* GR-1 growth except perhaps at higher concentrations of *Moringa* (1%) and when sugar is also present in the probiotic yogurt (Hekmat, Morgan, Soltani, & Gough, 2015). The possible growth enhancing effects of *Moringa* are significant because in order to produce health benefits, bacterial colony formation cannot fall below 10^6 CFU/mL (Kechagia et al., 2013) and (and). Hence *Moringa* could potentially enhance the shelf-life of the probiotic yogurt (Van Tienen et al., 2011). However, the sensory properties (appearance, flavour, texture and overall quality) of yogurt samples without *Moringa* were strongly preferred over samples with *Moringa.* No difference in sensory properties was seen when rating samples with different concentrations of *Moringa* (Van Tienen et al., 2011).

Past research in East Africa has tried to improve both consumer acceptability and nutrient density of the probiotic yogurt served by the yogurt kitchens. One study found that participants preferred yogurt products that were sweet, subtly flavoured, and maintained a smooth, creamy, homogenous texture (Irvine & Hekmat, 2011). Irvine and Hekmat, 2011 tested 5 different samples of probiotic yogurt with added fruits and vegetables in Tanzania and found that a mashed sweet potato sample that also contained cooked onions and garlic received the best overall score and had the least inter-subject variation. Another sample containing banana, papaya and honey was also well received by participants. When 5% sugar was added to probiotic yogurt with low concentrations of Moringa (0.5%) it was rated acceptable as a yogurt, but when a concentration of 1% *Moringa* was used with 5% sugar it still had a strong, undesirable flavour (Hekmat et al., 2015). By adding local fruits and vegetables to *Moringa*-probiotic yogurt, it is hypothesized that the sensory properties may be improved. This has the potential to further improve the health of those receiving probiotic yogurt from the community kitchens in East Africa, especially those with HIV, because of the extra micronutrients provided by *Moringa*. The objective of this study was to evaluate the acceptability of local fruits and vegetables in *Moringa*- probiotic yogurt.

2. Materials and Methods

2.1 Probiotic Mother Culture Preparations

The *Lactobacillus rhamnosus* GR-1 probiotic mother culture was prepared at the National Institute of Medical Research (NIMR) in Mwanza. The probiotic microorganisms were added to de Man, Rogosa and Sharp (MRS) broth and incubated anaerobically using BBL gas packs at 37°C overnight (17-18 hours). Milk (3.5% fat) was sterilized the next day in an autoclave at 121°C and 15 psi for 15 minutes followed by the addition of 2% of the incubated MRS. This was then incubated anaerobically at 37°C for 18 hours.

2.2 Probiotic Yogurt Productions

Standardized milk (3.5% fat) was heated to 87°C and the temperature maintained for 30 minutes under constant stirring. The milk was cooled to 37°C and 2% yogurt and 4% probiotic mother culture were added to the pasteurized milk. Raw banana and avocado were washed, peeled, pureed and placed into the bottom of separate containers. The sweet potato was boiled and cooled prior to blending. Raw banana and avocado were used to prevent the loss of vitamins and minerals (Severi, Bedogni, Manzieri, Poli, & Battistini, 1997) and to create a product that is easily replicable for the local women working in the yogurt kitchens. To prevent contamination, all fruits and vegetables were thoroughly washed and peeled before use. The pasteurized milk was poured over top of the pureed fruits and vegetables in the containers. A total of 250ml of fruit or vegetable were added per l000ml of yogurt. These were kept at 37°C for 6 hours to allow for adequate fermentation and a pH comparable to similar studies (Clark et al., 2009). After this, the product was refrigerated at 4°C for 12 hours to allow cooling and to stop bacterial growth (Clark et al., 2009). Next, 17.09g of dried *Moringa* leaves per 1L of yogurt were stirred into the chilled yogurt and fruit/vegetables samples prior to serving. The *Moringa* was not heat treated or introduced into the pasteurized milk with the pureed fruits and vegetables because it interfered with yogurt thickening. This amount of *Moringa* was chosen based on previous research (Van Tienen et al., 2011) because one liter of yogurt containing *Moringa* corresponds to 19% of the RDA of vitamin A for women and 15% of the RDA of vitamin A for men, making it a "good source" of vitamin A (Government of Canada, 2012). Table 1 lists the nutrient content of 17.09g of *Moringa* (Van Tienen et al., 2011) and the RDA reported by Health Canada (2005).

2.3 Consumer Test

2.3.1 Consumers

The Western University Research Ethics Board approved the research protocol. All sensory testing took place in the district of Mabatini in Mwanza, Tanzania. Inclusion criteria for participation consisted of understanding the intent of the study, being over 18 years of age, and the ability to provide consent. Exclusion criteria included lactose intolerance and/or food allergies to milk, *Moringa*, banana, avocado, or sweet potato. Participants were recruited via posters hung in the community one week before the evaluation. On the day of the evaluation, posters were handed out as an additional recruitment effort. Eligible participants received a letter of information and signed a consent form prior to participation. All forms were in Swahili and the consumer test took place in Swahili through a translator. As compensation for participation, participants received a voucher for 500ml of probiotic yogurt.

2.3.2 Sample Preparation

Five samples were selected for the consumer test. The selected yogurt samples included the probiotic yogurt (control), *Moringa* probiotic yogurt, *Moringa*-banana probiotic yogurt, *Moringa*-sweet potato probiotic yogurt, and *Moringa*-avocado probiotic yogurt. The consumer test occurred the day after yogurt preparation. Samples were presented in standardized amounts in clear cups in a counterbalanced order. Enough of each sample was given to allow for re-tasting if desired. Each sample was designated a random three-digit code, which appeared on the corresponding cups.

2.3.3 Consumer Test

The consumer test took place in the quiet back room of the Mabatini community yogurt kitchen. Two consumer tests took place simultaneously. After reading the letter of information and providing informed consent, participants were given five samples of probiotic yogurt in a random counterbalanced order, an evaluation form, a pencil, a glass of water, and a plastic spoon for each sample. Participants were instructed not to communicate with each other and to rinse their mouth between samples with water. Instructions were given through a translator in Swahili. The same instructions were listed at the top of each evaluation form in Swahili. Participants assessed each sample on a scale of 1 (dislike extremely) to 9 (like extremely) for appearance, taste, texture and overall quality. Further questions were asked to explain their evaluations.

2.4 Statistical Analysis:

The mean and standard deviation were used to summarize the ratings for each sensory characteristic (texture, flavour, appearance, and overall quality) for all yogurt samples. Repeated measures analysis of variance (ANOVA) was used to compare the within-group mean change for each category. Tukey's Honestly Significant Difference (HSD) was then used to determine how the means of each yogurt sample compared to each other. A p-value <0.05 was considered statistically significant.

3. Results

3.1 Consumer Test

3.2.1 Demographics and Product Usage Questionnaire

A total of 40 participants took part in the evaluation but the data of 3 participants were excluded from analysis due to incomplete consumer test forms. Of the 37 participants who completed the study, the majority were male (75%) and consumed yogurt on a daily basis (77%). A vast majority of participants were male due to the nature of the local culture. Most females tend to be at home during the day while males work outside the home within the community. All participants had previously consumed yogurt. The basic demographic information of participants is listed in Table 2.

Based on the 9-point hedonic scale, the results of the mean ratings for appearance, taste, texture and overall quality can be found in table 3. Significant differences ($p<0.05$) between mean sensory scores were found for appearance, flavour, texture and overall quality. The control sample had the highest score for appearance, flavour, texture and overall quality, while the *Moringa* sample had the lowest score for all four categories. The *Moringa*-banana sample contained significantly higher scores in all sensory categories except texture than all other samples except for the control.

3.2.2 Appearance

While the control sample was white in colour and had a thick consistency, the four remaining samples had a soft-green colour but held a similar consistency. The mean scores for appearance ranged from dislike slightly (*Moringa*) to like moderately (control). The *Moringa*-banana sample (6.9±2.0) and the control sample (7.1±1.8) had significantly higher ratings ($p<0.05$) for appearance than the *Moringa* sample (4.6±2.8), *Moringa*-sweet potato sample (5.4±2.2) and *Moringa*-avocado sample (5.5±2.6). However, there was no significant difference ($p=0.991$) between the control and *Moringa*-banana sample.

3.2.3 Flavour

The mean scores for flavour ranged from neither like nor dislike (*Moringa*-avocado) to like moderately (control). The *Moringa*-banana sample (6.7±2.1) and the control sample (6.9±2.1) had significantly higher ratings ($p<0.05$) for flavour than the *Moringa* sample (5.4±2.3) and *Moringa*-avocado sample (5.1±2.4). No significant difference ($p>0.05$) existed between the control, *Moringa*-banana sample and the *Moringa*- sweet potato (5.7±2.5) sample.

3.2.4 Texture

The mean scores for texture ranged from neither like nor dislike (*Moringa*) to like moderately (control). The *Moringa*-banana sample (6.2±2.4) and the control sample (6.7±2.0) had significantly higher ratings ($p<0.05$) for texture than the *Moringa* sample (4.8±2.4). No significant difference ($p>0.05$) existed between the control, *Moringa*-banana sample, *Moringa*- sweet potato sample (5.6±2.1) and the *Moringa*-avocado sample (5.3±2.4).

3.2.5 Overall Quality

The mean scores for overall quality ranged from neither like nor dislike (*Moringa*) to like moderately (control). The overall quality score for the control sample (7.2±2.3) and *Moring*-banana sample (6.2±2.5) were significantly higher ($p<0.05$) than the *Moringa* sample (4.5±2.6). The control sample was also significantly higher than the *Moringa*- sweet potato sample (5.2±2.7) and *Moringa*-avocado sample (5.4±2.6). No significant difference ($p=0.383$) existed between the control and *Moringa*-banana sample.

Table 2. Sex, age and product-usage of participants (n=37)

Participant characteristics		
Sex	Male	76%
	Female	24%
Age	18-24 years	43%
	25-50 years	41%
	>50 years	16%
Product-usage	1/day	84%
	1/week	5%
	1/month	8%
	Never	0%

Table 3. Panelists (n=37) mean sensory score (standard deviation) for appearance, flavour, texture and overall quality on a 9-point hedonic scale for each sample

Sample	Appearance	Flavour	Texture	Overall Quality
Moringa	4.6 ±2.8^{b}	5.4 ±2.3^{b}	4.8 ±2.4^{b}	4.5 ±2.6^{b,c}
Moringa- banana	6.9 ±2.0^{a}	6.7 ±2.1^{a}	6.2 ±2.4^{a}	6.2 ±2.5^{a,c}
Moringa-sweet potato	5.4 ±2.2^{b}	5.7 ±2.5^{a,b}	5.6 ±2.1^{a,b}	5.2 ±2.7^{c}
Moringa- avocado	5.5 ±2.6^{b}	5.1 ±2.4^{b}	5.3 ±2.4^{a,b}	5.4 ±2.6^{c}
Control	7.1 ±1.8^{a}	6.9 ±2.1^{a}	6.7 ±2.0^{a}	7.2 ±2.3^{a}

Note. Where 1=dislike extremely, 2= dislike very much, 3= dislike moderately, 4=dislike slightly, 5= neither like or dislike, 6= like slightly, 7= like moderately, 8= like very much, and 0= like extremely; $p< 0.05$ for appearance, flavour and overall quality. For each characteristic, treatments with the same letter are not statistically different by a Tukey's Test at the significance level of $P<0.05$.

4. Discussion

The objective of this study was to observe the effects of adding fruits and vegetables on the sensory qualities of probiotic yogurt supplemented with *Moringa.* We hypothesized that the addition of fruits and vegetables would improve the sensory qualities, resulting in a more acceptable product. Like previous studies, *Moringa*-probiotic yogurt was liked significantly less than the control sample for appearance, taste, texture and overall quality. This study showed that by adding banana to *Moringa*-probiotic yogurt it can be made comparable to probiotic yogurt alone. *Moringa*-banana probiotic yogurt had the second highest mean score in all categories and had significantly higher ratings than the *Moringa*-probiotic yogurt for appearance, taste, texture and overall quality. Furthermore, no significant difference existed between it and the control sample, indicating that the two samples are comparable. Likewise, we found no difference between the control sample and the *Moringa*-sweet potato sample for flavour and texture, and the *Moringa*-avocado sample for texture, suggesting that they are also comparable.

The findings of this study are important because they show that *Moringa*-probiotic yogurt could become an acceptable alternative to probiotic yogurt in East African communities when supplemented with banana. *Moringa*-probiotic yogurt is nutritionally superior to probiotic yogurt alone due to its high nutrient content (Moringanews network, 2007). Those living with HIV/AIDS would especially benefit because the increased nutritional needs caused by the HIV virus (Mangili, Murman, Zampini, Wanke, & Mayer, 2006; Kosmiski, 2011) would be better met by this novel yogurt when compared with probiotic yogurt alone. Furthermore, all samples tested incorporated only simple, locally available and cost-effective foods, enabling the local communities through the yogurt kitchen micro-enterprises to easily replicate the samples with little additional costs involved. *Moringa* grows readily in East Africa so these facilities could grow their own *Moringa* to incorporate into the probiotic yogurt. These samples could therefore be produced and sold in the yogurt kitchens.

A limitation of this study is that the majority of participants were men (75%) and were recruited from only one region in Mwanza. Because food preferences are partially determined by cultural forces, it was important that the

consumer test be conducted in the region where the product would be sold (Robalino & Robson, 2013).

5. Conclusion

Our study showed that by adding banana, sweet potato or avocado to *Moringa*-probiotic yogurt, it can be made comparable to probiotic yogurt alone. This product may offer additional health benefits over probiotic yogurt alone due to the added nutrients. Future studies should investigate the impact of *Moringa*-probiotic yogurt consumption on the health of consumers, especially those living with HIV. Studies should also look at further improving the sensory qualities of *Moringa*-probiotic yogurt in order to make it superior to probiotic yogurt alone and should include a sample with a proportional amount of female and male participants.

Acknowledgments

The authors would like to thank the National Institute of Medical Research for helping to culture the probiotic microorganisms and for providing support; the Tukwamuane's Women's Group for the use of their facilities for data collection; Western Heads East for support and encouragement; and Brescia University College for providing funding for this project.

References

Anukam, K. C., Osazuwa, E. O., Osadolor, H. B., Bruce, A. W., & Reid, G. (2008). Yogurt containing probiotic Lactobacillus rhamnosus GR-1 and L. reuteri RC-14 helps resolve moderate diarrhea and increase CD4 Count in HIV/AIDS patients. *Journal of Clinical Gastroenterology, 42*(3), 239-243. http://dx.doi.org/10.1097/MCG.0b013e31802c7465

Clark, S., Costello, M., Drake, M., & Bodyfelt, F. (2009). *The consumer test of dairy products* (2nd ed.). http://dx.doi.org/ 10.1007/978-0-387-77408-4

Conly, J. M., & Johnston, L. B. (2004). Coming full circle: From antibiotics to probiotics and prebiotics. *Canadian Journal of Infectious Diseases, 15*(3), 161-163.

Food and Drug Administrations [FDA]. (2014). *CFR- code of federal regulations title 21*. Retrieved from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=131.200

Gilliland, S. E. (1979). Beneficial inter-relationships between certain microorganisms and humans: Candidate microorganisms for use as dietary adjuncts. *Journal of Food Protection, 42*(2)*,* 164-167.

Government of Canada. (2012). *Nutrient content claims: What they mean.* Retrieved from http://healthycanadians.gc.ca/eating-nutrition/label-etiquetage/nutrient_claims-allegations_nutriment-eng.php

Hadman, I. Y., Kunsman, J. E., & Deanne, D. D. (1971). Acetaldehyde production by combined yogurt cultures. *Journal of Dairy Science, 54*(7), 1080-1082. http://dx.doi.org/ 10.3168/jds.S0022-0302(71)85975-1

Health Canada. (2005). *Dietary reference intakes*. Retrieved from http://www.hc-sc.gc.ca/fn-an/nutrition/reference/index-eng.php

Hekmat, S., Morgan, K., Soltani, M., & Gough, R. (2015). Consumer test of locally-grown fruit purees and inulin fibre on probiotic yogurt in Mwanza, Tanzania and the microbial analysis of probiotic yogurt fortified with Moringa oleifera. *Journal of Health, Population and Nutrition, 33*(1), 60-68.

Hekmat, S., & Reid, G. (2006). Sensory properties of probiotic yogurt is comparable to standard yogurt. *Nutrition Research, 26*(4), 163-166. http://dx.doi.org/10.1016/j.nutres.2006.04.004

Irvine, S. L., & Hekmat, S. (2011). Evaluation of sensory properties of probiotic yogurt containing food products with prebiotic fibres in Mwanza, Tanzania. *Food and Nutrition Sciences, 2*, 434-439. http://dx.doi.org/10.4236/fns.2011.25061

Irvine, S. L., Hummelen, R., Hekmat, S., Looman, C. W., Habbema, J. D., & Reid, G. (2010). Probiotic yogurt consumption is associated with an increase of CD4 count among people living with HIV/AIDS. *Journal of Clinical Gastroenterology, 44*(9), 201-205. http://dx.doi.org/10.1097/MCG.0b013e3181d8fba8

Kosmiski, L. (2011). Energy expenditure in HIV infection. *American Journal of Clinical Nutrition, 94*(6), 16775-16825. http://dx.doi.org/10.3945/ ajcn.111.012625

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., & Fakiri, E. M. (2013). Health benefits of probiotics: A review. *International Scholarly Research Notices, 2013*, 1-7. http://dx.doi.org/10.5402/2013/481651

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier. *International Dairy Journal, 11*(1),

1-17. http://dx.doi.org/10.1016/S0958-6946(01)00036-X

Mangili, A., Murman, D. H., Zampini, A. M., Wanke, C. A., & Mayer, K. H. (2006). Nutrition and HIV infection: Review of weight loss and wasting in the era of highly active antiretroviral therapy from the nutrition for healthy living cohort. *Clinical Infectious Disease, 42*(6), *836-842*. http://dx.doi.org/10.1086/500398

Moringanews network. *(2007). The nutritional value of Moringa oleifera Lam leaves: What can we learn from figures?* Retrieved from http://www.moringanews.org/doc/GB/Posters/Broin_poster.pdf

Reid, G. (2010). The potential role for probiotic yogurt for people living with HIV/AIDS. *Gut Microbes, 1*(6), 411-414. http://dx.doi.org/10.4161/gmic.1.6.14079

Robalino, N., & Robson, R. J. (2013). Genes, culture, and preferences. *Biological Theory, 8*(2), 151-157. http://dx.doi.org/ 10.1007/s13752-013-0108-0

Severi, S., Bedogni, G., Manzieri, A. M., Poli, M., & Battistini, N. (1997). Effects of cooking and storage methods on the micronutrient content of foods. *European Journal of Cancer Prevention, 6*(1), 21-24. http://dx.doi.org/ 10.1097/00008469-199703001-00005

UNICEF. (2013). *Eastern and Southern Africa: HIV and AIDS.* Retrieved from http://www.unicef.org/esaro/5482_HIV_AIDS.html

Van Tienen, A., Hullegie, Y. M., Hummelen, R., Hemsworth, J., Changalucha, J., & Reid, G. (2011). Development of a locally sustainable functional food for people living with HIV in Sub-Saharan Africa: Laboratory testing and consumer test. *Beneficial Microbes, 2*(3), 193-198. http://dx.doi.org/10.3920/BM2011.0024

World Health Organization. (2003). *Nutrient requirements for people living with HIV/AIDS.* Switzerland, Geneva: World Health Organization.

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Effect of Tiger Nut Residue Flour Inclusion on the Baking Quality of Confectionaries

Eke-Ejiofor, J.[1] & Deedam, J. N.[1]

[1] Department of Food Science and Technology, Rivers State University of Science and Technology Nkpolu, P.M.B. 5080, Port Harcourt

Correspondence: Eke-Ejiofor, J. Department of Food Science and Technology, Rivers State University of Science and Technology Nkpolu, P.M.B. 5080, Port Harcourt, Nigeria. Tel: 234-703-311-7436. E-mail: joyekee@yahoo.co.uk

Received: July 19, 2015 Accepted: August 6, 2015 Online Published: September 21, 2015

doi:10.5539/jfr.v4n5p172 URL: http://dx.doi.org/10.5539/jfr.v4n5p172

Abstract

Cakes and biscuits were prepared from blends of wheat and tiger nut residue flours produced using raw and dry extraction method and substituted at 5%, 10%, 15% and 20% levels. The cakes and biscuits were analyzed for proximate composition, physical characteristics and sensory properties. Results for proximate composition indicated that moisture, fibre, ash, protein and fat contents increased with an increase in tiger nut residue substitution. Ash content for cake increased from 0.93% in sample A (100% wheat flour) to 1.40% in sample E (80% wheat flour: 20% tiger nut residue flour), while ash for biscuit increased from 1.35% in sample A (100% wheat flour) to 1.85% in sample B (95% wheat flour: 5% tiger nut residue flour). Fibre content for cake increased from 1.08% in sample A (100% wheat flour) to 3.15% in sample E (80% wheat flour:20% tiger nut residue flour) as substitution increased, while for biscuit fibre increased from 1.59% in sample A (100% wheat flour) to 3.13% in sample D (85% wheat flour:15% tiger nut residue flour). Moisture content for cake increased from 21.95% in sample A (100% wheat flour) to 28.49% in sample E (80% wheat flour: 20% tiger nut residue flour) while for biscuit moisture increased from 1.08% in sample A (100% wheat flour) to 2.78% in sample B (95% wheat flour: 5% tiger nut residue flour). There was a statistical significant difference in all samples with increase in substitution. Result for physical characteristics showed a significant ($p \leq 0.05$) increase in cake volume and spread ratio of biscuit with increase in level of substitution of tiger nut residue flour. Sensory evaluation result for cakes showed sample E (80% wheat flour:20% tiger nut residue flour) as most acceptable for cakes, in terms of color (4.3), aroma (3.95), taste (4.2), texture (4.05) and general acceptability (4.3) while for biscuits sample B(95% wheat flour:5% tiger nut residue flour) as most acceptable in terms of color (4.15) aroma, (4.0) taste, (4.15) texture (4.3) and overall acceptability (4.35).

Keywords: utilization, tiger- nut, residue flour, cakes, biscuits

1. Introduction

Confectionery is related to food item that are rich in sugar and often referred to as a confection. It is the art of creating sugar based desert (Magee, 2009). Cake is an item of soft sweet food made from baking a mixture of flour, eggs and sugar. It is a form of bread or bread-like food served alone, as a desert amongst other food items or taking in a picnic (Ohl et al., 1981). Biscuit is a small, flat crispy cake. They are nutritive snacks produced from palatable dough that is transformed into appetizing product through the application of heat in an oven (Kure et al., 1998). Biscuits are a rich source of fat and carbohydrates, hence are energy giving food and also a good source of protein (Kure et al., 1998).

Tiger nut (*Cyperus esculentus var sativa*) a lesser known and underutilized crops, many of which are potentially valuable as human and animal food, has been identified to maintain a balance between population growth and agricultural productivity particularly in the tropical and sub-tropical areas of the world (Adejuyitan, 2011) with reference to Nigeria. It has been reported to be high in carbohydrate, moderate protein, oleic acid, mineral, vitamin C and E contents (Esteshola & Oreadu, 1996; Omode et al., 2004). Tiger nut produces about 25% oil of high quality and protein about 8% of the nut, where valued for their nutritious content, and dietary fibre. Tiger nut can be eaten raw, dried, roasted or grated and used as flour, vegetable milk, cosmetics, fuel, hog feed. It is

pressed for its juice to make beverage called (chufa) which is equally obtained from the rhizome (Belewu & Belewu, 2007). Tiger nut is found to be rich in myristic acid, oleic and linoleic acid (Eteshola & Oraedu, 1996).

Flour is a powder which is made by grinding cereal grains, beans or other seeds or root like cassava. It is the main ingredient of bread, which is a staple food for many cultures. Wheat flour is generally the main ingredient for baked products world over, making the availability of the flour a major economic issue. Increase in population as well as increased consumption rate of wheat based products in Nigeria has posed a huge burden on the importation, utilization and cost of wheat based product. Tiger nut a lesser known and underutilized crop, which has been valued for its nutritious dietary fibre and carbohydrate content could be eaten raw, roasted, dried, baked or made into a refreshing drink with its residue discarded in most cases, thereby converting this ideal waste. It has also been reported that tiger nut with its inherent nutritional and therapeutic advantages could serve as good alternative to cassava in baking industry (Ade-Omowaye et al., 2008). Therefore the objectives of this study are to produce flour from tiger nut residue for value addition and to evaluate the effect of addition of tiger nut residue flour on the baking qualities of cakes and biscuits.

2. Materials and Methods

2.1 Materials

Tiger nuts (*Cyperus esculentus*) yellow variety used for this study was purchased from fruit garden market in Port Harcourt. Commercial wheat flour and other ingredients such as margarine, sugar, fresh eggs, salt, milk, flavor and sodium bicarbonate (baking powder) were purchased from Mile 1 market in Diobu, Port Harcourt. Rivers State, Nigeria.

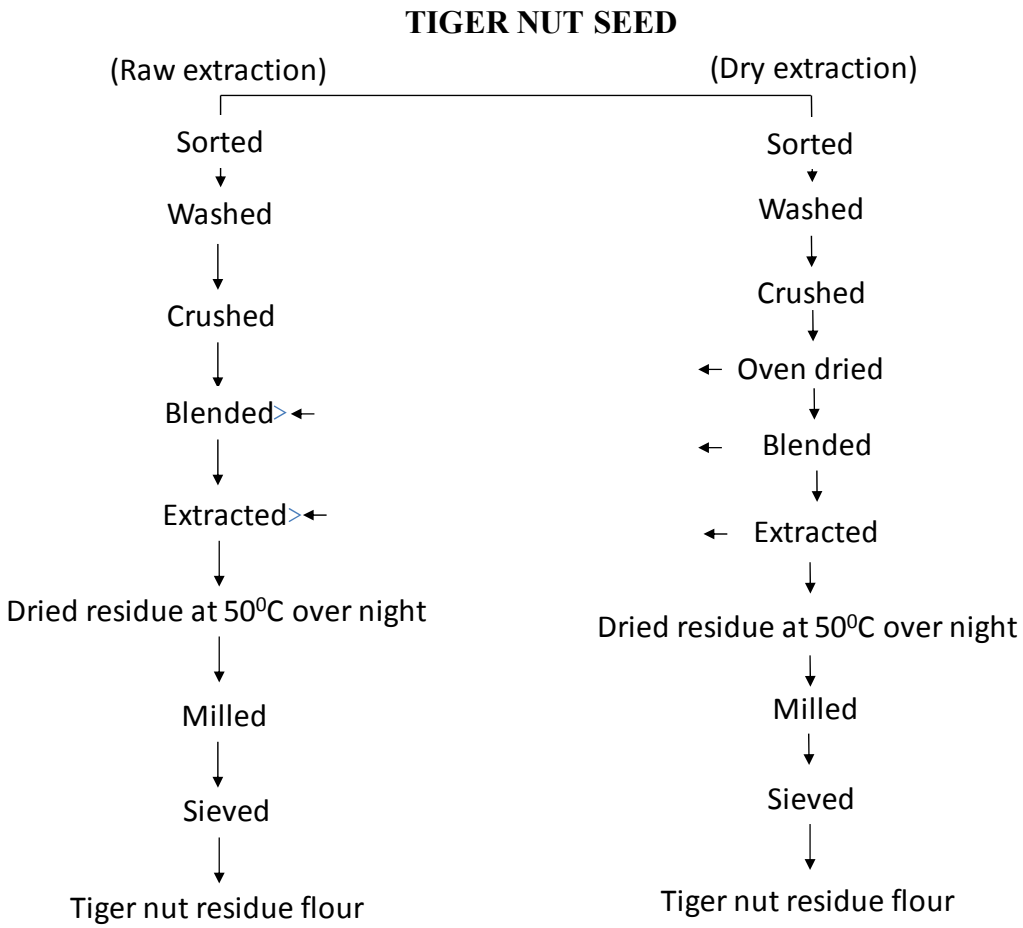


Figure 1. Flow chart illustrating the production of Raw and Dried tiger nut residue flour

Source: Oladele and Aina (2007).

2.2 Preparation of Tiger Nut Residue Flour

Tiger nut residue flour was prepared using two methods according to Figure 1. Seeds were sorted to remove defective seeds and carefully washed. Two kilograme freshly washed tiger nut seeds were divided into two (2) parts, the first part was crushed, blended and milk extracted leaving the residue, while the second part was crushed and oven dried at 50 ^{o}C for 24 hours before blending and extracting the milk before final drying. Dried tiger nut residues were milled into flour in a dry milling machine, cyclotec 1093 model, manufactured in Sweden by FOSS and sieved through a 50mm laboratory sieve to obtain flour of uniform particular size. The flour was then stored in a plastic air-tight container with lid at room temperature (37 ^{o}C) for further analysis and preparation of cakes and biscuits.

2.3 Wheat/Tiger Nut Composite Flour

Seven blends of both raw and dry extracted tiger nut residue flour were prepared by mixing varying proportions of tiger nut residue flour with wheat flour. Flour ratios of 5, 10, and 15% for biscuit and up to 20% for cake, were used for baking.

2.4 Recipe for Cake and Biscuit Production

Flour 200g, margarine 125g, sugar 50g, salt 0.02g, baking powder 2g, egg for cake 3, milk 75ml, 15ml water.

2.5 Preparation of Products

Cake was prepared using the creaming method as described by Victor et al. (1995) while biscuit was prepared by the rubbing-in- method as described by Oyewole et al. (1996).

2.6 Sensory Evaluation

Cake and biscuit samples were subjected to sensory evaluation within 24 hours of production. The following attributes namely, appearance, aroma, taste, texture and overall acceptability were assessed on cakes and biscuits samples, respectively using a 5 point hedonic scale with 5 as like extremely and 1 as dislike extremely. Twenty (20) trained panelists familiar with cake and biscuit, who were not hungry, neither sick nor allergic to baked products, were involved in the assessment. The panelists were instructed to rinse their month with water after tasting each cake or biscuit sample. Evaluation was done between 9-11am.

2.7 Statistical Analysis

Results were analyzed statistically using analysis of variance (ANOVA) and means were separated by least significant different (LSD) procedure, using the statistical package for social sciences (SPSS) version 20.0 soft ware 2007

2.8 Chemical Analysis of Cake and Biscuit

Chemical analysis of the samples such as moisture, ash, fat and protein were determined using the AOAC method (1990).While total available carbohydrate (TAC) was determined by the method described by Osborne and Voogt (1978) and fibre calculated by difference of moisture, ash, fat, protein and carbohydrate.

2.9 Determination of Spread Ratio

The spread ratio of biscuit sample was calculated using the formula below, as described by Oyewole et al. (1996):

$$Spread\ ratio = \frac{Diameter}{Height}$$

2.10 Determination of Cake Volume

Cake volume of samples were calculated using the formula below as described by Oyewole et al. (1996):

$$V=1/3\ nr2_h$$

Where r = radius, h = height.

3. Results and Discussion

3.1 Proximate Composition of Cakes from Wheat/Tiger Nut (Raw/Dry) Residue Composite Flour

Table 1 shows the proximate composition of cakes from wheat/tiger nut (raw and dry) residue flour. Moisture content ranged from 21.95%-28.49% for the raw and 21.95%- 24.82% for the dry residue flour cakes respectively, with sample A (100% WF-Wheat flour) having the lowest and sample E (80%WF: 20% TNRF-Tiger nut residue flour) the highest in both cases. This result of the present study is higher than the findings of

Eke-Ejiofor (2013) who reported a moisture content of 21.97%-22.26% for cake produced from African breadfruit/sweet potato-wheat composite flour blend with sample E showing the highest moisture content, which may be attributed to the increase in the addition of raw tiger nut residue flour. The study showed that the processing method used for the production of the flour affected the moisture content of the samples with the dry extracted tiger nut residue flour cake having the lowest moisture, indicating better keeping quality of the sample.

Ash content ranged from 0.72%-1.45% for the raw extracted flour and 0.82%-1.06% for the dry tiger nut residue flour, with sample B (95%WF and 5% TNRF) having the lowest in both cases and samples D (85%WF:15%TNRF) and E (80%WF:20%TNRF) as highest for raw and dry tiger nut residue flour respectively. Ash content for raw residue cake ranging from 0.72% to 1.45% agrees with the findings of Salve et al. (2011) with a value of 1.40%. While for dry residue flour cake, ash content ranging from 0.82% to 1.06% is lower than the findings of Ajani et al. (2012) with a value of 1.43%. Eke-Ejiofor (2013), reported ash values of 2.12-2.25% for cake produced from African breadfruit/sweet potato-wheat composition flour blend. The result of this study showed that ash content increased with an increase in substitution level.

Protein content of cake ranged from 8.53%-10.38% in the raw residue flour, with sample C (90%WF: 10%TNRF) as the lowest and samples A and E as the highest, while cakes from dry extracted residue flour had protein ranging from 8.69%-10.44% with sample D (85%WF: 15%TNRF) having the lowest and sample B having the highest (95%WF and 5% TNRF). Protein content of the raw and dry residues in the present study are higher than the findings of Oderinde and Tahir (1988) with a value of 7%, Akobundu et al. (1998) with a value of 9.51% and Eke-Ejiofor (2013) with a value of 5.78- 7.34% for African breadfruit/sweet potato-wheat composite flour blends. Protein content decreased with increase in substitution of tiger nut residue flour. This may be as a result of the increase observed in carbohydrate and fiber as substitution level increased.

Fat content of cakes ranged from 18.81%-21.68% in the raw residue with sample C (90%WF: 10%TNRF) having the lowest and 17.03%-24.62% in the dry residue with sample D (85%WF: 15%TNRF) having the lowest and sample E (80%WF: 20%TNRF) having the highest in both cases. Fat content for raw extracted tiger nut residue cake ranging from 18.82% to 21.68%, falls below the findings of Eke-Ejiofor (2013) with a value of 18.93 –21.79% for African breadfruit/sweet potato-wheat composite cake. The result of the study showed that fat content increased with an increase in substitution level. The higher fat content of the dry extracted residue cake may be as a result of the processing method which made fat more available.

Carbohydrate content of cakes ranged from 34.90%-45.85% in the raw and 37.14%-48.84% for the dry tiger nut residue flour, with sample E (80%WF: 20%TNRF) having the lowest and sample A (100%WF) as the highest respectively. This result agrees with the findings of Eke-Ejiofor (2013) with a carbohydrate content of 47.76-48.79% for African breadfruit/sweet potato-wheat composite cake. Carbohydrate content for raw residue in this study (39.66% to 45.85%) is higher than the findings of Algeria-Toran and Farre-Rovira (2003) with a value of 43.30%, but less than the value for dry tiger nut residue cake (37.14% to 41.65%).

Fibre content ranged from 1.00%-3.15% in the raw and 1.08-2.80% in the dry tiger nut residue cakes with sample B (95%WF and 5% TNRF) and A (100%WF) as lowest and sample E (80%WF: 20%TNRF) as highest, respectively. Eke-Ejiofor (2013) reported a value (1.00 -1.82%) for African bread fruit/sweet potato-wheat composite cake. Fibre content for raw residue ranging from 1.08% to 3.15% and dry residue ranging from 1.08% to 2.80% is higher than the findings of Eke-Ejiofor (2013). The result of this study showed that fibre content increased with an increase in the level of substitution. Raw extracted tiger nut residue cake had more fibre when compared with the dry extracted sample. Ash, total available carbohydrate, fat and fiber all increased with an increase in the substitution of tiger nut residue flour with samples showing significant ($p<0.05$)

3.2 Proximate Composition of Biscuit from Wheat/Tiger Nut (Raw/Dry) Residue Composite Flour

Table 2 shows the proximate composition of biscuits from wheat/tiger nut (raw and dry) residue flour. Moisture content of biscuits ranged 1.08%-2.78% for the raw and 1.08%- 1.85% for the dry sample Moisture content of the biscuits is lower than the findings of Eke-Ejiofor (2013) with a value of 3.00%-6.79% and Olaoye et al. (2007) with a value of 5.19%. The result of this study showed that biscuit from the dry residue flour contained lower moisture content which indicates better storage life and keeping quality of the biscuit. While ash content of the biscuits ranged from 1.34%-1.84% for the raw and 1.35% - 1.60% for the dry residue flour with sample A (100%WF) as lowest and sample B (95%WF: 5%TNRF) as highest respectively. Eke-Ejiofor (2013) reported ash content of 1.05 - 1.17% for African bread fruit/sweet potato-wheat composite biscuit samples, which is lower than the result of the present study. Ash content of raw tiger nut residue biscuits (1.35%to 1.84%) agrees with the findings of Ayo and Nkana (2003) with a value of 1.82% and that of dry residue flour ranging from 1.35% to

1.60% but lower than the findings of Olaoye et al. (2007) with a value of 1.90%. The result of the present study showed that ash content increased with an increase in the level of substitution.

Fat content ranged from 24.89% - 29.26% in the raw sample and from 24.89 - 38.99% in the dry residue with sample A (100%WF) as lowest and sample D (80%WF: 20%TNRF) as highest. This result is higher than that reported by Eke-Ejiofor (2013) of 21.93 - 27.37%. The result showed that fat content increased with an increase in the level of substitution.

Protein content ranged from 7.31% - 9.46% in the raw residue biscuit and 8.69% - 9.56% in the dry residue biscuit with sample B (95%WF: 5%TNRF) as lowest and sample A (100%WF) as highest. This result is lower than the findings of Olaoye et al. (2007) with a value of 15.83%. Biscuit produced from blends of African bread fruit/sweet potato-wheat composite flour reported a protein content of 5.59 - 7.08% which is lower than the present result (Eke-Ejiofor, 2013). Protein content increased with an increase in the substitution of tiger nut residue flour.

Fibre content of biscuits ranged from 1.06% - 3.13% in the raw residue sample and from 1.59% - 3.20% in the dry residue, with sample A (100%WF) as lowest and sample D (85%WF: 15% TNRF) as highest. Olaoye et al. (2007) reported a value of 0.62%, while Oluwanukomi et al. (2011) reported a value of 0.46%, both of which are lower than the findings of this work. The result of this present study showed that fibre improved when compared with previous works. Fibre content increased with an increase in the substitution of tiger nut residue flour.

Carbohydrate content of biscuit samples ranged from 56.15% - 69.61% in the raw residue and from 45.44 - 61.63% in the dry residue product, with sample A (100%WF) as highest and sample D (85%WF: 15%TNRF) as lowest. The result of the biscuit from the raw tiger nut residue ranging from 56.15% to 61.63% is higher than the findings of Bamishaiye and Bamishaiye (2011) with a value 54.7%. While carbohydrate content for dry residue ranging from 45.44% - 61.63% is lower than the findings of Eke-Ejiofor (2013) with a value 57.68% - 65.24%. The result of this present study indicates that carbohydrate content decreased with an increase in the level of inclusion of tiger nut residue flour.

3.3 Physical Characteristics of Cakes from Wheat/Tiger Nut (Raw and Dry) Residue Composite Flour

Table 3 shows the volumes and spread ratio of cakes and biscuits produced from raw and dry tiger nut residue flour inclusion up to 20% and 15% for cakes and biscuits respectively. Cakes produced from raw and dry residue flour ranged from 598.58 – 781.46cm^3, and from 598.58 -741.84cm^3 in volume with sample A (100%WF) as the lowest and E (80%WF: 20%TNRF) as highest in both cases. Cake volume increased with increase in substitution of tiger nut residue flour, and showed a significant difference ($P \leq 0.05$) in all its parameters respectively.

While spread ratio of biscuit produced from raw tiger nut residues ranged from 6.72- 9.88, with sample D (85%WF: 15%TNRF) as lowest and B (95%WF: 5%TNRF) as the highest. Spread ratio of biscuit produced from dry tiger nut residue ranged from 6.73-7.32 with sample A (100%WF) as the lowest and D (85%WF: 15%TNRF) as the highest. Biscuits showed an increase in spread ratio with an increase in the level off substitution and there was significant difference ($P \leq 0.05$) in the entire sample respectively.

3.4 Sensory Evaluation Result for Cakes Produced from Wheat/Tiger Nut (Raw/Dry) Residue Flour Composite

Table 4 shows the sensory evaluation result of cakes from wheat/tiger nut (raw/dry) residue flour. Color/appearance for cakes (raw residue) ranged from 3.50-4.55 and 3.7-4.2 for (dry residue) with sample A (100%WF) as most preferred and sample E (80%WF: 20%TNRF) least preferred in both cases. The decrease in color may be attributed to the creamy color of tiger nut residue flour. Aroma for cakes (raw residue) ranged from 3.45- 4.0 with sample E (80%WF: 20%TNRF) as the least preferred and showed no significant difference ($p>0.05$) with increase in substitution. While aroma for dry residue cakes ranged from 3.6 - 3.95 with all samples very much acceptable. Taste for cake (raw residue) ranged from 3.55 - 4.30 with sample C (90%WF: 10%TNRF) as most preferred while taste for cakes (dry residue) ranged from 3.9-4.3 with sample D (85%WF: 15%TNRF) and E (80%WF: 20%TNRF) as most preferred. General acceptability scores for cake (raw residue) ranged from 3.5-4.15 with sample is showing no significant difference ($p>0.05$). While general acceptability scores for dry tiger nut residue cake ranged from 3.9 - 4.25 with sample D (85%WF: 15%TNRF) and E (80%WF: 20%TNRF) as most preferred. The result for cakes showed significantly difference ($P<0.05$) for attribute like color, taste, texture and general acceptability. While aroma showed no significant difference ($P>0.05$) for cake samples. Substitution up to 20% was generally acceptable for cake.

3.5 Sensory Evaluation Result for Biscuit Produced from Wheat/Tiger Nut (Raw/Dry) Residue

Table 5 shows the sensory evaluation result of biscuits from wheat/ tiger nut (raw/dry) residue flour. Color/appearance of biscuits (raw residue) ranged from 3.6-4.15 while (dry residue) ranged from 3.4-4.03. Aroma for biscuit (raw residue) ranged from 3.5-4.0, while biscuit (dry residue) aroma ranged from 3.3-3.65. Taste for biscuit (raw residue) ranged from 3.70-4.15 and biscuit (dry residue) taste ranged from 3.00-4.0. Texture/crispness of biscuit (raw residue) ranged from 3.5-4.3, while texture/crispness for biscuit (dry residue) ranged from 3.20-3.55. General acceptability of biscuit (raw residue) ranged from 3.6-4.35, while that of biscuit from (dry residue) ranged from 3.0-3.95. In all the cases, sample B (95% WF: 5% TNRF) was most preferred for the raw extracted residue samples. The result for biscuit showed significant difference ($P<0.05$) in all the samples, for attribute like color, aroma, taste, texture and general acceptability. Substitutions up to 15% were generally acceptable for biscuit.

Table 1. Proximate composition (%) of wheat/tiger nut (Raw and Dry residue) cake

Sample		Moisture Content	Ash	Protein	Fat	TAC	Fibre
A	a	21.95^{e}	0.93^{b}	10.38^{b}	18.82^{d}	45.85^{c}	1.08^{e}
	b	21.95^{e}	0.93^{b}	10.38^{b}	18.82^{d}	45.84^{c}	1.08^{e}
B	a	21.72^{b}	0.72^{a}	8.66^{a}	19.64^{b}	42.23^{d}	1.00^{d}
	b	24.66^{c}	0.82^{e}	10.44^{c}	20.32^{b}	41.65^{b}	1.87^{e}
C	a	28.05^{c}	1.29^{b}	8.53^{d}	18.81^{c}	41.01^{b}	2.22^{b}
	b	22.87^{b}	0.93^{b}	8.81^{d}	19.87^{c}	45.11^{c}	2.41^{c}
D	a	26.67^{d}	1.45^{c}	10.16^{e}	19.61^{e}	39.66^{a}	2.45^{c}
	b	22.14^{d}	1.01^{a}	8.69^{b}	17.03^{b}	49.24^{d}	2.08^{b}
E	a	28.49^{a}	1.40^{a}	10.38^{c}	21.68^{a}	34.90^{e}	3.15^{a}
	b	24.82^{a}	1.06^{d}	9.56^{a}	24.62^{a}	37.14^{e}	2.80^{a}

Means with different superscript in the same column are significantly different ($P<0.05$).

a= Raw residue
b= Dry residue
A= 100 % wheat flour (control)
B= 95% wheat flour, 5% tiger nut residue flour
C= 90% wheat flour, 10% tiger nut residue flour
D= 85% wheat flour, 15% tiger nut residue flour
E= 80% wheat flour, 20% tiger nut residue flour
TAC = Total available carbohydrate
TNRF= Tiger nut residue flour

Table 2. Proximate composition (%) of wheat/tiger nut (Raw and Dry Residue) biscuit

Sample		Moisture content	Ash	Protein	Fat	TAC	Fibre
A	a	1.08^{d}	1.35^{b}	9.46^{b}	24.89^{d}	69.61^{b}	1.06^{d}
	b	1.08^{d}	1.35^{b}	9.46^{b}	24.89^{d}	61.63^{a}	1.59^{d}
B	a	2.78^{a}	1.84^{a}	7.31^{d}	26.76^{b}	61.63^{a}	2.23^{c}
	b	1.85^{c}	1.60^{a}	8.69^{d}	35.74^{b}	47.33^{b}	1.74^{c}
C	a	1.61^{c}	1.34^{b}	7.40^{c}	27.36^{c}	59.08^{c}	2.68^{b}
	b	1.52^{c}	1.37^{d}	8.94^{c}	38.78^{c}	50.09^{c}	2.34^{b}
D	a	2.10^{b}	1.57^{b}	7.79^{a}	29.26^{a}	56.15^{d}	3.13^{a}
	b	1.39^{d}	1.42^{b}	9.56^{b}	38.99^{a}	45.44^{d}	3.20^{a}

Means with different superscript in the same column are significantly different ($P<0.05$).

a= Raw residue
b= Dry residue
A= 100 % wheat flour (control)
B= 95% wheat flour, 5% tiger nut residue flour
C= 90% wheat flour, 10% tiger nut residue flour
D= 85% wheat flour, 15% tiger nut residue flour
TAC = Total available carbohydrate
TNRF= Tiger nut residue flour

Table 3. Physical properties of cakes and biscuits from wheat/tiger nut (raw/dry)residue composite flour

	(Raw residue)	(Dry residue)	(Raw residue)	(Dry residue)
	Cake		Biscuit	
Sample	**volume**	**volume**	**spread ratio**	**spread ratio**
A	598.58	598.58^{d}	6.73^{c}	6.73^{b}
B	741.04^{a}	652.99^{b}	6.72^{c}	7.04^{b}
C	764.77^{b}	683.10^{c}	7.08^{b}	7.20^{b}
D	764.14^{a}	729.65^{a}	9.88^{a}	7.32^{a}
E	781.43^{a}	741.84^{a}		

Means with different superscript in the same column are significantly different (P<0.05).

A= 100 % wheat flour (control)
B= 95% wheat flour, 5% tiger nut residue flour
C= 90% wheat flour, 10% tiger nut residue flour
D= 85% wheat flour, 15% tiger nut residue flour
E= 80% wheat flour, 20% tiger nut residue flour

Table 4. Mean sensory evaluation result of cakes from (raw and dry) Tiger-nut residue composite flour

Sample/code		Color/Appearance	Aroma	Taste	Texture	General Acceptability
A	a	4.55^{a}	4.00^{a}	3.90^{b}	3.75^{a}	4.15^{a}
	b	4.20^{a}	3.60^{a}	3.65^{c}	3.45^{d}	3.75^{b}
B	a	4.25^{b}	3.65^{a}	3.90^{b}	4.00^{a}	4.05^{a}
	b	3.70^{b}	3.55^{a}	3.45^{c}	3.65^{c}	3.65^{b}
C	a	3.85^{c}	3.70^{a}	4.30^{a}	3.85^{a}	4.10^{a}
	b	3.90^{b}	3.65^{a}	3.90^{b}	3.08^{c}	3.90^{b}
D	a	3.75^{c}	3.85^{a}	3.55^{c}	3.75^{a}	3.95^{a}
	b	3.85^{b}	3.85^{a}	4.30^{a}	4.00^{a}	4.25^{a}
E	a	3.50^{b}	3.45^{b}	3.60^{c}	3.45^{b}	3.50^{b}
	b	4.30^{a}	3.95^{a}	4.20^{a}	4.05^{a}	4.30^{a}

Means with different superscript in the same column are significantly different (P<0.05).

WF = Wheat flour
a= Raw residue
b= Dry residue
A= 100 % wheat flour (control)
B= 95% wheat flour, 5% tiger nut residue flour
C= 90% wheat flour, 10% tiger nut residue flour
D= 85% wheat flour, 15% tiger nut residue flour
E= 80% wheat flour, 20% tiger nut residue flour
TNRF= Tiger nut residue flour

Table 5. Mean sensory evaluation result of biscuits from (raw and dry) Tiger-nut residue composite flour

Sample		**Color**	**Aroma**	**Taste**	**Texture**	**General Acceptability**
A	a	3.60^{c}	3.25^{c}	3.35^{c}	3.70^{b}	3.60^{c}
	b	3.55^{b}	3.30^{b}	4.00^{a}	3.55^{a}	3.95^{a}
B	a	4.15^{a}	4.00^{a}	4.15^{a}	4.30^{a}	4.35^{a}
	b	4.05^{a}	3.30^{b}	3.05^{b}	3.25^{b}	3.25^{b}
C	a	3.85^{b}	3.50^{b}	3.75^{b}	3.50^{b}	3.80^{b}
	b	3.35$_{b}$	3.35^{c}	3.00^{c}	3.25^{b}	3.00^{b}
D	a	3.90^{b}	3.90^{a}	3.70^{b}	3.60^{b}	3.55^{c}
	b	3.60^{b}	3.65^{a}	3.15^{c}	3.20^{b}	3.25^{b}

Means with different superscript in the same column are significantly different (P<0.05).

a= Raw residue
b= Dry residue
A= 100 % wheat flour (control)
B= 95% wheat flour, 5% tiger nut residue flour
C= 90% wheat flour, 10% tiger nut residue flour
D= 85% wheat flour, 15% tiger nut residue flour
WF: Wheat flour
TNRF= Tiger nut residue flour

4. Conclusion

Result from this study has shown the possibility of producing cakes and biscuits of acceptable quality from lesser known and underutilized crop like tiger nut. Cakes and biscuits from tiger nut residue flour composite with wheat were more acceptable nutritionally as they contain significantly more fibre and ash. Wheat flour could be substituted with tiger nut residue flour up to 20% inclusion level for cakes and 15% in biscuit without altering the original nature, in terms of color, aroma, taste and texture. The results further showed that in terms of acceptance, the substitution level for biscuit production could depend on the method of processing (raw/dry) the flour. Wheat/tiger nut residue composite cakes and biscuits have proved to be beneficial in term of improved nutrition such as protein, fibre, and ash content of the products. It is therefore a viable substitute for wheat flour since a huge percentage of our foreign reserve is utilized in the importation of wheat. Additionally, tiger nut residue, which is an expected waste, has been turned to wealth and diversification in terms of usage. Products from dry residue flour was more acceptable to the raw residue since products containing raw residue had more moisture content, which has an implication in the shelf life and keeping quality of the end product.

References

Adejuyitan, J. A. (2011). Tiger nut processing: its food uses and health benefits. *AM food Technol, 6*(3), 197-201. http://dx.doi.org/10.3923/ajft.2011.197.201

Ade-Omowaye, B. I. O., Akinwande, B. A., Bolarinwa, I. F., & Adebiyi, A. O. (2008). Evaluation of tigernut (cyperus esculentus)- Wheat Composite flour and bread. *Afr J. Food Sci., 2*, 87-91.

AOAC. (1990). *Association of Official Analysis chemist* (15th Ed.). Washington, DC.

Ajani Alice, O., Oshundahunsi, O. F., Akinoso Rahman, A. K. A., & Abiodun Aderibigbe, D. (2012). Proximate composition and sensory Qualities of snacks procedure from Breadfruit flour. *Global journal of science fronitier Research biological science, 12*(7), 1-9.

Akobundu, E. N. T., Ubbaonu, C. N., & Ndupuh, C. E. (1998). Studies on the baking potential of non-wheat composite flours. *J. Food Sci.Tech., 25*, 211-214.

Alegria-Toran, A., & Farre-Rovira, R. (2003). Horchata y salud: Aspectos Nutricionales y dieteticos. In Fundacion. Valenciana da Estudios Avanzados, editor. Jornada chufa y Hotchata: Tradicion y salud. Valencia Spain: Conselleria de Agricultura, Pesca y Alimentacion p.55-70. View at google scholar.

Ayo, J. A., & Nkana, L. (2003). Effect of acha (D.exilis) flour on the physiochemical and sensory qualities of biscuits. *J. of Nutriton and food science, 33*(3), 125-130.

Bamishaiye, E. I., & Bamishaiye, O. M. (2011). Tiger nut: As a plan its derivatives and benefits. *Afric J. Food Agric. Nutr. Develop., 11*, 5157-5170. http://dx.doi.org/10.4314/ajfand.v11i5.70443

Belewu, M. A., & Belewu, K. Y. (2007). Comparative physico- chemical evaluation and preparation of tiger nut, soybean and coconut milk sources. *Int. J Agric. Biol, 9*, 785-787.

Eke-Ejiofor, J. (2013). Proximate and sensory properties of African Breadfruit and sweet potato-Wheat composite flour in Cakes and Biscuits. *International Journal of Nutrition and food sciences, 2*(5), 232-236. http://dx.doi.org/10.11648/j.ijnfs.20130205.13

Esteshola, E., & Oreadu, A. C. I. (1996). Fatty acids composition of Tiger nut tubers (cyperusesculentusL.), baobab seeds (Adansoniadigitata L.) and their mixtures. *J. American oil chemists society, 73*, 255-257. http://dx.doi.org/10.1007/BF02523905

Kure, O. A., Bahago, E. J., & Daniel, E. A. (1998). Studies on the proximate composition and effect of flour particle size on acceptability of Biscuit produced from blends of Soybeans and plantain flours. *Afri J. Food nutrition Devt., 7*(1), 1-22.

Magee, E. (2009). Sugar. What kinds to Eat and when. Retrieved July 11, 2009, from WebMD.com (Health and Cooking) 28 January 2009.

Oderinde, R. A., & Tahir, O. A. (1988). Chemical investigation of Nigerian Cyperus esculentus (L) tuber for possible industrial application. *Nig-J. Sci., 22*, 70-73.

Ohl, S. S., Roberta, L. D., & Hasler, D. (1981). *Guide to modern meals (Cakes and frostling)* (3rd ed., pp. 466-475). A Webster and McGraw Hill book company.

Oladele, A. K., & Aina, J. O. (2007). Chemical composition and functional properties of flour produced from two varieties of tiger nut (cyperus esculentus). *Afr. J. Biotechnol, 6*, 2473-2476.

Olaoye, O. A., Orilude, A. A., & Idowu, O. A. (2007). Quality characteristics of bread produced from composite flour of wheat, plantain and soybean. *Afr. J. Biotechnol., 5*(112), 1106.

Oluwanukomi, M. O., Adeyemi, I. A., & Oluwana, I. B. (2011). Effects of soybean enrichment on the physicochemical and sensory properties of biscuits. Application. *Tropical agriculture, 10*, 44-49.

Omode, A. A., Fatoki, O. S., & Olaogun, K. A. (2004). Physicochemical properties of someunderexplioted and nonconventional oil-seeds. *Agric food chem., 43*, 2850-3. http://dx.doi.org/10.1021/jf00059a015

Oyewole, O. B., Sanni, L. O., & Ogunjobi, M. A. (1996). Production of Biscuit using cassava flour. *Nig. Food, Journal, 3*, 24-28.

Osborne, D. R., & Voogt, P. (1978). The analysis of nutrient in food (p. 240). London, U.K.: Academic press.

Salve, R. V., Mehrajfatema, Z. M., Kadam, M. L., & More, S. G. (2011). Formulation, Nutritional Evaluation and storage study of supplementary food (Panjiri). *J food process Technol., 2*, 131.

Victor, C., Ronald, K., & David, F. (1995). *Practical cookery, (cakes, small Cakes, basic mixtures)* (8th Ed., pp. 706-707). Euston Road, London: Holder and Stoughlon educational.

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Proximate Composition, Physical and Sensory Properties of Cake Prepared from Wheat and Cocoyam Flour Blends

Yetunde E. Alozie[1] & Chiemela E. Chinma[2]

[1] Department of Human Ecology, Nutrition and Dietetics, University of Uyo, Nigeria

[2] Department of Food Science and Technology, Federal University of Technology, Minna, Nigeria

Correspondence: Yetunde E. Alozie, Department of Human Ecology, Nutrition and Dietetics, University of Uyo, Nigeria. Tel: 234-803-938-5198. Email: tundealozie@yahoo.com

Received: July 5, 2015 Accepted: September 5, 2015 Online Published: September 21, 2015

doi:10.5539/jfr.v4n5p181 URL: http://dx.doi.org/10.5539/jfr.v4n5p181

Abstract

The effect of replacing wheat flour with cocoyam (*Xanthosoma sagittifolium)* flour on the proximate composition, physical, sensory properties of cake were investigated. Baking trials were conducted at different levels of substitution (0, 20, 40, 50, 60, 80 and 100%). Cocoyam flour had higher values ($p < 0.05$) in all functional characteristics evaluated except for emulsifying capacity. Cake weights and volume increased with increasing cocoyam flour substitution while volume index decreased. Protein and fat contents of cake samples decreased ($p< 0.05$) with increasing levels of cocoyam flour substitution while ash and fibre contents increased. There was no significant ($p > 0.05$) difference in crust colour of samples. In terms of textural preference, cake prepared from 100% cocoyam flour was significantly higher than 100% wheat cake. Utilization of wheat-cocoyam flour blends in composite cake preparation will be beneficial to the nutrition of vulnerable groups considering the higher ash and fiber contents of of composite cake, and reduced carbohydrate and fat contents than control.

Keywords: Cocoyam flour, cake, composite, wheat flour, physical and sensory properties

1. Introduction

Consumption of various bakery and confectionary products is the demand of time due to change in food habit of the people. Cake is one of the relished and palatable baked products prepared from flour, sugar, shortening, baking powder, egg, essence as principal ingredients. Preparation of plain cakes from wheat flour is the conventional practice however, in tropical countries, wheat production is limited and importation of wheat flour to meet local demand is a necessity (Giami et al., 2004). Nowadays, the use of composite flour in which flour from locally grown crops replaces a portion of wheat flour is common in many developing countries to meet the high demand for functional foods with health benefits as well as decreasing the demand for imported wheat and stimulating production and use of locally grown non-wheat agricultural products (UNECA 1985).

Roots and tubers contribute about 20-48 % of the total calories and 7.1% protein to the diets of the people of sub-Saharan Africa (Nnabuk et al., 2012). Cocoyam is the third most important tuber crop in Nigeria after cassava and yam. Its cultivation spreads from the coastal areas to the savannas. They account for over 50% of the caloric intake of the people of the south. Cocoyam is fair in protein (7-9%), yam has less than 6% protein, cassava is a poor source of protein (<3%) while sweet potatoes are poor in protein but fair in their supply of the B-vitamins. Thus cocoyam has some nutritional advantages over other root and tubers crops (Nnabuk et al., 2012). Cocoyam is herbaceous perennial plant belonging to the araceae family and constitutes one of the six most important root and tuber crops world-wide (Ekanem & Osuji 2006). They are among the major crops grown in wetlands with minimal inputs and offer high potential for alleviating food insecurity and income constraints. The most popular and widely consumed species of cocoyam are the *Colocasia, xanthosoma, alocasia, caryotosperma, chamissonis* and the a*mophophallus* (Eze & Okorji 2003). Nutritionally, cocoyam supplies easily digestible starch (Sefa-Dedeh & Sackey, 2002) and are known to contain substantial amounts of protein, vitamin C, thiamine, riboflavin, niacin and significant amounts of dietary fibre (Niba, 2003). The leaves are cooked and eaten as vegetable. They contain β-carotene, iron and folic acid which protects against anemia (FAO, 1990; Sukamoto, 2003). Cocoyam is a rich source of carbohydrate, dietary fiber, but low in fat, protein and ash content (Owusu-Darko et al., 2014). Moisture content of cocoyam ranges from 67.1 to 69.17%

depending on the specie (Bradbury & Holloway, 1998). It has been widely reported that cocoyam possesses the smallest starch grain-size relative to other roots and tubers. This makes cocoyam suitable for several food products for potentially allergic infants, persons with gastro-intestinal disorders as well as diabetic patients (Onyenobi et al., 2010). The smallest starch molecules of cocoyam have been associated with increased digestibility over other crops, making it suitable for feeding invalids, production of confectioneries and baby foods (Eneh, 2013). The carbohydrate fraction of cocoyam consists of 2.6 per cent pentosans which makes it a possible alternative for industrial pentosans used in confectionery (Ubalua & Chukwu, 2008).

Despite the importance and nutritive value of cocoyam in Nigeria and many other nations, its industrial potential as well as its contribution to food security has been grossly under-estimated (Ekwe *et al*., 2009) as it had been regarded as "poor man's food" or "women crop".

Flours milled from other crops such as maize, millet, sorghum, cassava, plantains, potatoes and rice had been added to wheat flour to extend the use of the local crops and reduce the cost of wheat importation. Several flour samples such as African yam bean, tigernut, moringa and chickpea have been incorporated into wheat flour for cake preparation to provide better overall essential amino acid balance, combat protein energy malnutrition, enhance sensory property and reduce total dependence on imported wheat flour (Alozie et al., 2009; Eke et al., 2009; Chima et al., 2013). Composite flour incorporating cocoyam has been used in extruded products such as noodles and macaroni (FAO, 1990). Opportunities and support for the use of cocoyam flour for production of baked goods if feasible would help to lower the dependency of developing nations on imported wheat. This study was aimed at investigating the proximate composition, physical and sensory properties of cakes prepared from wheat and cocoyam flour blends.

2. Materials and Methods

2.1 Source of Raw Materials

The study was conducted in the laboratories of the Department of Human Ecology, Nutrition and Dietetics, University of Uyo, Akwa Ibom State, Nigeria. Cocoyam cornels (variety), Wheat Flour (Golden Penny, Nigeria Flour Mills Plc) and cake ingredients (margarine, eggs, granulated sugar, salt and baking powder) were purchased from a local market (Akpanadem) in Uyo metropolis of Akwa Ibom State, Nigeria.

2.2 Production of Cocoyam Flour (CF)

Fresh corms were thoroughly washed with tap water, peeled using a stainless steel knife, rewashed and cut into 0.5 cm thick slices. The slices were blanched at 75°C for 15 minutes in potable water. The slices were dried to a constant weight in an oven (Gallenkamp, England) set at 60°C for nine hours before milling into flour using a grinder fitted with a 500 μm mesh sieve. Flour obtained was packed in Ziploc bags and stored at 4° C ready for preparation of composite flours (Njintang et al., 2008**)**.

2.3 Formulation of Blends

Wheat flour was substituted with cocoyam flour at substitution levels of 20, 40, 50, 60, 80 and100% labeled A, B, C, D, E and F. Sample with 100% wheat flour labeled G served as control. A Kenwood mixer was used for mixing flour samples at speed 6 for five minutes to achieve uniform mixing.

2.4 Proportion of Ingredients

The proportion of ingredients used consists of flour (400 g), sugar (150g), margarine (250g), eggs (300 g), baking powder (10g), salt (5g), browning (10ml) and vanilla essence (10ml) as described by Ceserani and Kinton (2008) with slight modification (reduction) in the quantity of sugar.

2.5 Preparation of Cakes

The method of Ceserani and Kinton (2008) was adopted for the preparation of cake. The margarine and sugar were creamed manually for 10min in a stainless steel bowl until light and fluffy. The egg was beaten for three min and vanilla essence added. It was added to the creamed mixture gradually while beating continued. Flour samples from various composite blends were separately sieved, with salt and baking powder and gradually folded into the mixture with a metal spoon. Browning was added and mixed thoroughly until a soft consistency batter was formed. The batter was transferred to a six inch greased baking pan and baked in a preheated oven at 200˚C for 30 min and a further 20min at a reduced temperature of 170˚C. A skewer was inserted into the center of the cake to ascertain it is cooked. When cooked, the cakes were allowed to cool in the tin for three minutes before turning out on wire racks for further cooling and analysis.

2.6 Functional Properties Determination

Functional properties of flour such as bulk density was determined using the method of Okezie and Bello (1998), water absorption capacity, swelling capacity and gelatinization temperature were evaluated using the method of Okaka et al. (1997).

2.7 Proximate Analysis

Proximate parameters: moisture, protein, crude fibre, fat, ash, carbohydrate and energy values of cake samples were determined according to AOAC (2005). The carbohydrate content was determined by difference while Atwater factors were used to calculate the energy value of the samples. All analysis was carried out in triplicates.

2.8 Determination of Physical Properties of Cakes

Volume of cake was determined by seed displacement method, as described by AACC (2000). Volume index of cake samples was measured according to AACC (2000). The cakes were cut vertically through the center and the heights of the cake sample were measured at three different points (B, C and D*)* along the cross-sectioned cakes using the template (AACC, 2000). According to the method, volume index was determined by the following formula: Volume index = B + C + D, where B and D are the heights of the cake at the points 2.5 cm away from the center toward the left and right sides of the cake respectively while C is the height of the cake at the center point. Weight of cake samples was determined using an electronic digital balance.

2.9 Sensory Evaluation

The sensory properties of cakes were evaluated using twenty semi-trained panelists consisting of staff and students of the Department of Human Ecology, Nutrition and Dietetics, University of Uyo, Nigeria. Cakes were evaluated for crust color, flavor, texture, appearance and overall acceptability using nine point Hedonic scale (where 1 = liked extremely and 9 = disliked extremely) (Iwe, 2002). A slice of cake from each blend was presented to panelists. Each panelist was provided with a glass of tap water to rinse the mouth between evaluations.

2.10 Statistical Analysis

Data obtained was subjected to analysis of variance (ANOVA) (Steel & Torrie, 1980). Significance difference was accepted at 5 % probability level

3. Results and Discussion

3.1 Functional Properties of Flours

The functional properties of flours play important role in the manufacturing of products as it determines the application and use of food materials for various food products (Adeleke & Odedeji, 2010). The result of functional properties of wheat and cocoyam flour is presented in Table 1. Result showed significant differences in functional parameters of the flours except loose bulk density. Cocoyam yam flour had higher values for water and oil absorption capacities, packed bulk density, swelling capacity and gelatinization temperature with low emulsifying capacity compared to wheat flour.

Table 1. Functional properties of wheat and cocoyam flour

	Water absorption capacity (g/100g)	**Swelling capacity (%)**	**Emulsifying capacity (%)**	**Gelatinization Temperature**	**Oil absorption capacity (g/100g)**	**Loosed bulk density (g/ml)**	**Packed bulk density (g/ml)**
Wheat flour	174.00±0.00^{b}	1.60±0.15^{b}	17.13±0.19^{a}	59.13±0.29^{b}	167.0±0.0^{b}	0.40±0.00^{a}	0.70±0.00^{b}
Cocoyam flour	182.0±0.03^{a}	1.85±0.05^{a}	15.23±0.16^{b}	69.24±0.29^{a}	176.67±0.28^{a}	0.40±0.02^{a}	0.76±0.00^{a}

Mean values with different superscript in a column are significantly ($p \leq 0.05$) different from each other.

Water absorption capacity of flour is an important property in foods. The ability of protein in flours to physically bind water is a determinant of its water absorption capacity (Ikpeme et al., 2010). Flours with high water absorption capacity have been reported to be good ingredients in bakery applications as they improve handling characteristics and lead to improved freshness of baked products (Ma et al., 2011). The values of the water absorption capacity was high in cocoyam flour which may be attributed to its protein subunits structure which

dissociates on heating in agreement with the findings of Apotiola and Fashakin (2013) that soybean flour protein subunits have more water binding sites.

Oil absorption capacity is an important parameter of flour used in baking (Ikpeme et al., 2010) since it reflects the emulsifying capacity (Kaur et al., 2007) from the industrial viewpoint. Higher oil absorption capacities suggest better mouth feel and flavor retention. The gelling ability of a sample is due to the nature of the starch and protein and their interaction during processing. Variations in the gelling properties of flours have been attributed to the relative ratio of protein, carbohydrates and lipids that make up the flours and interaction between such components (Sathe et al., 1982).

Swelling capacity is often related to protein and starch contents (Woolfe, 1992). Higher protein content in flour may cause the starch granules to be embedded within a stiff protein matrix, which subsequently limits the access of the starch to water and restricts the swelling power (Aprianita et al., 2009). The amylopectin is primarily responsible for granule swelling. Thus, higher swelling capacity of cocoyam flour suggests higher amylopectin content than wheat flour (Tester & Morrison, 1990). Moorthy and Ramanujam (1986) reported that the swelling power of granules is an indication of the extent of associative forces within granule.

Bulk density is an indication of the porosity of a product and a function of flour wettability which influences packaging design and could be used in determining the required type of packaging material (Iwe & Onalope 2001; Akubor, 2007). It is also a reflection of the load the sample can carry if allowed to rest directly on another (Onimawo & Asugo, 2004). The packed bulk density of cocoyam flour in the present study (0.76 g/ml) is similar to that observed for taro flour (0.57–0.71 g/ml) by Njintang et al. (2007) but higher (0.43–0.49 g/ml) than that reported (0.43–0.49 g/ml) by Tagodoe and Nip (1994) for taro flour.

3.2 Physical Properties of Cakes

The weight and volume of cake samples (Table 2) ranged from 30.0 to 34.6g, and 225.5 to 259.5cm^3 respectively. Cake weights and volume increased with increasing cocoyam flour levels. This may be attributed to the high packed bulk density of the cocoyam flour. Weights and volumes of baked products have been reported to be dependent on bulk density of the flour blends used (Chinma et al., 2007). Also, increased cake volume could be due to high gas retention thus high expansion of the product which is attributable to high peak viscosity of flour (Chinma et al., 2010). On the other hand, cake volume index of 100% wheat cake was significantly higher than composite cakes.

Table 2. Physical characteristics of cakes prepared from wheat-cocoyam flour blends

Wheat: cocoyam	Cake weight (g)	Cake volume (cm^3)	Cake volume index
100:0	30.0± 0.02^{d}	225.50± 0.01^{e}	102.5± 0.02^{a}
0:100	34.6± 0.15^{a}	259.50± 0.10^{a}	82.32± 0.01^{e}
80:20	30.8± 0.22^{c}	231.25± 0.18^{d}	94.35± 0.05^{b}
60:40	32.8± 0.12^{b}	246.50± 0.02^{c}	87.45± 0.04^{c}
50:50	33.0± 0.06^{b}	247.85± 0.15^{c}	87.42± 0.03^{c}
40:60	33.6± 0.23^{b}	253.84± 0.32^{b}	84.65± 0.01^{d}
20:80	34.2± 0.03^{ab}	256.78± 0.16^{ab}	83.95± 0.05^{d}

Mean values with different superscript in a column are significantly ($p \leq 0.05$) different from each other.

3.3 Proximate Composition of Cakes

There were no significant differences ($p>0.05$) in moisture, protein, fat, ash, fibre, carbohydrate and energy contents between 80: 20 (wheat: cocoyam) cake and control (Table 3). It was observed that protein and fat contents decreased ($p<0.05$) with increasing levels of substitution while ash and crude fibre contents increased. Ash is the total mineral content of a food item. The increase in ash content of composite cakes could be attributed to the higher level of ash content in cocoyam flour than wheat flour. The higher crude fibre content of composite cakes may be attributed to higher crude fiber content of cocoyam flour than wheat flour (Ikpeme et al., 2010).

Table 3. Proximate composition of cakes prepared from wheat-cocoyam flour blends (g/100g)

Parameter	100WF	100CF	80WF:20CF	60WF:40CF	50WF:50CF	40WF:60CF	20WF:80CF
Moisture	18.31[a]	20.25[a]	19.74[a]	21.45[a]	18.23[a]	21.66[a]	20.06[a]
Protein	8.27[a]	6.75[b]	8.04[a]	7.86[c]	7.62[c]	7.23[d]	6.61[b]
Fat	19.77[a]	18.15[b]	19.18[a]	18.84[c]	18.68[c]	18.43[c]	18.33[bc]
Ash	1.54[b]	1.86[a]	1.59[b]	1.60[b]	1.65[b]	1.76[a]	1.82[a]
Fibre	1.24[a]	1.48[a]	1.27[a]	1.33[a]	1.36[a]	1.40[a]	1.45[a]
Carbohydrate	50.87[a]	48.51[a]	49.48[a]	47.92[a]	51.16[a]	47.45[a]	48.73[a]
Energy(Kcal)	414.49[a]	396.39[a]	405.50[a]	396.68[a]	408.44[a]	392.87[a]	398.33[a]

Mean values with different superscript in a row are significantly ($p \leq 0.05$) different from each other.

WF= Wheat flour; CF= Cocoyam flour.

3.4 Sensory Properties of Cakes

The result of analysis of selected sensory properties of cakes is shown in Table 4. Values obtained for sensory characteristics of all test products were acceptable by consumers. There was no significant difference in crust color of cake samples. This may be attributed to the color of cocoyam flour which has a brown appearance with increasing amount added to the mixtures. The appearance score of the cakes ranged from 2.15 to 3.95, with cake samples from 100% cocoyam flour having the highest score while samples from 80:20 wheat-cocoyam flour blends had the lowest and most desirable score. There was significant difference in appearance between control sample and the composite cakes except when 80% cocoyam flour was substituted. In terms of textural preference, only 100CF was significantly higher than 100WF. Acceptable cakes can be made with up to 50% cocoyam flour substitution.

Table 4. Sensory properties of cakes prepared from wheat and cocoyam flour blends

Parameter	100WF	100CF	80WF:20CF	60WF:40CF	50WF:50CF	40WF:60CF	20WF:80CF
Crust Color	1.05[a]	1.25[a]	1.00[a]	1.10[a]	1.15[a]	1.20[a]	1.20[a]
Taste	3.35[a]	2.75[b]	2.55[b]	2.60[b]	2.80[b]	2.80[b]	2.85[b]
Appearance	2.85[b]	3.95[a]	2.15[c]	2.20[c]	2.30[c]	2.35[c]	2.95[b]
Texture	2.90[b]	3.45[a]	2.85[b]	2.60[c]	2.55[c]	2.45[c]	2.30[c]
Acceptability	2.70[b]	3.35[a]	2.35[c]	2.40[c]	2.50[c]	2.80[b]	2.80[b]

Mean value different superscript in a row are significantly ($p \leq 0.05$) different from each other.

WF= Wheat Flour and CF= Cocoyam Flour.

4. Conclusion

The result of the study revealed that substitution of wheat flour with cocoyam flour increased the ash and fibre contents of cake samples. Acceptable cake was produced by substituting wheat flour with cocoyam flour at 20 and 50 per cent vice versa, and could be used for commercial production. This study served as a preliminary investigation on and confirmed the possibility of producing an acceptable cake from wheat and cocoyam flour blends. Acceptable cakes can be prepared with up to 50% cocoyam flour substitution. Studies are on-going in our laboratory on the nutritional qualities of the composite cakes.

References

AACC. (2000). Approved methods of the American Association of Cereal Chemists (17th ed.). American Association of Cereal Chemists International, St. Paul, MN.

Adeleke, R. O., & Odedeji, J. O. (2010). Functional properties of wheat and sweet potato flour blends. *Pak. J. Nutr., 9*, 535-538. http://dx.doi.org/10.3923/pjn.2010.535.538

Akubor, P. I. (2007). Chemical, functional and cookie baking properties of soybean/maize flour blends. *J Food Sci Technol., 44*(6), 619-622.

Alozie, Y. E., Udofia, S., Lawal, O., & Ani, I. F. (2009). Nutrient composition and sensory properties of cakes made from wheat and African yam bean flour blends. *Journal of Food Technology, 7*, 115-118.

AOAC. (2005). Association of Official Analytical Chemists (18th ed.). Washington D.C. USA: A.O.A.C.

Apotiola, Z. O., & Fashakin, J. F. (2013). Evaluation of cookies from wheat flour, soybean flour and cocoyam flour blends. *Food Science and Quality Management, 14*, 17-21.

Aprianita, A., Purwandari, U., Watson, B., & Vasiljevic, T. (2009). Physico-chemical properties of flours and starches from selected commercial tubers available in Australia. *International Food Research Journal, 16*, 507-520.

Bradbury, J. H., & Holloway, W. D. (1988). *Chemistry of tropical root crops: Significance for nutrition and Agriculture in the Pacific.* Australian Centre for International Research, Canberra.

Ceserani, V., & Kinton, R. (2008). Practial Cookery (10th ed.). John Wiley and Sons, New York.

Chinma, C. E., Abu, J. O., & Abubakar, Y. A. (2010). Effect of tigernut (*Cyperus esculentus*) flour addition on the quality of wheat-based cake. *International Journal of Food Science and Technology, 45*, 1746-1752. http://dx.doi.org/10.1111/j.1365-2621.2010.02334.x

Chinma, C. E., Gbadamosi, K. B., Ogunsina, B. S., Oloyede, O. O., & Salami, S. O. (2014). Effect of addition of germinated *Moringa* seed flour on the quality attributes of wheat-based cake. *Journal of Food Processing and Preservation, 38*(4), 1737-1742. http://dx.doi.org/10.1111/jfpp.12136

Chinma, C. E., Ingbian, E. K., & Akpapunam, M. A. (2007). Processing and acceptability of fried cassava balls (''Akara-akpu'') supplemented with melon and soyabean flour. *Journal of Food Processing and Preservation, 31*, 143-156. http://dx.doi.org/10.1111/j.1745-4549.2007.00117.x

Ekanem, A. M., & Osuji, J. O. (2006). Mitotic index studies on edible cocoyams (*Xanthosoma and Colocasia* spp). *African Journal of Biotechnology, 5*(10), 846-849.

Eke, J., Sanni, S. A., & Owuno, F. (2009). Proximate and sensory properties of banana cakes. *Nigerian Food Journal, 27,* 102-106. http://dx.doi.org/10.4314/nifoj.v27i2.47479

Ekwe, K., Nwosu, K., Ekwe, C., & Nwachukwu, L. (2009). Examining the under explained values of cocoyam (*Colocasia* and *Xanthosoma spp*). Nutrition and Economy in Nigeria. In: Jarnieka H., Ganry J., Zeledon Hoeshie I. and Kahara R. (eds). *Proceedings of International symposium on underutilized plants for food security, Income and sustainable Development*. *Acta Horticulture, 86*, 71-78

Eneh, O. C. (2013). Towards food security and improved nutrition in Nigeria: Taro (*Colocasia antiquorum*) grit as carbohydrate supplement in energy food drink. *African Journal of Food Science, 7*(10), 355-360. http://dx.doi.org/10.5897/AJFS2013.1068

Eze, C. C., & Okorji, E. C. (2003). Cocoyam production by women farmers under improved and local technologies in Imo state, Nigeria. *Africa J. Sci., 5*(1), 113-116.

FAO. (1990). Roots, Tubers, Plantains and Bananas in Human Nutrition. Food and Agricultural Organisation, Food and Nutrition series, No.24.

Giami, S. Y., Amasisi, T., & Ekiyor, G. (2004). Comparison of breadmaking properties of composite flour from kernels of roasted and boiled African breadfruit (*Treculia africana decne*) seeds. *Journal of Raw Material Research, 1***,** 16-25.

Ikpeme-Emmanuel, C. A., Osuchukwu, N. C., & Oshiele, L. (2010). Functional and sensory properties of wheat (*Aestium triticium*) and taro flour (*Colocasia esculenta*) composite bread. *Afr J Food Sci., 4*, 248-253

Iwe, M. O. (2002). *Handbook of Sensory methods and analysis*. Enugu, Nigeria: Rejoint Com. Services Ltd.

Iwe, M. O., & Onalope, O. O. (2001). Effect of extruded full-fat soy flour into sweet potato flour on functional properties of the mixture. *J. Sustain, Agric. Environ., 3*, 109-117.

Kaur, M., Singh, N., & Sandhu, K. S. (2007). Preparation and characterization of protein isolates from different lentil (*Lens culinaris*) cultivars. *J Food Sci Technol., 44*, 327-329.

Ma, Z., Boye, J. I., Simpson, B. K., Prasher, S. O., Monpetit, D., & Malcolmson, L. (2011). Thermal processing effects on the functional properties and microstructure of lentil, chickpea, and pea flours. *Food Research International, 44*, 2534-2544. http://dx.doi.org/10.1016/j.foodres.2010.12.017

Moorthy, S. N., & Ramanujam, T. (1986). Variation in properties of starch in cassava varieties in relation to age of the crop. *Starch/Starke, 38*, 58-61. http://dx.doi.org/10.1002/star.19860380206

Niba, L. L. (2003). Processing effects on susceptibility of starch to digestion in some dietary starch sources. *Int. J. of Food Sci. Nutrition, 54*, 97-109. http://dx.doi.org/10.1080/0963748031000042038

Njintang, N. Y., Carl, M. F., Facho, B., Pierre, K., & Joel, S. (2008). Effect of taro (*Colocasia esculenta*) flour addition on the functional and alveographic properties of wheat flour and dough. *Journal of the Science of Food and Agriculture, 88*, 273-279. http://dx.doi.org/10.1002/jsfa.3085

Njintang, Y. N., Mbofung, C. M. F., Moates, K. G., Parker, L., Fauld, C. B., & Smith, A. C. (2007). Functional properties of five varieties of taro flour and relationship to creep recovery and sensory characteristics of achu (taro based paste). *J Food Eng., 82*, 114-120. http://dx.doi.org/10.1016/j.jfoodeng.2006.12.023

Nnabuk, O. E., Essien, E., Ebenso, E. E., & UKPE, R. A. (2012). Industrial potential of two varieties of cocoyam in bread making. *E-Journal of Chemistry, 9*(1), 451-464. http://dx.doi.org/10.1155/2012/635894

Okaka, J. C., Anosike, G. N., & Okaka, A. N. C. (1997). Effect of particle size profile of sun-dried and oven dried cowpea flours on their physical and functional characteristics in model system. *J. Food Sci. Technol., 3*, 65-74.

Okezie, B. O., & Bello, A. B. (1988). Physicochemical and functional properties of winged bean flour and isolate compared with soy isolate. *J. Food Sci., 53*(2), 450-454. http://dx.doi.org/10.1111/j.1365-2621.1988.tb07728.x

Onimawo, I. A., & Asugo, S. (2004). Effects of germination on the nutrient content and functional properties of pigeon pea flour. *J Food Sci. Technol., 41*(2), 170-174.

Onyenobi, V. O., Chukwu, G. O., Asumogha, G. N., Okoye, B. C., Ewuziem, J. E., Ogbonna, M. C., & Akpinpelu, A. O. (2010). Marketing of Edible Cocoyam, *Xanthosoma spp* (Tannia) in Abia State, Nigeria. Cocoyam Research Programme. 2010 Annual Report and Programme of work. NRCRI, Umudike, pp. 69-73.

Opara, L. U. (2002). Edible Aroids: Postharvest Operation. In: AGST/FAO: Danilo, M. (Ed.). Massey University, New Zealand.

Owusu-Darko, P. G., Paterson, A., & Omenyo, E. L. (2014). Cocoyam (Corms and Cormels)- An under exploited food and feed resource. *Journal of Agricultural Chemistry and Environment, 3*(1), 22-29. http://dx.doi.org/10.4236/jacen.2014.31004

Sakamoto, L. A. (2003). Development of Early maturing and Leaf Blight Resistant Cocoyam (Colocasia esculenta (L) Schott) with Improved Taste. Proceedings of a Final Research coordination Meeting. Organized by the Joint FAO/IAEA Division of Nuclear Technology in Food and Agriculture Held in Pretoria, South Africa (pp. 19-23).

Sathe, S. K., Deshpande, S. S., & Salunkhe, D. K. (1982). Functional properties of winged bean proteins. *Journal of Food Science, 47*, 503-508. http://dx.doi.org/10.1111/j.1365-2621.1982.tb10112.x

Sefa-Dedeh, S., & Sackey, E. K. (2002). Starch structure and some properties of cocoyam (*Xanthosoma sagittifolium* and *Colocasia esculentum*). *Food Chemistry, 79*, 435-444. http://dx.doi.org/10.1016/S0308-8146(02)00194-2

Tagodoe, A., & Nip, W. K. (1994). Functional properties of raw and precooked taro (*Colocasia esculenta*) flours. *Int. J. Food Sci. Technol., 29*, 457-462. http://dx.doi.org/10.1111/j.1365-2621.1994.tb02087.x

Tester, R. F., & Morrison, W. R. (1990). Swelling and gelatinisation of cereal starches: Effect of amylopectin, amylose and lipids. *Cereal Chemistry, 67*, 551-559.

Ubalua, A. O., & Chukwu, L. I. (2008). Potentials and Constraints of Cocoyam Production in Nigeria Proc. 42nd. Conf. Agricultural Society of Nigeria (ASN) October 19 – 23 2008 (pp. 288-302). Ebonyi State University, Abakaliki, Nigeria.

UNECA. (1985). *Technical compendium on composite flours* (pp. 67-81). Ethiopia: United Nations Economic Commission for Africa.

Woolfe, J. (1992). *Sweet potato: An untapped food resource* (pp. 1-13, 366-372). Cambridge University Press, London.

Journal of Food Research; Vol. 4, No. 5; 2015
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Reviewer Acknowledgements

Journal of Food Research wishes to acknowledge the following individuals for their assistance with peer review of manuscripts for this issue. Their help and contributions in maintaining the quality of the journal are greatly appreciated.

Journal of Food Research is recruiting reviewers for the journal. If you are interested in becoming a reviewer, we welcome you to join us. Please find the application form and details at http://www.ccsenet.org/reviewer and e-mail the completed application form to jfr@ccsenet.org.

Reviewers for Volume 4, Number 5

Adrian Hernandez-Mendoza, Research Center for Food and Development, Mexico

Akshay Kumar anugu, Ingredion Incorporated, United States

Antonello Santini, University of Napoli "Federico II", Italy

Bojana Filipcev, University of Novi Sad, Serbia

Cemil KUREKCI DVM, Mustafa Kemal University, Turkey

Djilani Abdelouaheb, Badji Mokhtar University, Algeria

Elke Rauscher-Gabernig, Austrian Agency for Health and Food Safety, Austria

Fengmei Zhu, Hebei Normal University of Science and Technology, China

Fernando Sanches de Lima, University of Londrina, Brazil

Giuseppe Zeppa, Università degli Studi di Torino, Italy

Hrvoje Pavlović, Faculty of Food Technology Osijek, Croatia

Isabela Mateus Martins, State University of Campinas, Brazil

James Owusu-Kwarteng, University for Development Studies, Ghana

Jelena Dragisic Maksimovic, University of Belgrade, Serbia

Jose M. Camina, National University of La Pampa and National Council of Scientific and Technical Researches (CONICET), Argentina

Juarez Vicente, Federal Rural of Rio de Janeiro University, Brazil

Juliano De Dea Lindner, Federal University of Santa Catarina (UFSC), Brazil

Liyan Chen, South Dakota State University, United States

Lizhe WANG, Nanjing Agricultural University, China / FOODAWA Food Technology, Germany

Magdalena Polak-Berecka, University of Life Sciences, Poland

Mahantappa Halimani, Brigham and Women's Hospital, USA

Maria Rosana Ramirez, National Council for Scientific and Technologic Research- UNLPam, Argentina

Natália Alencar, Campinas University, Brazil

Nela Nedic Tiban, Faculty of Food Technology Osijek, Croatia

Call for Manuscripts

Journal of Food Research is an international, double-blind peer-reviewed, open-access journal, published by the Canadian Center of Science and Education. It publishes original research, applied, and educational articles in all areas of food science and technology. The scopes of the journal include, but are not limited to, the following fields: history and culture of food, novel foods and sensory analysis, food quality and safety, food chemistry and toxicology, food microbiology, food nutrition and health, nutraceuticals and functional food, food engineering and physical properties, food laws and regulations, food economics and marketing, concise reviews and hypotheses in food science, and so on. The journal is available in electronic form in conjunction with its print edition. All articles and issues are available for free download online.

We are seeking submissions for forthcoming issues. All manuscripts should be written in English. Manuscripts from 3000–8000 words in length are preferred. All manuscripts should be prepared in MS-Word format, and submitted online, or sent to: jfr@ccsenet.org

Paper Selection and Publishing Process

a) Upon receipt of a submission, the editor sends an e-mail of confirmation to the submission's author within one to three working days. If you fail to receive this confirmation, your submission e-mail may have been missed.

b) Peer review. We use a double-blind system for peer review; both reviewers' and authors' identities remain anonymous. The paper will be reviewed by at least two experts: one editorial staff member and at least one external reviewer. The review process may take two to three weeks.

c) Notification of the result of review by e-mail.

d) If the submission is accepted, the authors revise paper and pay the publication fee.

e) After publication, the corresponding author will receive two hard copies of the journal, free of charge. If you want to keep more copies, please contact the editor before making an order.

f) A PDF version of the journal is available for download on the journal's website, free of charge.

Requirements and Copyrights

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the authorities responsible where the work was carried out, and that, if accepted, the article will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher. The editors reserve the right to edit or otherwise alter all contributions, but authors will receive proofs for approval before publication.

Copyrights for articles are retained by the authors, with first publication rights granted to the journal. The journal/publisher is not responsible for subsequent uses of the work. It is the author's responsibility to bring an infringement action if so desired by the author.

More Information

E-mail: jfr@ccsenet.org

Website: www.ccsenet.org/jfr

Paper Submission Guide: www.ccsenet.org/submission

Recruitment for Reviewers: www.ccsenet.org/reviewer

The journal is peer-reviewed
The journal is open-access to the full text
The journal is included in:

AGRICOLA	Global Health	Standard Periodical Directory
AGRIS	Google Scholar	SHERPA/RoMEO
CAB Absracts	JournalTOCs	Ulrich's
Chemical Abstracts Service	PKP Open Archives Harvester	
EBSCOhost	ProQuest	

Journal of Food Research

Bimonthly

Publisher Canadian Center of Science and Education
Address 1120 Finch Avenue West, Suite 701-309, Toronto, ON., M3J 3H7, Canada
Telephone 1-416-642-2606
Fax 1-416-642-2608
E-mail jfr@ccsenet.org
Website www.ccsenet.org/jfr

ISSN 1927-0887