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The Relationship between Children's Noncognitive Skills toward Food and Their Food Habits in a Cross-Sectional Study

Tomoko Osera[1, 2], Setsuko Tsutie[3], Misako Kobayashi[2] & Nobutaka Kurihara[1]

[1]Hygiene and Preventive Medicine, Graduate School of Life Science, Kobe Women's University, Japan

[2]Takakuradai Kindergarten attached to Kobe Women's University, Japan

[3]Clinical Nutrition Management, Graduate School of Life Science, Kobe Women's University, Japan

Correspondence: Nobutaka Kurihara, Graduate School of Life Science, Kobe Women's University, 2-1 Higashisuma-Aoyama, Suma, Kobe, Japan. Tel: 81-78-737-2417. E-mail: kurihara@suma.kobe-wu.ac.jp

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Abstract

Our previous study suggested that children's food preferences were related to their concern about food and respect for food. In general, concern and respect were sorts of non-cognitive skills, which are useful for future life if acquired during childhood. The aim of this study was to make clear how concern about food and respect for food were related to their food habits and life style. We investigated the relationship between children's non-cognitive skills and their food habits in a cross-sectional study. From 2007 to 2016, 2,408 Japanese kindergarten children aged 3 to 5 years were included in the study. The distribution was categorized into two patterns of their non-cognitive skills based on whether a particular the guardians answered children's non-cognitive skills, concern about food and respect for food. The high and low of non-cognitive skills toward food were related to lifestyle, food preferences and food habits. High non-cognitive skills toward food may be associated with to take good food habits and their preferences in kindergarten children.

Keywords: respect for food, concern about food, kindergarten children

1. Introduction

Non-cognitive skills during childhood are useful for future life. Non-cognitive skills have been broadly defined as representing the "patterns of thought, feelings and behavior" (Les et al, 2008). In general, concern and respect included in non-cognitive skill. Our previous study suggested that children's food preferences were related with their concern about food and respect for food (Osera et al, 2016a).

Childhood is a crucial period for developing food acceptance patterns (Cashdan, 1998). For example, children are exposed to unhealthy food choices, which may have contributed to the increase in the prevalence of overweight observed among youth in the past several years (St-onge, 2003). Less dislike for certain foods in high school students was related to good food habits at the present day as well as a lower numbers of disliked food items in childhood (Osera, 2017a). The development and long-term health of children are linked to food habits established from early childhood (Scaglioni, 2008). The aim of this study was to make clear how concern about food and respect for food were related to their food habits and lifestyle.

Non-cognitive skills have been studied by some economists and pedagogs. Hickman et al. (2006) showed that both cognitive and non-cognitive abilities determine social and economic success. Our demonstration that non-cognitive skills are important in explaining a diverse array of behaviours helps to explain why early childhood programs, such as Head Start and the Perry Preschool Program, are effective (Heckman, 2006). The Perry Preschool research study showed that high-quality preschool programs for young children living in poverty contributed to their intellectual and social development in childhood and their success in school and economic performance, and reduced incidence of crime in adulthood (Schweinhart, 2005). Therefore, non-cognitive skills during childhood are very important for future life (Duffy, 2000). During childhood is important to take high non-cognitive skills. In the study, we would like to demonstrate the relationship between non-cognitive skills toward food and their food habits and life style.

We investigated the relationship between children's "concern about food" and "respect for food" and their food habits and lifestyle in a cross-sectional study.

2. Method

2.1 Study Design and Subjects

A study was using data from children in one kindergarten obtained over one decade, we conducted a study to determine whether or not the children's health was changed. From 2007 to 2016, 2,408 children aged 3 to 5 years from a kindergarten in Japan were included in the study. The questionnaire was distribution by every April in each year. The distribution was every children but the response rate was 98.0±3.1 during 10 years (Table 1). After we summed up all the data in cross-sectional study, we cleared no changes during one decade. These data were secondary usage.

Table 1. The response rate of the each study

Year	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Response rate(%)	98.8	100.0	99.6	99.2	99.6	97.2	97.7	97.6	100.0	89.7

2.2 Questionnaire

The main outcomes were height, weight, "respect for food", "concern about food" and food preferences. Because our previous study suggested that "respect for food" and "concern about food" were very important item for children's lifestyle (Osera, 2016a), we adopted these items. Therefore, we examined the relationship between the two non-cognitive skills (concern about food and respect for food) and 11 items. Of the 11 items, 2 lifestyle habits (waking time and sleeping time) and 9 behaviour related to food habits (help set the table, help with cooking, go shopping at the supermarket with parents, frequency of eating breakfast, talk about food while eating, talk about taste while eating, enjoy school lunch, food preference and number of foods preferred. Waking time and sleeping time were scored on a four-point scale, with higher scores indicating better habits. All other questions were scored on a five-point scale, with higher scores indicating better habits, except for food preference. Food preference was scored on a two-point scale, indicating presence or absence. 'Presence' means that the child disliked more than one food; 'absence' means that the child disliked no food. The guardians answered children's non-cognitive skills, concern about food and respect for food. In addition, all outcomes were self-reported by parents.

If a child disliked a food, we asked what kind of food from a list of 55 foods. These foods were all available in regular school lunches and were often disliked by children, as shown in our previous study (Osera, 2016a).

2.3 Definitions of Overweight and Obesity

For children, overweight and obesity were defined based on the Kaup score. Kaup score is use to define children's health, especially to check tendency of thin or obese in Japan. Overweight was defined as a kaup score of 16.5 to 18.5, and obesity was defined as a kaup score of 18.5 or higher (Imamura, 1983).

2.4 Statistical Analysis

Differences in rates of mother's working style between years were examined using Fisher's exact probability test. Three-way analysis of variance (ANOVA) was used to change each item (e.g. height, weight) between age, sex and during one decade. P values < 0.05 were considered to indicate statistical significance.

The distribution was categorised into two patterns of children's non-cognitive skills based on whether a particular the guardians answered children's non-cognitive skills, concern about food and respect for food. "Concern about food" was related with weight and healthy food habits in adolescents (MacFarlane et al, 2010). In the study non-cognitive skills toward food are defined as "concern about food" plus "respect for food." To address this definition, the maximum total score for concern about food and respect for food was 10, the minimum was 1 and the mean and standard deviation were 7.6±1.6. So, we distributed two groups. According to this procedure compared with high and low of two non-cognitive skills toward food of respect for food and concern about food. Three-way ANOVA was used to change each item (e.g. waking time, sleeping time, et al.) between age, sex and level of non-cognitive skill. P values < 0.05 were considered to indicate statistical significance. The data were analyzed using SPSS for Windows, Version 23 (IBM, New York, NY. USA).

2.5 Ethical Approval

Guardians were informed about the objects and methods of this study and guardians answered the questionnaire only if they desired, in the absence of any compelling force and with the right of free withdrawal. Individual privacy was strictly protected thorough the investigation. Each study was approved by the president of the kindergarten. All guardians provided informed consent for participation in the each study. Under these conditions,

the mothers agreed to cooperate in the scientific investigations in the kindergarten, including this study, when their children entered. The present study is based on the data which some studies with all participantes' permission. This work is a part of the study which has been approved by the Kobe Women's University Ethics Committee Regarding Human Subjects; H29-2.

3. Results

3.1 Participant Characteristics

Table 2 shows the age and sex distribution of each study sample. Differences in rates of mother's working style between years were examined using Fisher's exact probability test (Table 3). The proportion of mothers who were housewives was significantly lower in 2016 than in 2012 (75.3% vs. 81.6%; P < 0.001, Fisher's exact probability test). The rate of obesity was 0.3%, and the rate of overweight was 2.2% in the average of all data (data not shown).

Table 2. Distribution of the study samples by age, sex and survey year

Year	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Boy										
Three years old	31	19	40	30	28	47	28	28	21	23
Four years old	44	48	33	53	37	32	60	34	28	18
Five years old	50	46	48	34	54	36	33	58	35	29
Girl										
Three years old	27	25	31	27	26	36	35	33	34	30
Four years old	44	63	42	56	49	51	55	40	39	37
Five years old	54	44	63	43	53	50	51	55	41	38
N=number										

Table 3. Differences between mother's working style and year

Year	2012	2013	2014	2015	2016	P value
House wife	81.6	76.3	74.9	76.0	75.3	< 0.001

Note. Fisher's exact probability test: * P < 0.05, ** P < 0.01, *** P < 0.001

The ratio was Mother's housewives. The data before 2012 are not available, because we examined this question from 2012.

3.2 Relationship between Five Items (Height, Weight, Food Preferences, Concern about Food and Respect for Food) and Age and Sex During 10 Years

Figure 1 shows the mean height according to age, sex and survey year. There were significant differences by age and sex, but there were no significant differences by year (age, P < 0.001; sex, P < 0.001; year, N.S.; three-way ANOVA) (Figure 1).

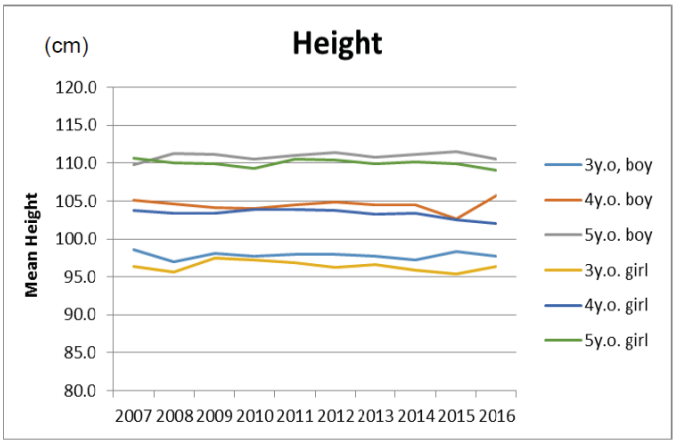

Figure 1. Height of study subjects by sex, age and survey year in the study population, 2007-2016

Note. Mean hight during one decade in terms of gender, age were assessed using Three way ANOVA; * P < 0.05, ** P < 0.01, *** P < 0.001. Significant differences, age *** P < 0.001, sex *** P < 0.001, year N.S.

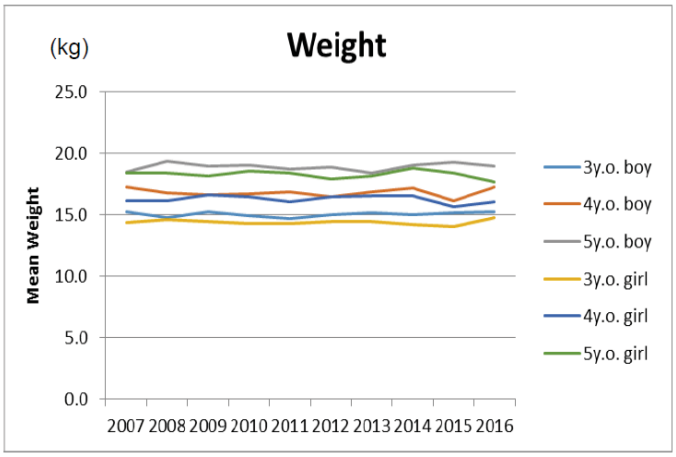

Figure 2. Weight of study subjects by sex, age and survey year in the study population, 2007-2016

Note. Mean weight during one decade in terms of gender, age were assessed using Three way ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significant differences, age *** $P < 0.001$, sex *** $P < 0.001$, year N.S.

The results for weight showed almost the same tendency as those for height. Figure 2 shows the mean weight according to age, sex and survey year. There were significant differences by age and sex, but there were no significant differences by year (age, $P < 0.001$; sex, $P < 0.001$; year, N.S.; three-way ANOVA) (Figure 2).

Figure 3 shows the mean food preference scores according to age, sex and survey year. There were no significant differences by age, sex or year (age, NS; sex, NS; year, NS; three-way ANOVA) (Figure 3).

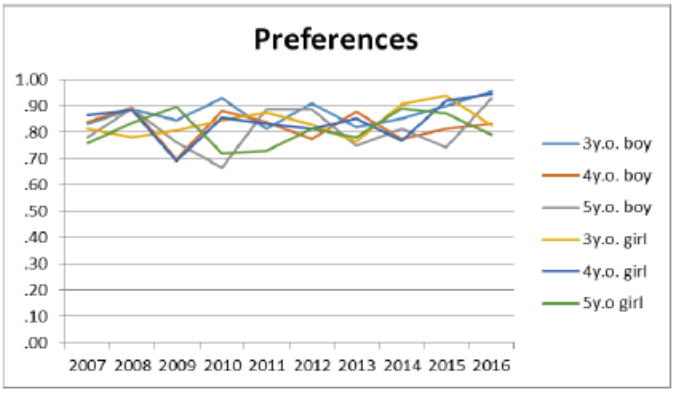

Figure 3. Preferences of study subjects by sex, age and survey year in the study population, 2007-2016.

Note. Children's food preferences during one decade in terms of gender, age were assessed using Three way ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significant differences, age N.S., sex N.S., year N.S. Two point scale up to 0 to 1. 1 was presence and 0 was absence.

Figure 4 shows the mean scores for concern about food according to age, sex and survey year. There were significant differences by age, sex and year (age, $P < 0.05$; sex, $P < 0.01$; year, $P < 0.05$; three-way ANOVA) (Figure 4). The results for respect for food showed almost the same tendency as those for concern about food.

Figure 5 shows the mean scores for results for respect for food according to age, sex and survey year. There were significant differences by age and sex, but there were no significant differences by year (age, $P < 0.001$; sex, $P < 0.05$; year, N.S.; three-way ANOVA) (Figure 5).

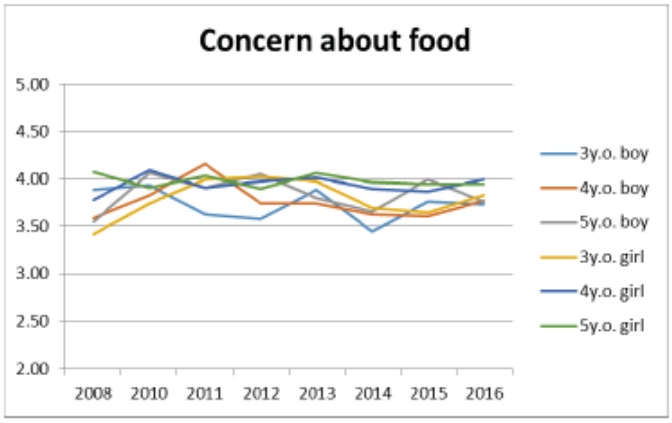

Figure 4. "Concern about food" of study subjects by sex, age and survey year in the study population, 2008-2016

Note. Mean "concern about food" during one decade in terms of gender, age were assessed using Three way ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significant differences, age * $P < 0.05$, sex ** $P < 0.01$, year * $P < 0.05$ Five point scale up to 1 to 5.

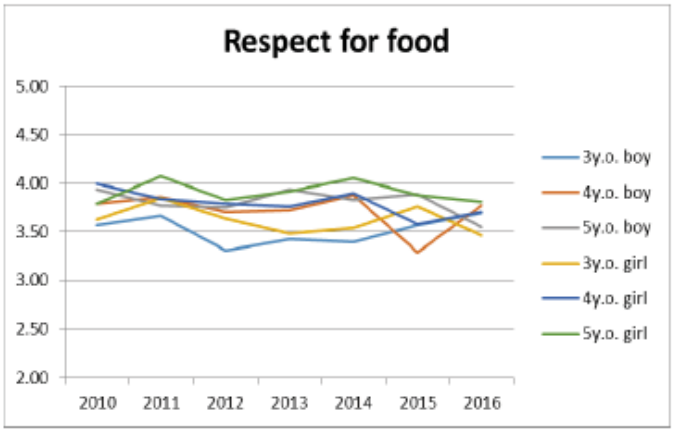

Figure 5. "Respect for food" of study subjects by sex, age and survey year in the study population, 2010-2016

Note. Mean "respect for food" during one decade in terms of gender, age were assessed using three way ANOVA; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significant differences, age *** $P < 0.001$, sex * $P < 0.05$, year N.S. Five point scale up to 1 to 5.

3.3 Noncognitive Skills toward Food Related With Life Style and Food Habits

Table 4 shows the results for waking time and sleeping time. There were significant differences in non-cognitive skills toward food compared with high and low but there were no significant differences by age and sex. Frequency of eating breakfast, talking about food, talking about taste and food preferences showed the same tendency. There were significant differences in non-cognitive skills toward food compared with high and low but there were no significant differences by age and sex (Table 4).

Helping set the table, cooking and shopping were significant differences in non-cognitive skills toward food, age and sex (Table 4 and supplemental data, the data was not shown in the manuscript).

Enjoying school lunch and number of food preferences were significant differences non-cognitive skills and age but there were no significant differences by sex (Table 4). The score for enjoying school lunch increased with age from 4.1 ± 0.9 at 3 years, to 4.3 ± 0.8 at 4 years, to 4.4 ± 0.9 at 5 years (mean ± SD). The score for number of food preferences decreased with age from 9.0 ± 7.9 at 3 years, to 6.6 ± 6.1 at 4 years, to 5.0 ± 4.6 at 5 years (mean ± SD, data not shown).

Table 4. Relationship between high and low noncognitive skills toward food and their food habits

	High				Low				P value		
				N				N	skill	sex	age*
Waking time[+]	3.3	±	0.6	762	3.2	±	0.7	597	< 0.001	N.S.	N.S.
Sleeping time[+]	2.5	±	0.7	757	2.3	±	0.7	587	< 0.001	N.S.	N.S.
Help set the table[++]	4.1	±	0.8	329	3.7	±	1.0	288	< 0.001	< 0.01	< 0.001
Cooking	3.7	±	1.0	910	3.4	±	1.1	691	< 0.001	< 0.001	< 0.001
Shopping[$]	4.4	±	0.8	707	4.2	±	1.0	521	< 0.001	< 0.001	< 0.001
Enjoying school lunch	4.6	±	0.7	757	3.9	±	0.9	595	< 0.001	N.S.	< 0.001
Eat breakfast	4.0	±	0.1	909	4.0	±	0.3	691	< 0.01	N.S.	N.S.
Talk about food	4.2	±	0.7	761	3.8	±	0.8	596	< 0.001	N.S.	N.S.
Talk about taste	4.3	±	0.7	480	3.9	±	0.8	376	< 0.001	N.S.	N.S.
Food preference [+++]	0.8	±	0.4	893	0.9	±	0.3	675	< 0.001	N.S.	N.S.
Number of food preferences[++++$$]	4.9	±	4.6	370	8.9	±	7.8	338	< 0.001	N.S.	< 0.001

Note. The relationship between non-cognitive skills toward food, age and sex were assessed using three way ANOVA;* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; The maximum total score for concern about food and respect for food was 10, the minimum was 1 and the mean and standard deviation were 7.6±1.6. Based on this, we devided into two groups. According to this procedure compared between high and low of two non-cognitive skills toward food, respect for food and concern about food. The data showed including all age children.* Mean of each age data was not shown. + Up to 4 point scale. Good habits indicate 4 point. ++ Up to 5 point scale. Good habits indicate 5 point. +++'Presence' means that students dislike more than one foods, 'Absence' means that students disliked no food. 1 was 'presence' and 0 was 'absence'. ++++The foods student disliked, which were chosen themselves from a list of 55 foods. $ "Shopping" means "go shopping for dinner with guardians". $$ The number was described mother choose children's disliked food.

4. Discussion

The first, we checked changes in the same kindergarten over 10 years. Children's height, weight, food preferences and respect for food did not significantly change, and concern about food showed a slightly significant change (Figures 1–5). Height, weight and non-cognitive skills toward food (respect for food and concern about food) showed significant differences by age and sex (Figures 1, 2, 4, 5). During 3 to 5 years old children, there are large differences in physical and psychological characteristics according to age and sex. On the other hand, there were no significant differences in food preferences by age, sex and survey year (Figure 3). There were no changes in food preferences than physical and psychological by age and sex during 10 years.

We assessed non-cognitive skills summed up as 'concern about food' and 'respect for food'. Parental concern about adolescent weight was associated with lower intake of energy-dense snacks and less home availability of these food items (MacFarlane et al, 2010). In addition, our previous study suggested that the amount of vegetables in packed lunches may be related to mothers' concern for vegetables and children's preferences (Osera et al, 2017b). The present study showed the same tendency as MacFarlane's study and our previous study; the mother answered children's high and low of non-cognitive skills toward food were significantly related to their lifestyle and food habits in children of all ages (Table 4). Waking time, sleeping time, frequency of eating breakfast, talk about food, talk about taste and food preferences were significantly related to non-cognitive skills toward food but not to age and sex. Eating breakfast every day is associated with having a healthy body weight, likely due to a more even distribution of energy intake across meals throughout the day (Dubois et al, 2008). Omitting breakfast affected children's appetite ratings (Kral et al, 2011). In addition, compared with decreased sleep duration, increased sleep duration in school-age children resulted in lower reported food intake, lower fasting leptin levels and lower weight (Hart et al, 2013). Not only eating breakfast but also sleep duration is very important for children's food habits and correct food intake. Knowledge, attitude and behaviour (KAB) is an important theoretical model for health education, which asserts that behaviour change is affected by knowledge and attitude (Xu et al, 2010). According this theory, children and mothers important to take high concern about food and respect for food, after behaviour will be change and eating breakfast every day.

The study showed that children's consumption of soybean products was related to whether or not family members had conversations about food at meals and whether or not mothers told their children about Japanese foods (Osera et al, 2016b). The study showed that talking about food, talking about taste and food preferences were related to non-cognitive skills toward food. Conversation between mothers and children during meals may be important for the development of children's non-cognitive skills toward food.

Helping to set the table, cooking and shopping for dinner with guardians were related to non-cognitive skills toward food and to children's age and sex. Utter et al. (2016) suggested that learning to cook and having the opportunity to cook may provide a unique means for adolescents to develop life skills and contribute positively to their families. We believe that this also applies to children. Because these three items are related to age, they can do any house work. The proportion of mothers who were identified as housewives significantly decreased over 10 years. In Japan, housewives do most of the cooking, washing and cleaning. These habits may have influenced the results of this study. Girls' scores were higher than boys' scores on these three items. In addition, these scores increased with increasing age (Table 4 and supplemental data, the data was not shown in the manuscript). Thus, the results suggested that high and low of non-cognitive skills toward food and housework related to cooking were significantly related to the child's sex.

Enjoying school lunch and number of food preferences were related to non-cognitive skills toward food and to age. The number of food preferences decreased with increasing age. Enjoying school lunch was significantly related to decreasing food preferences in our previous study (Osera et al, 2014). The kindergarten has school lunches, and kindergarten school lunch and nutrition education may be useful to decrease the number of food preferences. This opinion was supported by Lambert et al., who implied that teachers and school health professionals should improve the nutritional content of foods allowed in the classroom (Lambert et al, 2016). In the detail of the result, in food preferences, our result showed presence or absence of food preferences were significant differences by high and low of non-cognitive skills. There were no significant differences by age and sex. But the numbers of food preferences were significant differences by high and low of non-cognitive skills and age. In this study, food preference and the number of food preferences did not differ significantly by sex. Our previous study showed that the number of food preferences was significantly decreased during childhood and adolescence, as children grew up (Osera et al, 2016a). That study and other studies suggested that food preferences differed between boys and girls, and that these gender differences varied among elementary, middle and high school students (Osera et al, 2016a; Caine-Bish & Scheule, 2009). Therefore, it could not still conclude but we consider that only during childhood were not significant differences by gender in food preferences. Nutrition education and school lunches are very useful to improve non-cognitive skills toward food in kindergarten children.

A limitation of the current study is that it was a cross-sectional study and therefore could not explain cause and effect. In the next study, we will try to clear using a retrospective study. It is important to take higher non-cognitive skills toward food during childhood and may influence future health. In the future, we will examine the cause and effect relations of children's non-cognitive skills toward food to nutrition education and school lunches. High non-cognitive skills toward food may be associated with to take good food habits in kindergarten children. In addition, since these data were of the secondary usage, it may be highly likely to have selection bias. Local data are needed because there are also differences in determinants of factors across cultures, places and ages.

5. Conclusion

Higher non-cognitive skills toward food during childhood are important because it may influence their food habits and lifestyle during childhood.

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Author Contributions

T.O., S.T., and N.K. developed the standardized protocol and structured questionnaire. M.K. conducted the focus group research in the kindergarten. T.O. drafted the manuscript. All authors critically revised the article for important intellectual content and approved the final manuscript.

Conflicts of Interest

This work was supported by JSPS KAKENHI Grant Number JP17K12925 for Tomoko Osera. The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Appendix A

Appendix A. Relationship between noncognitive skills toward food, age and sex

	Boy							Girl									
	High			Low				High			Low				P value		
				N			N				N			N	skill	sex	age*
Waking time^{+}	3.3	±	0.7	318	3.3	±	0.7	300	3.3	±	0.6	444	3.1	±	0.6	298	< 0.001	N.S.	N.S.
Sleeping time^{+}	2.5	±	0.8	316	2.3	±	0.7	296	2.5	±	0.7	441	2.3	±	0.8	292	< 0.001	N.S.	N.S.
Help set the table^{++}	4.0	±	0.8	129	3.5	±	1.1	145	4.1	±	0.8	200	3.8	±	0.9	145	< 0.001	< 0.01	<0.001
Cooking	3.6	±	1.1	386	3.1	±	1.1	348	3.8	±	0.9	523	3.6	±	1.0	344	< 0.001	< 0.001	<0.001
Shopping$^{\$}$	4.2	±	0.9	312	4.1	±	1.0	267	4.5	±	0.6	394	4.3	±	0.9	255	< 0.001	< 0.001	<0.001
Enjoying school lunch	4.5	±	0.8	318	3.9	±	0.9	298	4.6	±	0.6	439	3.9	±	0.9	298	< 0.001	N.S.	<0.001
Eat breakfast	4.0	±	0.1	386	4.0	±	0.2	348	4.0	±	0.1	522	3.9	±	0.3	344	< 0.001	N.S.	N.S.
Talk about food	4.2	±	0.7	317	3.8	±	0.8	300	4.2	±	0.6	444	3.7	±	0.8	297	< 0.001	N.S.	N.S.
Talk about taste	4.3	±	0.7	197	3.9	±	0.8	185	4.3	±	0.7	283	3.9	±	0.7	191	< 0.001	N.S.	N.S.
Food preference $^{+++}$	0.8	±	0.4	378	0.9	±	0.3	337	0.8	±	0.4	514	0.9	±	0.3	337	< 0.001	N.S.	N.S.
Number of food preferences$^{++++\$\$}$	5.6	±	5.7	145	9.1	±	8.3	164	4.4	±	3.7	225	8.5	±	6.7	173	< 0.001	N.S.	<0.001

Note. The relationship between non-cognitive skills toward food, age and sex were assessed using three way ANOVA;* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; The maximum total score for concern about food and respect for food was 10, the minimum was 1 and the mean and standard deviation were 7.6±1.6. Based on this, we devided into two groups. According to this procedure compared between high and low of two non-cognitive skills toward food, respect for food and concern about food. The data showed including all age children.* Mean of each age data was not shown. + Up to 4 point scale. Good habits indicate 4 point. ++ Up to 5 point scale. Good habits indicate 5 point. +++'Presence' means that students dislike more than one foods, 'Absence' means that students disliked no food. 1 was 'presence' and 0 was 'absence'. ++++The foods student disliked, which were chosen themselves from a list of 55 foods. $ "Shopping" means "go shopping for dinner with guardians". $$ The number was described mother choose children's disliked food.

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Enzymatic Kinetics of Enzymatically Extruded Degerminated Maize Using Glucamylase

Chengye Ma[1, 2], Shuangshuang Yu[1], Yuyan Fan[1], Shuhua Wu[1], Zhehao Zhang[1], Yuedong Song[1] & Hongjun Li[1, 2]

[1]School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, 255000, China

[2]Key Laboratory of Shandong Provincial Universities for Technologies in Functional Agricultural Products, Zibo, 255000, China

Correspondence: Chengye Ma, School of Agricultural Engineering and Food Science, Shandong University of Technology, Zibo, China. E-mail: mcycn2002@163.com

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Abstract

In this study, the reaction rates of native degerminated maize, extruded degerminated maize and enzymatically extruded degerminated maize using glucoamylase were evaluated and the extrudate models were investigated. The effects of enzyme concentration, substrate pH, temperature and incubation time on the reaction rates were studied. The Lineweaver–Burk equation was used in order to obtain the parameters of the kinetics equation of catalysed hydrolysis. The results show that NDM's vm is 0.0845g/(mL·min) and km is 0.0032, EDM's vm is 0.6251g/(mL·min) and km is 0.0167, EEDM's vm is 1.897g/(mL·min) and km is 0.0240. The reaction rate of EEDM is quicker than those of NDM and EDM. The kinetics equation of EEDM is in accordance with the Michaelis–Menten equation.

Keywords: extrusion, degerminated maize, glucoamylase, enzymatic kinetics

1. Introduction

Maize is a major grain crop rich in linoleic acid, minerals and vitamins, and has a high nutritional and medicinal value. Maize has been used in many processing sectors including food processing, feed processing and deep processing, and starch syrup is a very important component of deep processing. Starch produced using the wet method of degerminated maize compared with using the dry method has many advantages, including reducing the equipment investment cost, shortening the process flow, and reducing sewage discharge and energy consumption. The combined application of extrusion and enzymatic technology is an effective method of biological and mechanical degradation, accelerating the rate of hydrolysis of amylase and improving the utilization rate of starch (Shen et al. 2010).

Enzymatic kinetics can be described as a science based on enzyme catalysis and the factors which affect it (Román et al. 2016). The parameters and equation of enzymatics kinetics can be obtained by analysing different actors such as substrate concentrations, and varying the pH, temperature and reaction time (Baks et al. 2008; Raphaelides et al. 2012). Literature regarding enzymatic kinetics has been reported (Stephen and Wang 2009) and an enzymatic kinetics model has been built by analysing the influence of the above-mentioned parameters(Gao et al. 2017). The parameters and equation of enzymatic kinetics of maize starch have been obtained by analysing the effects of different concentrations of enzymes, and varying the pH, temperature and time ofthereaction rate of β-amylase (Zhao et al. 2009).

Extrusion technology is a process involving transporting, mixing, smashing, shearing and pumping, which has the advantages of energy saving and enabling high quality and yield (Morales et al. 2015). Enzyme technology combined with extrusion activation is an effective method of the biological and mechanical degradation of starch, accelerating the reaction rate of amylase and increasing the ratio of starch. During the extrusion process, the effect of moisture, heat and mechanical shear ruptures hydrogen bonds, the crystalline structure and starch grains; hence, the enzymatic kinetics equation of extrudates needs to be investigated.

The aim of this study was to compare the reaction rate of enzymatically extruded degerminated maize with degerminated maize and extruded degerminated maize, and to build an enzymatic kinetics model using the

Lineweaver–Burk method, as the rules and a model of enzymatic kinetics can provide support for the production of starch syrup.

2. Material and Methods

2.1Materials

The raw material used in this study was degerminated maize with a 12.58% moisture content, 74.46% starch content, 7.96% protein content and 0.96% fat content, which was purchased from Tianjin Food Co., LTD. Glucoamylase, 20000u/g, was purchased from Beijing AoBoXing Biological Technology Co., LTD. Thermostable α-amylase, 40000u/mL, was purchased from Shandong LongDa Biological Engineering co., Ltd. All other chemicals used were of analytical grade.

2.2 Instruments and Equipment

Figure 1 is the single-screw extruder .It was made by Shandong University of Technology, which consisted of a modular barrel (three pieces) and screw (four pieces), with a productivity of 100 kg/h. The screw rotation speed varies from 0 to 1200 rpm, the barrel is continuously adjustable at a temperature range between 0~300℃and the extruder is equipped with an automatically controlled closed-loop digital instrumentation system. The die diameter of the extruder and clearance between the templates and screw top are adjustable.

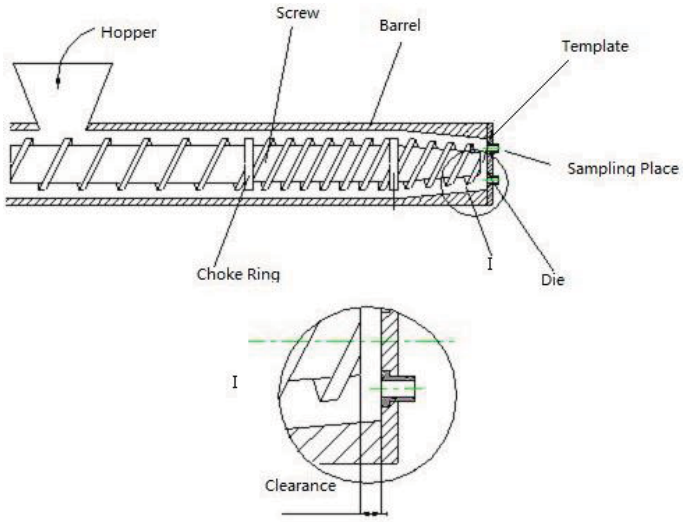

Figure 1. Schematic diagram of single-screw extruder

A UV-2102PCS ultraviolet and visible spectrophotometer (Ke Instrument Co., LTD, Shanghai, China) was used for analysis.

2.3 Preparation of Enzymatically Extruded Degerminated Maize

Degerminated maize with a moisture content was 30.0% was ground to flour and thermos tableα-amylase was added. The system parameters of the single-screw extruder are shown in Table 1.

2.4 Preparation of Liquid Glucose and Determination of Reducing Sugar Content

6.00g samples were added to 38.0mL acetic acid-sodium acetate buffer in tubes. After 10 minutes in a water bath at 55 ℃,a specific amount of glucoamylase was added and the solution was left in the water bath at 55℃ for30min. The reaction was stopped by increasing the water bath temperature to 100℃ for 10min. Finally, the solution was centrifuged at 4000rpm for 20min to separate the liquid supernatant from the reaction slurry.

The consumption volume of the sugar solution was measured and the reducing sugar concentration was determined on the basis of direct titration of GB/T5009.7-2008(Chinese national standards).

Table 1. System parameters of the single extruder

No.		Diameter of die nozzle/amount mm	Temperature at end of discharge ℃	Speed of screw r/min	Moisture content %	Thermostable α-amylase contentu/g	Remarks
1	Native degerminated maize	/	/	/	/	/	NDM
2	Extruded degerminated maize	φ12×3	60.0	110.0	30.0	0	EDM
3	Enzymatically extruded degerminated maize	φ12×3	60.0	110.0	30.0	10.0	EEDM

2.5 The Measurement of the Kinetics

Constant Enzymatic Kinetics was Analysed Using the Michaelis–MentenEquation and the Parameters of Kinetics were Obtained Using Double-Reciprocal Analysis(BP et al. 2006). Different concentrations of the three samples were taken and the reaction rates were measured. The Michaelis–Menten equation is shown below.

$$v = \frac{v_{\max}[s]}{k_m + [s]}$$

The reciprocal of the Michaelis–Menten equation is:

$$\frac{1}{v} = \frac{k_m + k_m[s]}{v_{\max}[s]}$$

The reaction rate was calculated and the reciprocal of the Michaelis–Menten equation was obtained.

2.6 Statistical Analysis All Experiments were Performedin Triplicate and Data are Expressed as Means

Statistical analysis was performed using SAS9.1, and comparisons between the reaction rate and time were performed using ANOVA; statistical significance was considered as $p<0.05$. The figures were processed by origin8.0 and Vmax and Km were calculated according to the Lineweaver–Burk plot.

3. Results and Discussion

3.1 The Solution of the Michaelis Constant and the Maximal Reaction Rate

The Lineweaver–Burk equation was used to determine the kinetic parameters and the plots obtained are presented in Fig. 2; the least square method was used to perform linear fitting(Jukić et al. 2007). The results show a linear relationship between 1/S and 1/V in the control, and the correlation coefficients of equations were above 0.9500 and equations were highly significant.

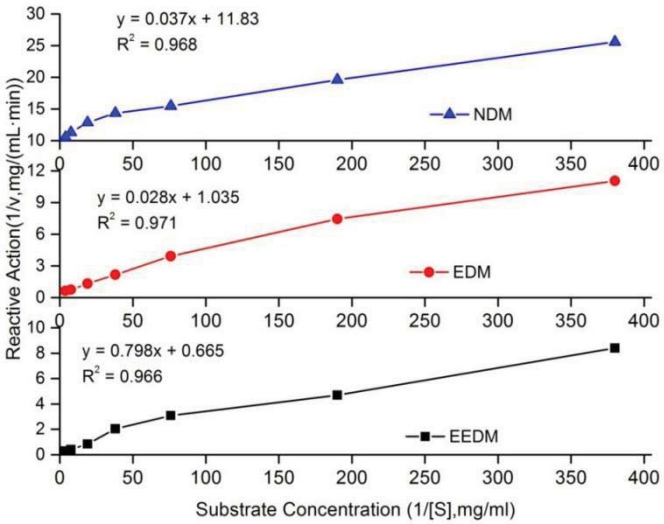

Figure 2. Lineweaver–Burk plot for the glucoamylase catalysed hydrolysis of enzymatically extruded degermed maize

The equation, coefficient of association, Vmax and Km are shown in Table 2. It can be seen that R2 is above 0.96 and there action rules of all three materials follow the Lineweaver–Burk equation and exhibited good correlation. A higher Km value indicates higher affinity and according to the value of Km, EEDM had the strongest affinity with glucoamylase and NDM had a lower affinity than the two other materials used. Enzymatic extrusion can make degerminated maize gelatinized and decrease the degree of polymerization; however, the contact area of degerminated maize and glucoamylase was increased. The advantages of using the Lineweaver–Burk graph method is that it is convenient and fast (Baks et al. 2006b), and the results are accurate. This method is governed by substrate concentration as a low concentration of substrate resulted in a low enzymatic hydrolysis rate and influenced the accurate measurements of Vmax and Km. Generally, the result was accurate when the substrate concentration was 0 to 10 mg/mL or from 0.33 to 2.0 Km (Zhang et al. 2007).

Table2.Kinetics Equation of Catalysed Hydrolysis Using the Lineweaver–Burk Plot

No.	Samples	Equations	R^2	V_{max} mg/(mL·min)	K_m (mg/mL)
1	Degerminated maize	y_1=0.0327x_1+10.4530	0.9680	0.0845	0.0032
2	Extruded degerminated maize	y_2=0.0202x_2+0.8406	0.9768	0.6251	0.0167
3	Enzymaticallyextruded degerminated maize	y_3=0.7980x_3+0.7750	0.9660	1.1897	0.0240

3.2 Effect of the Enzyme Concentration of Feed Materials on Reaction Rate

As shown in Fig. 3, the reaction rates increased with an increase in enzyme concentration. Under the conditions of sufficient substrate, higher enzyme concentrations exhibited faster reaction rates, resulting in more product. This was ascribed to the fact that the increased enzyme concentration provides more active sites, increasing the probability of an enzyme-substrate collision and subsequent reaction, leading to a higher reaction rate (Raphaelides et al. 2012).The reaction rate of NDM increased slowly upon increasing the enzyme concentration, while EEDM increased the quickest. Without squeezing, degerminated maize kept its original crystalline structure and was not easy to hydrolyse; however, extrusion gelatinized the degerminated maize and destroyed the crystalline texture, which led to an accelerated rate of hydrolysis (Zhang et al. 2015). On this basis, glucoamylase hydrolysed the α-1,4 glycosidic and α-1,6 glycosidic linkages, which led to maize producing glucose molecules exhibiting a low degree of polymerization; hence, the reaction rate of EEDM was the fastest (Han 2009). Under the conditions of unchanged temperature and pH and sufficient substrate concentration, the higher the enzyme concentration was, the faster the reaction rate (Zhao et al. 2009).

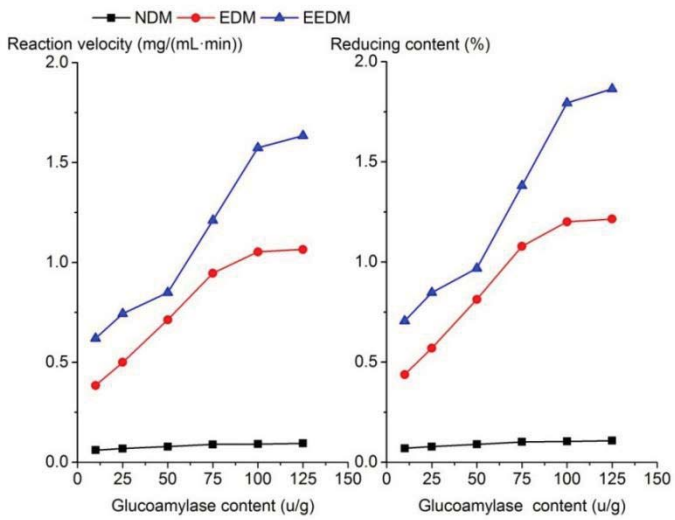

Figure 3. The effects of different substrate enzyme concentrations on reaction rates

3.3 Effect of Temperature on the Reaction Rate

As observed in Fig. 4, the optimum reaction temperature for NDM was 40℃. For the other two materials, the optimum reaction temperature was 50℃. Enzyme activity was enhanced upon increasing the temperature over a range of temperatures; however, when the reaction temperature increased up to 50℃, the reaction rate was lower, due to the fact that high temperatures can prevent gelatinization and lower enzyme activity. Studying the enzymatic kinetics of α-amylase during the saccharification process shows the degree of hydrolysis increased upon increasing the temperature, whereas the enzyme activity decreased; 60℃ was found to be a good temperature for both the degree of hydrolysis and enzyme activity (Baks et al. 2006a).

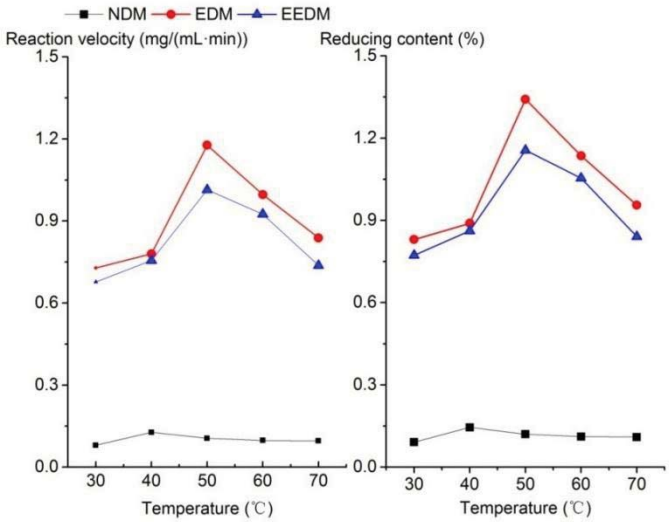

Figure 4. The effects of temperature on reaction rates

3.4 Effect of Reaction Time on Reaction Rate

Figure 5 shows the reaction rate over the reaction time. The initial hydrolysis reaction rate, at the same concentration levels, was highest in EEDM, followed by EDM, and lastly NDM. As the reaction process progressed, the reaction rate gradually slowed down, with the fifth reaction rate of three materials being the fastest. The reaction rate was slowed down by the decrease of raw material concentration and increasing time (Polakovič and Bryjak 2004).

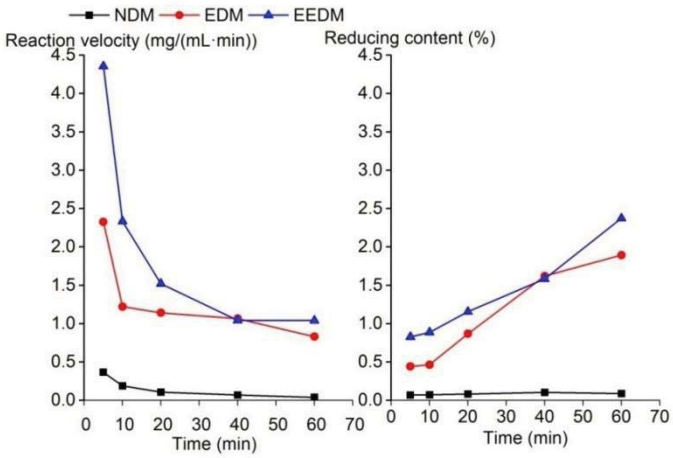

Figure 5. The effect of time on the reaction rate

4. Conclusion

These results show that the degree of hydrolysis and reaction rate of EEDM was higher than EDM and NDM. When the substrate pH was 5.0, the temperature was 50℃ and the reaction time was 5 minutes, the higher the concentration of substrate and enzyme present, the faster the reaction rate was. The Vmax and Km of NDM were 0.0845 and 0.0032, respectively; the Vmax and Km of EDM were 0.6251 and 0.0167, respectively and the Vmax and Km of EEDM were 1.1897 and 0.0240, respectively. Enzymatic kinetics of enzymatically extruded degerminated maize with glucamylase followed the basic rules of the Michaelis-Menten equation, which can be used to perform data fitting.

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Proximate Composition and Sensory Properties of Complementary Food Formulated From Malted Pre-Gelatinized Maize, Soybean and Carrot Flours

Patience C. Obinna-Echem[1], Lucretia I. Barber[1] & Confidence I. Enyi[1]

[1]Department of Food Science and Technology, Rivers State University, P.M.B 5080, Port Harcourt, Rivers State, Nigeria

Correspondence: Patience C. Obinna-Echem, Department of Food Science and Technology, Rivers State University, P.M.B 5080, Port Harcourt, Rivers State, Nigeria. E-mail: patience.obinna-echem@ust.edu.ng

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Abstract

The nutrient and sensory properties of malted pre-gelatinized maize supplemented with varying amounts of soy and carrot flour was evaluated. The blends (Malted pre-gelatinized maize flour : Soy flour : Carrot flour) in grams were: **A** (80: 20: 0), **B** (73.125: 23.125: 3.75), **C** (66.250: 26.250: 7.50), **D** (65.625: 23.125:11.25), **E** (65: 20:15), **F** (63.125: 33.125: 3.75), **G** (63.125:25.625: 11.25), **H** (60: 25: 15) and **I** (100:0:0). There were significant ($P \leq 0.5$) differences in the proximate composition of the blends. The moisture content ranged between 3.55 - 8.10%. The protein content of the samples increased ($P \leq 0.5$) with the increase in soy substitution and varied from 11.61% for the control (sample I) to 21.53% for sample F. The fat, ash and crude fibre content of the blends varied from 1.68 - 10.86, 1.45 - 2.8 and 0.20 - 4.40% respectively. The control had significantly ($P \leq 0.5$) the highest carbohydrate content of 75.61%, while it varied between 55.30 and 71.60 % for others. The energy values varied from 360.43 - 405.00 Kcal/g. The sensory scores were based on a 9-point hedonic scale, with 1 and 9 expressed as dislike extremely and like extremely. The assessors' likeness for the sensory attributes (colour, texture, taste, aroma and general acceptability) was below neither like nor dislike. This study revealed that substitution with soybeans and carrot flours increased the nutrient composition of the malted pre-gelatinized maize, soybean and carrot flour blends. Particularly the soy flour as sample F with the highest soy flour substitution (33. 123g) had significantly the highest protein (21.53%), fat (10.86%) and energy (405 Kcal/g) values. This would be recommended for good quality porridge. Although, the sensory analysis revealed the need for further investigation on processing methods especially the malting process as to enhance the overall acceptability of the product.

Keywords: complementary food, malted pre-gelatinized maize, carrot soybean flour, proximate composition, sensory analysis

1. Introduction

Majority of maize products in developing countries are for human consumption while in the developed world it is mainly used for industrial purposes and animal feed (FAO, 2012). Maize (*Zea mays L., Poaceae*) is the most important cereal in the world after wheat and rice with regard to cultivation areas and total production. It has an average chemical composition of 10.3% protein, 60.5% starch, 1.2% sugar, 2.5% crude fibre and other substances (Addo-Quaye, Darkwa & Ampiah, 2011). It also contains high level of dietary fiber (12.19%) but low in 'trace minerals and ascorbate (Hornick & Weiss, 2011). Maize protein content varies in common varieties from about 8 to 11% of the kernel weight (FAO, 2014). The protein is relatively fair in the sulphur containing amino acids, methionine and cystine but low in lysine and very low in tryptophan (Okoh, 2014).

Maize is prepared and consumed in a variety of ways and its economic value is increased through the development of technologies which process it into value added products and thus promoting its production and consumption. Fermentation of maize has some advantages. It helps in the introduction of probiotic bacteria so by consuming fermented foods, beneficial bacteria and enzymes are being added to overall intestinal flora for important health benefits (Kalui, Mathara & Kutima, 2010). The breakdown of some of the sugars and starches in food during fermentation makes for easy digestibility of fermented foods (Elyas, El Tinay, Yousif & Elsheikh,

2002; Lei, Friis &Michaelsen, 2006). Other advantages of fermentation include the increase in availability of vitamins and minerals and the removal of some natural compounds that interfere with the absorption of nutrients (Towo, Matuschek & Svanberg, 2006).

One of the principal ways of using maize is by fermenting it into ogi/akamu (a cereal gruel that is often used as complementary food). Maize starch is also used for products such as custard (vanilla flavoured corn starch) which is often given as complementary food to infants. The disadvantage of ogi is that the traditional production process has been implicated in nutrient losses (Obinna-Echem, Kuri & Beal 2014). On the other hand, germination (malting or sproulting) is a simple and traditional method that can be used at home. It involves hydrating and holding the grains at ambient temperature to sprout. During germination, both endogenous and newly synthesized enzymes begin to modify seed constituents, starch, protein and fat are hydrolysied by amylolytic, proteolytic and lipolytic enzymes, respectively (Katina, Liukkonen, Kaukovirta-Norja, Adlercreutz, Heinonen, Lampi, Pihlava, & Poutanen, 2007). This non-thermal process has the advantages of: reduction of anti-nutritional factors, including phenolic compounds, phytic acid, trypsin inhibitors and oligosaccharides **(**Shimelis & Rakshit, 2007), enhancement of organoleptic qualities due to a softening of texture and an increase in the flavour of various cereals (Subba Rao & Muralikrishna, 2002), improvement of the functional properties (water absorption, foaming and gelation) of rice flour (Moongngarm, Moontree, Deedpinrum, & Padton, 2014), generation of bioactive components such as riboflavin, thiamine, biotin, pantothenic acid, niacin, vitamin C, tocopherols and phenolic compounds, and also increase their availability (Moongngarm & Saetung, 2010) increase in the contents of free amino acids and the improvement of protein digestibility (Afify, El-Beltagi, Abd El-Salam & Omran, 2012).

Soybean (*Glycine max*) is a legume species native to East Asia, widely grown for its edible bean which has numerous uses (Anders, 2013). Soy beans have a relatively low content of carbohydrates and a relatively high content of proteins, and furthermore, contain a number of health promoting compounds. Together, soybean oil and protein content account for about 60% of the dry beans by weight (protein at 40% and oil at 20%). The remainder consists of 35% carbohydrate and about 5% ash. Soybean consists of approximately 8% seed coat or hull, 90% cotyledons and 2% hypocotyl axis or germ (Anders, 2013).

Carrot (*Dacus carota*) is the most important crop of *Apiaceae* family. It is a root vegetable that has worldwide distribution. They were first used for medical purposes and gradually used as food. Written records in Europe indicated that they were cultivated prior to the tenth century. The colours of carrot flesh may be white, yellow, orange, red, purple, or very dark purple (Joao, 2014). They are an excellent source of antioxidant compounds, and the richest vegetable source of the pro-vitamin A carotenes. Carrots' antioxidant compounds help protect against cardiovascular disease and cancer and also promote good vision, especially night vision. They have a unique combination of three flavonoids: kaempferol, quercetin and luteolin (Lila, 2004). They are also rich in other phenols (Gonçalves, Pinheiro, Abreu &Silva, 2010).

Complementary foods are foods other than breast milk or infant formula (liquids, semisolids, and solids) introduced to an infant to provide nutrients (USDA, 2009). Complementary foods are mostly produced from food which include, cereals, such as wheat, maize and rice, roots, tuber, legumes such as soybeans, cowpeas etc. Formulation of complementary foods can be made by using one or combination of more than one plant product (cereal such as maize, millet and sorghum combined with legume such as cowpea, groundnut, soybeans etc) (Akpapunam & Dedeh, 1995).

Complementary foods are expected to be high in energy density, containing all essential amino acids, required vitamins and minerals and safe level of antinutritional components while retaining the qualities for palatability (Abeshu, Lelisa, & Geleta, 2016). Animal-source foods such as milk are important for complementary feeding as they provide high quality protein, bioavailable micronutrients, and have low levels of anti-nutrients and fibre. However, these are unaffordable for majority of the population in sub-Saharan African countries like Nigeria. Most nursing mothers use local alternatives to milk which include cereals such as maize or millet in combination with legumes such as soybean and groundnut. Cereals that are commonly used in the preparation of infant complementary food are limiting in lysine and methionine which can be supplemented by the addition of legume such as soybean. The gelatinization of starch is associated with the disruption of granular structure causing starch molecules to dissolve in water and many food products contain partially cooked starch granules that contribute to their functional and structural properties (Ratnayake & Jackson, 2006). Pre-gelatinization will result in partially cooked starch which can enhance the functional quality of the complementary food. Germination with its earlier stated advantages can also enhance the quality of complementary foods. Hence adequate processing and judicious blending of locally available foods could result in improved intake of nutrients. This studied was therefore, aimed at the production and evaluation of the proximate and sensory properties of complementary food from

pre-gelatinised malted maize, soy and carrot.

2. Method

2.1 Preparation of Malted Pre-Gelatinized Maize, Soybean and Carrot Flour

Yellow variety of maize (*Zea mays*), soybean (*Glycine max)* and carrot (*Dacus carota*) were bought from a local market (Mile 3) in Diobu, Port Harcourt, Rivers State, Nigeria.

Five kilograms of sorted and washed maize were spread on jute bags and allowed to sprout at room temperature. The sprouts were removed, the grains washed and boiled with 4 litres of water until tender and easily crushed between the thumb and index finger. Excess water was drained out of the boiled maize and the malted pre-gellatinized grains were dried in a hot oven at 60^0C overnight. The dried grains were milled with a hammer mill and sieved to obtain the malted pre-gellatinized maize flour (M). The flour was packaged in a transparent plastic container with lid and stored in the deep freezer until required for use.

The soy and carrot flour were prepared as described by Barber, Obinna-Echem & Ogburia (2017). Briefly, 3kg of sorted and washed soybeans were blanched at 85^{o}C for 2 minutes, and then soaked in 6 L of water for 24 h with a change of water after every 6 h. The soybean seed testae were removed, the seeds washed and dried in a cabinet at 50^{o}C for 24 h. The product was milled with hammer mill and sieved to obtain the fermented soy flour (S). Fresh carrots were washed and the outer layers scraped. About 3 kg of the carrot were grated, dried at 50^{o}C for 8 h and blended with hammer mill to obtain carrot flour (C). The prepared flours were packaged separately in well labelled plastic containers and preserved in a deep freezer until required for use.

2.2 Recipe Formulation for the Malted Pre-Gelatinized Maize, Soybean and Carrot (MSC) Complementary Food

The malted and pre-gelatinized maize flour was supplemented with different proportions of the soybeans and carrot flour as shown in Table 1. To obtained a homogenous flour, the different combinations were individually homogenized in a rotary mixer (Philips, type HR 1500/A, Holland), and then stored in airtight plastic containers and preserved in a deep freezer until required for analyses. Malted and pre-gelatinized maize flour without any substitution served control.

Table 1. Recipe (in grams) for the formulation of malted and pre-gelatinized maize-soybean-carrot complementary food

Sample ID	Malted and pre-gelatinized maize flour (M)	Levels of Substitution	
		Soybean Flour (S)	Carrot Flour (C)
A	80.000	20.000	-
B	73.125	23.125	3.750
C	66.250	26.250	7.500
D	65.625	23.125	11.25
E	65.000	20.000	15.000
F	63.125	33.125	3.750
G	63.125	25.625	11.250
H	60.000	25.000	15.000
I	100	0	0

2.3 Proximate Analysis

Proximate analyses were carried out on the samples using standard AOAC (2005) methods. Moisture content was calculated after drying at 105^{o}C to constant weight in an air oven (Thermo Scientific-UT 6200, Germany). Lipids were estimated by exhaustive extraction of known weight of samples with petroleum ether using rapid Soxhlet extraction apparatus (Gerhardt Soxtherm SE- 416, Germany). Determination of protein was by Kjeldahl method. The efficiency of the nitrogen values were corrected with acetanilide values and multiplied by the factor of 6.25 to obtain the protein value. Ash was determined gravimetrically after incineration in a muffle furnace (Carbolite AAF-11/18, UK) for 24 h at 550^{o}C. Crude fibre was obtained by difference after the incineration of the ash-less filter paper containing the insoluble materials from the hydrolysis and washing of moisture free defatted sample (0.5 g). Carbohydrate content was determined by the difference: 100% - (% MC + % Ash + % Crude protein + % Fat + % Crude fibre). Energy (Kcal/g) was calculated using the Atwater factor of 4.0 Kcal/g for protein and carbohydrate and 9 Kcal/g for fat.

2.4 Sensory Analysis of the Malted and Pre-Gelatinized Maize, Soybean and Carrot (MSC) Complementary Porridge

Each of the various blends as shown in Table 1 was mixed with 200 mL of cold water to make slurry. Then equal part of boiling water was added to the slurry with continuous stirring to obtain the Malted and pre-gelatinized Maize-Soybean-Carrot (MSC) porridge. Sensory properties (colour, mouth-feel, taste, texture and overall acceptability) of the porridge pap from different soybean and carrot substitutions were carried out using a panel of 20 assessors consisting of nursing mothers, staff and students of the Department of Food Science and Technology, River State University, Port Harcourt. The assessors are regular consumers of fermented maize porridges. Mothers were preferred to infants as the mothers are the ones that make choices of what complementary food to feed their infants with. The aroma, colour, taste, texture and overall acceptability of the samples were evaluated in sensory evaluation boots where coded porridge samples were presented in random order with a ballot sheet for each sample. The scores were based on a 9 - point hedonic scale, with the degree of likeness of the product attribute expressed as: 1 - dislike extremely, 2 - dislike very much, 3 - dislike moderately, 4 - dislike slightly, 5 - neither like nor dislike, 6 - like slightly, 7 - like moderately, 8 - like very much and 9 - like extremely. Assessors were instructed to score colour first and water was provided for rinsing the mouth. Expectoration cups with lids were provided for panelists who did not wish to swallow the samples.

2.6 Statistical Analysis

Results were analyzed statistically by analysis of variance and difference between means separated using the Least Significance Difference (LSD) procedure. The non-parametric Friedman test and 2-sample t-test were employed in determining the statistical differences among the product sensory attributes.

3. Results and Discussion

3.1 Proximate Composition of Maize, Soybean and Carrot (MSC) Complementary food

The proximate compositions of the porridges are shown in Table 2. The moisture content of the samples varied significantly ($P \leq 0.5$) from 8.10 for sample H to 3.55 % for Sample I (Control without soybean and carrot). There was increase in the moisture content with the addition of soy and carrot flour. The moisture contents of the samples were within the acceptable limit of not more than 10% for long term storage of flour. Low moisture content would prevent the growth of mould and reduce moisture dependant biochemical reactions (Onimawo & Akubor, 2012).

The protein content of the samples increased ($P \leq 0.5$) with the increase in soy and carrot substitution. Sample F had significantly ($P \leq 0.5$) the highest protein content of 21.53% while the control had significantly ($P \leq 0.5$) the lowest protein content of 11.61 %. The increase in protein could be attributed to the soy flour. Maize is limiting in lysine and tryptophan. It is expected that the amino acid of soybean will complement that of cereal flour. The protein is important for tissue replacement, disposition of lean body mass and growth. Fat is important in the diets of infants and young children as it provides essential fatty acids, facilitates absorption of fat soluble vitamins, enhances dietary energy density and sensory qualities and the prevention of undesirable weight gain in infants. The fat content of the blends ranged from 10.86 to 1.68 % for Sample F and A respectively. Fat is important in the diet of infant and young children because it provides essential fatty acid, facilitates absorption of fat soluble vitamin, enhance dietary energy, density and sensory quality (FAO 2001). It has been recommended that, during the complementary feeding period (6 – 12 months) a child's diet should derive 30 – 40% of energy from fat (Michaelsen Weaver, Branca, & Robertson, 2000). According to a joint WHO/FAO/UNU The energy requirements for a 6-month-old female involved in moderate physical activity is 340 kJ kg^{-1} body weight (WHO, 2007), an infant weighing 7.34 kg would 2495 kJ of energy daily. The fat composition of this complementary food will only meet 2.5 – 16.4% of the energy requirement. This however can be enhanced with available oil to increase the recommended fat ratio. The samples had varying ash content from 2.8 – 1.45 % for sample C and G respectively. The general trend was that of an increase with increase in soy flour substitution. Ash is an indication of availability of minerals.

The crude fibre content was low and ranged between 4.40 and 0.20 for sample F and B respectively. This range is within the fibre content reported for maize, soy bean, and carrot (Arawande & Borokini, 2010; Butt & Batool 2010). Fibre plays a role in the increased the utilization of nitrogen and absorption of some other micronutrients. The low fibre content is in agreement with the report that food used for complementary feeding should contain low fibre as high fibre can lead to high water absorption and displacement of nutrient and energy needed for the growth of children less than two years (Klim, Isaac & Joseph 2001; Michaelsen, Weaver, Branca & Robertson, 2000).

The carbohydrate content of the samples varied significantly ($P \leq 0.5$). The control (100% maize flour) had significantly ($P \leq 0.5$) the highest carbohydrate content of 75.61%. The carbohydrate content for the malted and pre-gelatinized maize, soybean and carrot flour blends ranged between 55.30 and 71.60% for sample B and sample I, respectively. Maize is mostly a carbohydrate food hence it was out of place for the unsubstituded sample to have high carbohydrate content compared to others. The energy value in the formulation varied ($P \leq 0.5$) due to variation in protein and fat contents of the samples. The values varied between360.43 – 405.00 Kcal/g.

Table 2. Proximate Composition (%) of malted and pre-gelatinized maize, soy and carrot flour blend

Samples	Moisture	Ash	Fat	Protein*	Crude Fibre	Carbohydrate	Energy (kcal/g)
A	6.85±0.10^{b}	1.90±0.50^{ab}	1.68±0.50^{f}	19.15±0.05	3.24±0.10^{b}	67.19±1.04^{c}	360.43±0.50^{d}
B	5.77±0.13^{c}	2.59±0.90^{ab}	4.93±0.19^{de}	15.48±0.00	0.20±0.00^{e}	71.06±0.84^{b}	390.44±5.01^{b}
C	6.00±0.00^{c}	2.80±1.10^{a}	8.13±0.33^{b}	18.29±0.05	2.40±0.40^{c}	62.39±1.23^{d}	395.83±7.63^{a}
D	6.97±0.38^{b}	2.35±0.05^{ab}	5.22±0.68^{cde}	19.54±0.01	3.28±0.14^{b}	62.66±0.88^{d}	375.71±2.61^{c}
E	6.12±0.12^{bc}	2.15±0.25^{ab}	2.55±0.00^{f}	17.26±0.02	1.40±0.01^{d}	70.53±0.38^{b}	374.10±1.40^{c}
F	6.12±0.12^{bc}	1.80±0.00^{ab}	10.86±1.77^{a}	21.53±0.00	4.40±0.15^{a}	55.30±2.03^{e}	405.00±7.76^{a}
G	7.48±0.62^{a}	1.45±0.06^{b}	7.34±0.07^{bc}	17.83±0.00	1.48±0.29^{d}	64.44±0.34^{cd}	395.07±0.77^{ab}
H	8.10±0.00^{a}	1.99±0.10^{ab}	6.60±0.51^{bcd}	18.01±0.01	1.08±0.10^{d}	64.23±0.50^{d}	388.28±2.53^{b}
I	3.55±0.05^{d}	2.40±0.10^{ab}	3.67±0.49^{ef}	11.61±0.00	3.17±0.19^{b}	75.61±0.63^{a}	381.85±1.86^{bc}

Values with same superscript in the same column do not differ significantly ($p \leq 0.05$). N=3 ±SD.

*Protein content of the samples varied significantly ($p \leq 0.05$).

Blend (M:S:C); M = Malted pre-gelatinized maize flour, S = Soy flour, C = Carrot flour. **A** (80: 20: 0), **B** (73.125: 23.125: 3.75), **C** (66.250: 26.250: 7.50), **D** (65.625: 23.125:11.25), **E** (65: 20:15), **F** (63.125: 33.125: 3.75), **G** (63.125:25.625: 11.25), **H** (60: 25: 15) and **I** (100:0:0)

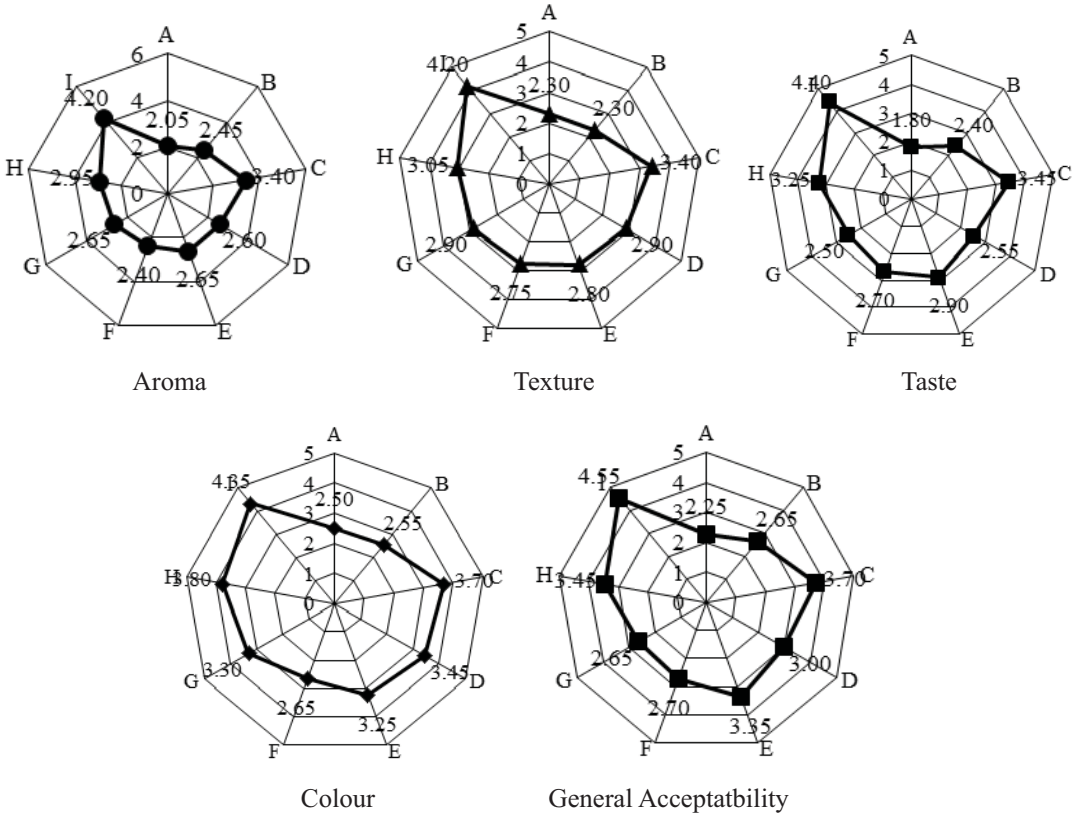

Figure 1. Sensory analysis of porridges from malted and pre-gelatinized maize, soy and carrot blend

Blend (M:S:C); M = Malted pre-gelatinized maize flour, S = Soy flour, C = Carrot flour

A (80: 20: 0), **B** (73.125: 23.125: 3.75), **C** (66.250: 26.250: 7.50), **D** (65.625: 23.125:11.25), **E** (65: 20:15), **F** (63.125: 33.125: 3.75), **G** (63.125:25.625: 11.25), **H** (60: 25: 15) and **I** (100:0:0).

Generally, the substitution with soybeans and carrot flours increased the nutrient composition of the malted pre-gelatinized maize, soybean and carrot flour blends. Particularly the soy flour as sample F with the highest soy flour substitution (33. 123g) had significantly ($P \leq 0.5$) the highest protein (21.53%), fat (10.86%) and energy (405 Kcal/g) values. According to a Joint WHO/FAO/UNU Expert Consultation report (WHO, 2007) the daily protein and energy requirements for a 6-month-old female involved in moderate physical activity are 1.12 g kg^{-1} and 340 kJ kg^{-1} body weight, respectively; therefore an infant weighing 7.34 kg would require 8.2 g of protein and 2495 kJ of energy daily. The consumption of 100 g of the porridges would meet more than 100% (189 - 263%) of the daily protein requirement, while the energy requirement would be met with the consumption of 147 - 166 g of the porridge.

3.2 Sensory Evaluation

There were significant differences ($P \leq 0.5$) among the sample in colour, texture, taste, aroma and general acceptability (Figure. 1). The control sample without any substitution had significantly ($P \leq 0.5$) the highest score (≥4.20) for all the attributes, while sample A had the least (≤1.80). The assessors' range of likeness for all the attributes were within dislike very much and neither like nor dislike. This could be attributed to the malting effect of the maize. The colour of the porridges may also have been affected by the addition of carrot. Although colour is less important for babies, mothers would play a vital role for any complementary food to be successfully utilized and accepted. The porridge from Sample F had a beany flavor as described by the panellists. This sample F had the highest soy flour substitution and the flavour could be attributable to the beany flavor of soybean. On the basis of nutrient composition, sample F could be recommended, while the sensory properties particularly the taste and flavour can be improved with the addition of sugar.

4. Conclusion

Maize, soybean and carrot are locally available and affordable raw materials that can be used by mothers as home-based complementary foods. There was increase in the nutrient content of the blends formulated in this study, particularly sample F with the highest soy flour substitution (33. 123g) that had significantly the highest protein (21.53%), fat (10.86%) and energy (405 Kcal/g) values. This would be recommended for good quality porridge. The sensory analysis revealed the need for further investigation as to enhance the overall acceptability of the product.

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Nutrient and Anti-nutrient Profile of a Local Formula from Sorghum, Peanut, Honey and Ghee (Metu2) for Treatment of Severe Acute Malnutrition

Amegovu K. Andrew[1], Peter Yiga[2], Kuorwel K. Kuorwel[3] & Timothy Chewere[4]

[1]Department of Food Technology, College of Applied and Industrial Sciences, University of Juba, P. O. Box 82, Juba, Republic of South Sudan

[2]Faculty of Science, Department of Food Technology, Kyambogo University, P.O. Box 1, Kampala, Uganda

[3]Department of Chemistry, College of Applied and Industrial Sciences, University of Juba, 82, Juba, Republic of South Sudan

[4]Andre Foods International(AFSS), P.O. Box 830 Entebbe, Uganda

Correspondence: Amegovu K. Andrew, Department of Food Technology, College of Applied and Industrial Sciences, University of Juba, 82, Juba, Republic of South Sudan. E-mail: kiri_andrew@yahoo.com

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Abstract

World over, we are still struggling with persistent acute malnutrition levels; an estimated 17 million preschool children suffer from SAM, roughly the same figures as reported in 2013, a trend depicting insufficient progress towards the 2025 World Health Assembly. One such affected area is Karamoja Region in North Eastern Uganda. Partly, the trend could be attributed to unsustainable interventions like RUTF. Formulas from locally available foods could provide not only an affordable but also a culturally acceptable and effective home based solution. Locally available sorghum, peanut, honey and ghee in North Eastern Uganda, is such a potential local formula. The nutritional and anti-nutritional profile of this local formula(metu2) was compared to plumpy-nut. Standard official analytical methods were used. Proximate composition was comparable and within the WHO recommendations for therapeutic formulas. Local formula(metu2) had a comparatively high energy content, 528kcal/100g to 509kcal in plumpynut. Vitamin A and K contents were below the WHO recommendations in local formula while Na, Mg and essential fatty acids were comparable and within the contents needed for SAM recovery. Zn was comparatively higher in plumpy-nut but levels in both formulas were below the recommendations. Trypsin inhibitors, phytates and condensed tannins were higher in local formula while aflatoxins were within the limits but not for plumpynut. Though lacking in critical K, Zn and Vitamin A, local formula(metu2) was comparable to plumpy-nut and its efficacy to sustain recovery from SAM needs to be studied.

Keywords: local formula(metu2) (sorghum, peanut, honey & ghee), plumpy-nut, severe acute malnutrition (SAM).

1. Introduction

Malnutrition is still among the main public health challenges of the 21st century despite copious advances and improvements in child health over the last decade (Black et al., 2013). A sizable wide-reaching burden of wasting exists, particularly severe acute malnutrition (SAM; weight-for-height *Z* score [WHZ] <–3). World over, an estimated 52 million preschool children are wasted of whom; 17 million suffer from SAM (UNICEF, World Health Organization [WHO] & World Bank Group, 2017) roughly the same figures as reported in 2013, a trend depicting insufficient progress towards the 2025 World Health Assembly target. SAM presents with an amplified risk of mortality and morbidity. An estimated 400,000 child deaths are attributed to SAM annually; i.e. the risk of death is about 10-fold greater compared to children with a z-score ≥ – 1(Black et al., 2013). Deaths from under nutrition are comparable to those resulting from infectious diseases (Black et al., 2013; Bryce, Boschi-Pinto, Shibuya, Black, & Group, 2005). Developing Sub Saharan Africa remains the most affected (Food Agricultural Organization [FAO], 2014). In majority of Sub Saharan Countries; South Sudan, Uganda, Kenya, Burundi and Malawi levels are consistently above the 10% emergency levels year in year out.

Management regimens for SAM have been available for some time, and programmatic evidence shows that they have been largely effective (WHO, 2013). For treatment of uncomplicated SAM cases (without need for stabilization, infections and fluid management), community based models using ready to use therapeutic foods (RUTF) are being applied (WHO, 2013) and programmatic evidence shows that they are as effective as the standard care (Bhutta et al., 2013). This along with the cost effectiveness, and wide reach, has made community based models to grow rapidly globally (Bhutta et al., 2013). Though largely effective, RUTF present with a number of challenges in developing country contexts. Ordinarily, RUTFs are expensive, not familiar to the local beneficiaries, making them un-sustainable in low income countries with recurrent under-nutrition. For example, a package of plumpy'nut costs US 6 cents, translating in to US$ 60 for a full two-month treatment of a child (Latham, Jonsson, Sterken, & Kent, 2011). Besides, costs associated with delivery and distributions are not included in the above estimate and these could push the figure further up. Program implementation is also affected by pipeline breakdowns. The set up franchises in strategic countries have done little to bring down the cost as some ingredients and packaging materials used are imported. For example in Malawi where RUTF has been produced using the same ingredients as in Plumpy'nut formulation, the milk powder cost constituted more than half of the final RUTF cost (Collins & Yates, 2003). Thus, without United Nations agencies and other International Non-governmental Organizations who are currently footing these costs, parents of affected children cannot afford RUTF's.

To address these challenges in Sub Saharan African Countries, community based solutions; where such therapeutic foods can be made in the community or at home using locally available foods are needed. This strategy could save costs, reduce child mortality, address sustainability gaps and improve efforts to effectively manage cases at community level. A number of studies, largely in Asian contexts have tested and found positive results as far as this strategy is in managing acute malnutrition. For example, for decades, Indian hospitals have successfully used local foods to come up with formulations to treat SAM (Latham et al., 2011). A topical systematic review by Schoonees, Lombard, Musekiwa, Nel, and Volmink (2013) did not find variances in clinical outcomes between SAM children treated using home-based RUTF and standard diet, concluding that either RUTF or flour porridge can be used depending on availability, affordability and practicality. According to Collins et al. (2006), basic ingredients for RUTF production are; a staple food preferably a cereal, a protein source; plant or animal based, vitamin and mineral mix and an energy enhancer to increase energy density. Thus, with locally available foods, communities have the capabilities to produce their own RUTF. Though evidently possible, few studies have started potential of local formulations to counter SAM in Sub-Saharan African contexts. Therefore, in this study, we formulated a diet (METU-2) from locally available foods in North Eastern Uganda; sorghum, peanut, ghee and honey and its nutrient and anti-nutrient profile was compared to that of plumpy'nut.

2. Materials and Methods

2.1 Development of Local Food Product(metu2)

Formulation; Nutri-survey software, employing linear programming, was used for formulation of the local product, METU-2 (Erhardt, 2004). The composition (quantity of each food component) of the formulation was based on WHO/UNICEF/WFP/SCN draft specifications for therapeutic foods. METU-2 contained 39% sorghum, 35% peanut, 13% honey and 13% ghee.

Preparation/Processing of ingredients; raw materials for the formulation of metu2 were locally procured from North Eastern Uganda. Sorghum, groundnuts, ghee and honey were obtained from Moroto district, Karamoja region. Sorghum and groundnuts were sun dried for five days to moisture levels below 10%. Low density material, particularly leaf, damaged kernels and stalk in sorghum were removed by winnowing. Dirt free sorghum was then milled into flour. Groundnuts were hand sorted to remove damaged kernels, foreign matter and the shriveled kernels. The groundnuts were then dry roasted to a white roast for 30 minutes using a charcoal stove before grinding to a paste. Milk from Karamajong Zebu cows was traditionally processed by fermenting it for three days in pots. Fermented milk was thereafter churned in a jerry can by hand until fat globules accumulated on top. Accumulated fat globules (ghee) were then scooped off, washed to remove the whey, and then matured for one week to develop the flavor. The ghee was then boiled using a charcoal stove for 30 minutes to remove impurities.

Plumpynut: Plumpy nut, presently used by UNICEF to treat SAM in Moroto District, Karamoja Sub-Region was used as a comparator. This Plumpy nut is manufactured by Fabrique par: JB. 24, rue Radama 1 er, BP207, Antananarivo, Madagascar.

2.2 Proximate Composition Determination

Determination of Crude Fat

Fat was determined using AOAC (2000) method number 920.39. This involved using a soxhlet apparatus to extract the fat from the dried sample (3.00g) using 60ml of petroleum ether as the extraction solvent. The percentage fat was then obtained as the ratio of the extracted fat to the original sample weight.

Determination of protein

The amount of protein was determined using Kjeldahl's method according to AOAC (2000) method number 984.13. Samples were weighed (1g) and digested in concentrated sulphuric acid with one Kjeldahl tablet followed by distillation in 40% sodium hydroxide. The resulting solution was titrated with 0.1N hydrochloric acid using a mixed indicator (methyl red and bromocresol green).

Determination of moisture

Moisture was determined using the oven drying method as described by AOAC (2000) method number 925.40. Samples were weighed (5g) in dry petri-dishes and heated in an electric oven at 105^{o}C for 5 hours. Dried samples were cooled in desiccators, and the weight taken. The difference in weight was then obtained.

Determination of crude fibre

Crude fiber was determined according to the ISO (2000) method number 6865. Samples were weighed (1g) and transferred to crucibles. Petroleum ether (30ml) was added and mixture filtered under vacuum. The residue was dried and quantitatively transferred to a beaker. Sulphuric acid (150 mL) was added and the mixture boiled under reflux for 30 minutes. The solutions were quickly filtered under suction and residues washed thoroughly with water until acid free. Residues were transferred back to beakers, to which 150mL of KOH was added and solution boiled under reflux for 30 minutes and quickly filtered under suction. Residues were washed with hot water until the rinsing was neutral. Residues were then thrice washed in acetone and transferred to crucibles. They were dried to a constant weight in an oven at 105^{o}C for 4 hours, cooled in a dessicator and weighed. Samples were then incinerated at 550^{o}C for 2 hours, cooled in dessicator and reweighed. Percentage crude fiber was then computed.

Determination of ash

Ash content was determined according to AOAC (1999) method number 972.15. Samples were weighed (5g) in dry crucibles, carbonized on a hotplate, and heated in a muffle furnace at 550^{o}C for 6 hours. Ash content was determined after cooling samples in the desiccators to ambient temperature.

Determination of carbohydrate and energy

Carbohydrate was determined by difference in ash, moisture, fat, crude fiber and protein while energy was calculated using assessed proximate composition and the corresponding Atwater factors.

Determination of mineral content

Mineral content was analysed using an atomic absorption spectroscopy as described by AOAC (2005b) method number 975.03. Samples (2 g) were digested with concentrated nitric acid and hydrogen peroxide. magnesium (Mg), iron (Fe), copper (Cu), zinc (Zn), sodium (Na) and potassium (K) were determined at wavelengths 317.9 nm, 285.2 nm, 259.9 nm, 324.7 nm, 213.9 nm, 589.6 nm, and 766.5 nm, respectively, using an air-acetylene flame. Sodium chloride and potassium chloride were used as standards for determination of Na and K. Standard solutions of magnesium oxide and ferrous ammonium sulphate were used for determining concentrations of Mg, Ca and Fe.

Determination of Vitamin A

Vitamin A was determined according to AOAC (2001) method number 2001.13. Samples were weighed (10g) in 250mL amber glass flat bottom round flasks. Ascorbic acid (0.5g) and ethanol (50mL) were added to the sample. A 0.5M sodium sulphite solution (4 mL) and KOH solution (10 mL) were added. Samples were saponified by boiling solution under reflux for 30 minutes. After hydrolysis, distilled water was added (20mL). Solutions were cooled to ambient temperature under a stream of cold water. Samples were transferred to 250 mL separation funnels. Flasks were rinsed with distilled water (10 mL) and diethyl ether (50 mL). Funnels were swirled and left to stand to allow phases to separate. Bottom layer was collected in a flask and diethyl ether phase transferred to a separation funnel. This extraction step with diethyl ether was done three times. Diethyl ether extracts were washed with distilled water (50mL) by inverting the funnel 5 times without shaking to avoid emulsions from forming. Diethyl ether extracts were drained in a clean flask by filtering over anhydrous sodium sulphate followed by rinsing of filter paper with diethyl ether (20mL). Sample extracts were concentrated by drying in

rotavapor at 40°C and then dissolved in n-hexane (10mL). Extracts were analyzed using thin layer chromatography (TLC Silica Gel F254 plate). Retinol ester was used for preparing reference solution. The reference solution contained 0.01 mg retinol/µl ester (3.3 International Units (IU) from each ester/µl) in cyclohexane. A mobile phase was a mixture of ether and cyclohexane (20:80 V/V) stabilized with 1 g/l solution of butylhydroxytoluene. About 3 µl of each solution was spotted on the plate. Spots were examined in ultraviolet light at 254nm. A principal spot from test sample was confirmed by corresponding with that of retinol in the chromatogram of reference solution.

Determination of fatty acids

Fatty acids profile was determined using gas chromatography (GC) (PerkinElmer, Norwalk, USA) in accordance with AOCS (1998) method number Ce 1b-89. Samples (10g) were mixed with chloroform (100mL) for 2 minutes with the Ultra-Turrax followed by centrifuging at 2000 rpm for 5 minutes. The mixture was filtered over a filter paper with anhydrous sodium sulphate and evaporated (20mL) under a stream of nitrogen at 40°C. Fat (0.5g) was dissolved in diethyl ether (2mL). A mixture of potassium hydroxide (KOH) in methanol (MeOH) solution (0.5 mL) was added to the dissolved fat solution. To the soap solution, water (2mL) and hexane (15 mL) were added. The mixture was shaken and left to stand to allow phases to separate after which the top layer was decanted. The mixture was washed four times with water (2mL) to remove residual hexane. Samples were dried using anhydrous sodium sulphate. Dried samples were transferred to a GC-auto sampler vial. Samples and standards were run on the GC.

Determination of phytates

Phytates content was determined using the Anion-Exchange method as enlisted by AOAC (2000) method number 986.11. Phosphate was used as a standard. Samples were weighed (2g) and transferred to Erlenmeyer flasks to which 2.4% HCL (40mL) was added. The mixture was homogenized for 3 hours. Columns were prepared by adding resin (0.5g) into the columns. After forming, resin beds were washed with 0.7M NaCL and distilled water. Homogenized samples were filtered and the filtrate (2mL) transferred to 25mL volumetric flasks. The Na_2EDTA-NaOH reagent (2mL) was added and the solution diluted to volume with water. The solution was mixed and transferred to the column and the eluate discarded. Water (15mL) and 0.1M NaCL (15mL) were eluted through column and eluate discarded. A 0.7M NaCL (15mL) was eluted through the column and eluate collected in digestion flasks. A mixture of concentrated H_2SO_4 acid (1mL) and HNO_3 acid (6mL) were added to the flasks, and digested until active boiling ceased. After cooling, water (10mL) was added; flasks swirled and heated at low temperature for 10 minutes to dissolve the salt. The cooled solution was transferred to a volumetric flask (50mL), molybdate solution (4mL) and solfunic acid (2mL) were added. The solution was diluted to volume with water, left to stand for 15 minutes and absorbance read at 880nm using atomic absorption spectrophotometer (PerkinElmer, Norwalk, CT, USA).

Determination of condensed tannins

Tannins were determined according to Vanillin-HCL method (Broadhurst and Jones, 1978) using atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT, USA). A standard curve was prepared using catechin (Sigma-Aldrich Chemical, St. Louis, MO, USA). Samples (0.2 g) were mixed with 70% acetone (10 ml). The tubes containing dissolved sample in acetone were homogenized in a bath containing ice and water for 10 min and then centrifuged at 3800rpm at 4°C for 30 min. The supernatant (original extract) was transferred into another tube without disturbing the residue, kept on ice and away from sunlight. The original extract (0.05 ml) was transferred into tubes and made up to 0.25 ml with 50% methanol. Vanillin (1.5 ml) and concentrated HCL (0.75 ml) were added. The tubes were homogenized and incubated at room temperature for 10 min after which absorbance was read at 500nm against a blank.

Determination of trypsin inhibitor content

The casein digestion method was used for determining trypsin inhibitor (Kakede et al., 1969). Samples (4 g) were weighed, and defatted using petroleum ether. Defatted samples were weighed (1 g) in Erlenmeyer flasks and the phosphate buffer added (20 ml). Contents were shaken on a shaker for 1 hour followed by centrifuging at 5000 rpm for 5 min. Supernatant (1 ml) was transferred to a 50 ml volumetric flask and diluted to volume with phosphate buffer. Sample aliquots (0.5 ml) were transferred to test tubes and distilled water added to make 1ml. Stock trypsin solution (1 ml) was added to each test tube and tubes placed in a water bath at 37°C. Casein solution (2%, 1 ml) previously brought to 37°C was added and then incubated at 37°C for 20 min. The reaction was then stopped by adding 6ml of 5% trichloroacetic acid (TCA). Suspensions were thoroughly mixed on a vortex mixer and left to stand at ambient temperature for 1 hour. Suspensions were filtered, and the absorbance of filtrate and trypsin standards measured at 280 nm using atomic absorption spectrophotometer (PerkinElmer,

Norwalk, CT, USA).

Determination of aflatoxins

Aflatest Fluorometer (VICAM V1 #4, Watertown, MA, USA) was used for determining aflatoxin in accordance with AOAC (2001) aflatest method number 991.31. A mixture of sample (50g) and salt, NaCl (5g) were placed in a blender jar, to which was added methanol: water solution, 80:20 (100ml), and then blended for 1 minute. Extract was filtered through a fluted filter paper and 10ml of filtered extract transferred to a clean vessel, to which purified water (20 ml) was added and homogenized. Dilute extract was filtered through a glass microfiber filter and 1 ml (equivalent to 0.167 g sample weight) of it passed through aflatest-P-affinity column at a rate of 1 to 2 drops per second, followed by 2ml purified water, 1ml at a time. The column was eluated with 1 ml HPLC grade methanol and sample eluate collected in a glass cuvette. Aflatest developer solution (1 ml) was added to eluate, mixed and then placed in a fluorometer to measure aflatoxin content.

Statistical analysis

Data was analyzed using STATA software version 12. Difference between means of proximate composition, fatty acid composition, minerals, vitamin A and anti-nutrients were tested for significance using the least significance difference (LSD) at 95% confidence level ($p<0.05$).

3. Results

3.1 Proximate Composition

Table 1 presents the comparison of the proximate profile of METU-2 to that of Plumpy nut. Analysis revealed comparable crude protein levels; 12.1% and 11.8% plumpy-nut and METU-2 respectively. Higher moisture levels, 9.8% were found in METU-2 compared to 2.7% in Plumpy nut. On the same note, crude fiber analysis revealed a higher content for METU-2, 1.24% compared to less than 0.01% levels found in plumpy nut. In terms of ash content, levels varied significantly from 3.6% in plumpy nut to 1.4% in METU-2. A superior carbohydrate profile was found for Plumpy-nut, 54.9% compared to 40.6% of METU-2. However, METU-2 had an expressively high fat content, 35.1% than Plumpy nut, 26.6%. Accordingly, a notable difference was noted in the total energy profile of the two samples; METU-2 had 528.2Kcal compared to 509 Kcal found in Plumpy nut.

Table 1. Proximate composition of Plumpy-nut and METU-2

Parameter	Plumpy-nut	METU-2	*P value*	*Recommended levels*
Moisture (%)	2.7±0.01 [b]	9.8±0.06 [b]	0.000	10
Protein (%)	12.1±0.15 [a]	11.8±0.95 [a]	0.05	10 to 12 % total energy
Fat (%)	26.9±0.06 [b]	35.1±0.21 [a]	0.0002	45 to 60 % total energy
Ash (%)	3.6±0.18 [a]	1.4±0.00 [b]	0.0016	
Crude fibre (%)	<0.01 [b]	1.2±0.93 [a]	0.0014	
Carbohydrate (%)	54.7±0.28 [a]	40.6±0.15 [b]	0.0001	
Total energy (kcal/100g)	509±1.09[b]	528.0±1.48[a]	0.0023	520-550

Results are expressed on dry basis except those of dietary fibre. Values in rows with different superscript letters are significantly different ($p<0.05$). Values are means of three replicates ± standard deviation

3.2 Micro Nutrient Profile

Table 2 presents an overview of the micro nutrient profile of the two therapeutic foods. Analysis showed that plumpy nut had higher levels of potassium (1437.5 – 420.95mg/100g), zinc (2.29 – 1.70mg/100g), copper (0.76 – 0.38mg/100g) and iron (9.23 – 5.53mg/100g) compared to METU-2. Plumpy-nut as well had superior sodium levels, 289.15mg/100g compared to 101.05mg/100g in METU-2. Both formulations statistically contained comparable magnesium contents; 119mg/100g and 114.32mg/100g plumpy-nut and METU-2 respectively. The concentration of vitamin A, 0.98mg/100g in plumpy-nut was significantly higher than that of METU-2, 0.52 mg/100g.

Table 2. Micronutrient Profile of Plumpy-nut and METU-2

Parameter (mg/100g)	Plumpy-nut	METU-2	*P value*	*Recommended levels (mg/100g)*
Potassium	1437.5±2.69[a]	410.95±8.13[b]	0.000	1100 to 1400
Magnesium	119.9±4.20 [a]	114.32±3.29 [a]	0.130	80 to 140
Zinc	2.29±0.09 [a]	1.70±0.02 [b]	0.006	11 to 14
Iron	9.23±0.18 [a]	5.53±0.06 [b]	0.000	10 to 14

Copper	0.76±0.17 [a]	0.38±0.010 [b]	0.046	1.4 to 1.8
Sodium	289.15±3.32 [a]	101.05±1.34 [b]	0.000	290 maximum
Vitamin A	0.98±0.01 [a]	0.52±0.14 [b]	0.000	0.8 to 1.1

Values in rows with different superscripts are significantly different (P<0.05). Values are averages of three replicates ± standard deviation.

3.3 Fat Acid Profile

Fatty acid profile of lipids extracted from plumpy-nut and METU-2 is presented in Table 3 below. Both formulations, contained comparable contents of PUFA's; METU-2 contained 2.82 g/100g of linoleic acid (18:2, n-6) while plumpy had levels of 2.87g/100g. Linolenic acid (18:3, n-3) levels ranged from 0.71g/100g to 0.75mg/100g for METU-2 and plumpy nut respectively. Oleic acid was also found in comparable levels in the two samples; 8.1396g/100g in METU-2 and 8.994g/100g in plumpy nut. On the other hand, Plumpy-nut contained expressively higher amounts of stearic acid (0.78 g/100g) and myristic acid (0.39g/100g) compared to levels found in METU-2 (0.01g/100g and 0.08g/100g stearic and myristic acid respectively). However, in terms of palmitic acid, METU-2 had significantly higher amounts, 0.35g/100g compared to Plumpy nut, 0.01g/100g.

Table 3. Fatty Acid Profile of Plumpy-nut and METU-2

Parameter (g/100g)	**Plumpy-nut**	**METU-2**	***P value***
Myristic acid	0.39±0.03[a]	0.079±0.18[b]	0.0008
Palmitic acid	0.35±0.02 [a]	0.04±0.00 [b]	0.0014
Stearic acid	0.78±0.20 [a]	0.01±0.02 [b]	0.0167
Oleic acid	8.99±0.21 [a]	8.14±0.63 [a]	0.1065
Linoleic acid	2.87±0.11 [a]	2.82±0.70 [a]	0.331
Gamma-linoleic acid	0.75±0.08 [a]	0.71±0.00 [a]	0.278
Arachidic acid	5.74±0.08 [a]	ND	
Erucic acid	3.73±0.43 [a]	0.85±0.008 [b]	0.0054
Lignoceric acid	0.57±0.04 [a]	0.74±0.09 [a]	0.0722

Values in rows with different superscripts are significantly different (P<0.05). Values are averages of three replicates ± standard deviation.

3.4 Levels of Anti-nutrients

All analyzed anti-nutrients; condensed tannins, trypsin inhibitors and phytates were significantly higher in METU-2 compared to plumpy-nut. Condensed tannin content ranged from 9.43mg/g in METU-2 to 6.55mg/g in Plumpy-nut while trypsin inhibitors varied from 2.54mg/g to 1.98mg/g respectively. Phytate levels ranged from 5.22mg/g in plumpy-nut to 6.72mg/g in Plumpy-nut.

Aflatoxin content: Plumpy-nut contained aflatoxin levels of 5.93ppb while METU-2 had 4.03 ppb.

Table 4. Anti-nutrient Profile of Plumpy-nut and METU-2

Parameter (mg/g)	**Plumpy-nut**	**METU-2**	***P value***
Condensed tannins	6.55±0.54[b]	9.43±0.09[a]	0.014
Phytates	5.22±0.02[b]	6.72±0.01[a]	0.000
Trypsin inhibitors	1.98±0.44 [b]	2.54±0.09 [a]	0.015

Values in rows with different superscript are significantly different (p<0.05). Values are averages of three replicates ± standard deviation.

4. Discussion

Proximate analysis showed comparatively high moisture content for METU-2. Scientifically, moisture levels above 10% provide a conducive environment for microbial and chemical reactions accelerating spoilage along with production of undesirable toxic components (Codex, 2006). Protein profile of both formulations was within the WHO recommendations of 10 to 12 % total energy for therapeutic diets (WHO, 2007). In SAM, mucosal enzymatic activity along with a number of transport systems within the body decrease and as these are protein based, sufficient protein is needed at rehabilitation stage to correct this deficiencies. While for plumpy nut, milk powder and peanut may equally contribute to quality of its protein profile, for METU-2, peanuts are the main contributor as protein in sorghum is not of adequate quality (Leder, 2004).

Though plumpynut had a superior carbohydrate profile, both formulations had adequate amounts to provide

sufficient energy for a child to recover from severe malnutrition. Carbohydrates are particularly needed for provision of glucose for brain functioning. About 95% of dry matter in honey is simple sugars; fructose and glucose, (Bogdanov, Jurendic, Sieber, & Gallmann, 2008).

Crude fiber levels were much higher in METU-2 and these were majorly attributed to sorghum. The milling process used for sorghum leaves much fiber. Malnourished children usually have a reduced mucosa surface and are therefore not able to fully absorb diets with high crude fiber content. Additionally, high fiber content complexes trace elements which are paramount in severely wasted children. Thus, the high crude fiber content of METU-2 is a detrimental factor.

Both formulations had a fat content within the 45 to 60 % total energy recommended amounts by WHO. However, METU-2 had a comparatively high fat content. A high fat content of METU-2 is majorly attributed to ghee. In addition to contributing profoundly to energy density, fat is also needed in the absorption of highly required vitamins, A and E (Michaelsen, 2009). Accordingly, the energy profile of METU-2 was higher than that of Plumpy nut and within the recommended 520- 550 Kcal/100g by WHO. Plumpynut was a fewer calories below this recommendation. Energy facilitates catch up growth in severely malnourished children in rehabilitation phase (Michaelsen, 2009), warranting energy dense dietary formulations.

4.1 Micronutrient Profile

For children suffering from SAM; zinc, potassium and magnesium along with vitamin A and folic acid are the most important while iron and sodium should be limited (Bhan, Bhandari, & Bahl, 2003).

Zinc is essential for the activity of more than 100 enzymes (Hotz & Brown, 2004). Deficiency impairs the working of immune system and has a direct consequence on the structure and function of mucosa (Bhan et al., 2003). Mucosa effect relates to mal-digestion. It is as well a vital component of intracellular superoxide dymutase, an enzymatic pathway system which keeps free radicals (involved in PEM manifestation) under control (Michael HN Golden & Ramdath, 1987). Zinc depletion occurs during cases of SAM (Fell et al., 1973). On this basis, WHO recommends that dietary formulations for SAM children contain zinc levels of 11 to 14 mg/100g. However, both formulations were below this recommendation. Particularly lower levels were found in METU-2. Sorghum is a good source of zinc but like other plant based foods, its bioavailability is low owing to anti-nutrients (Léder, 2004). A sustainable strategy to improve zinc content of these foods may include soil enrichment with zinc based fertilizers as research shows that zinc content of foods is largely soil dependent (Gibson, 2006).

During severe acute malnutrition cases, there is a considerable loss of potassium which in turn leads to sodium retention (Bhan et al., 2003). That is, severely wasted children have lower potassium levels, particularly intracellular concentration while total sodium levels, intracellular increase. When recovery starts, potassium concentration can drop dangerously, if adequate amounts aren't provided. In addition to promoting fluid retention leading to oedema, potassium deficiency translates in to intracellular acidosis, stimulating accumulation of Ca^{2+} deteriorates protein metabolism (M. Golden, 1988). It could as well lead to a decrease in contractile power of muscle fibers and together with hypokaliemia result in a reduction in cardiac output (M. Golden, 1988). For these reasons, higher potassium and lower sodium levels are recommended for SAM children compared normal children. While Plumpy nut contained potassium levels within the WHO recommended amounts of 1100 to 1400 mg/100g for therapeutic diets (WHO, 2007), levels in METU-2 were significantly lower. On a good note, sodium levels were below the 290 mg/100g maximum limits.

Magnesium content of the two formulations was comparable and within 80 to 140 mg/100g WHO recommendations (WHO, 2007). Peanuts contributed profoundly to the amounts in the two formulations (King et al, 2008). Sorghum is as well a good source (Léder, 2004). Magnesium is required by several enzymes involved in nucleic acid metabolism and thereby affects multiple physiologic processes (Rude, 1998). It is as well vital in ion transport systems; Ca^{2+}, pumps and Na-K ATPase. Deficiency is particularly sensitive for Na-K pump. Magnesium is frequently low in SAM and needs to be corrected for proper running of these sensitive physiological processes.

Copper is another important micronutrient in SAM treatment, owing to its role in controlling free radicals (intracellular superoxide dymutase) and its involvement in iron metabolism (transport and oxidation in the plasma) (Michael HN Golden & Ramdath, 1987). Despite peanuts being a rich source of copper (king et al., 2008), both formulations did not meet the recommended 1.4 to 1.8 mg/100g for SAM recovery.

In malnutrition cases, iron is sequestrated into storage sites, as SAM presents with infections and inflammations

yet iron promotes bacterial growth. Additionally, iron sustains radical generation process, presenting potential toxicity (Michael HN Golden & Ramdath, 1987). For these reasons, iron levels are always kept low even at the rehabilitation stage. Accordingly, WHO recommends levels of 10 to 14 mg/100g in therapeutic food formulations (WHO, 2007). However, METU-2 was way below this recommendation. Fortification explains levels found in plumpy-nut while those reported for METU-2, are largely attributed to sorghum. Sorghum is stated to have iron levels approximating 4mg/100g (Léder, 2004).

Vitamin A deficiency is common in SAM (Bhan et al., 2003). In addition to dietary deficiency, insufficiency is as well attributed to diminishing of the necessary enzymes for absorption and transportation. Dietary deficiencies of zinc, fat and proteins as well explain this deficiency. Children with SAM have more frequent infections, which increases their demand and interferes with the absorption at the gut level. Vitamin A serves a number of critical roles ranging from maintenance of the epithelial cellular integrity to promoting adequate functioning of the immune system. To realize morbidity reduction along with correction of the diminished mucosa in SAM, vitamin A is indispensable. Levels found in METU-2 were lower than those found in plumpynut and a little below the recommended 0.8 to 1.1 mg/100g WHO levels. Ghee used in METU-2 is a rich source of vitamin A.

4.2 Fatty Acid Profile

SAM affected children usually suffer essential fatty acid deficiency (EFA), depicted by the manifesting symptoms; skin changes, impaired resistance to infections, growth rate and development (Calder, 2013; Jones et al., 2015). Long chain poly unsaturated fatty acids (LC-PUFA) like docosahexaenoic acid (DHA) are chief constituent of neural lipid, and inadequacy during early childhood is linked to a range of neurodevelopmental abnormalities. Accordingly, SAM affected children are at risk of long-term cognitive and behavioral deficits. Additionally, essential fatty acid deficiency impairs nutrient absorption along with dietary calorie utilization (Calder, 2013). Thus, inadequate amounts during nutritional rehabilitation may escalate these deficits. Long chain PUFA's can be biosynthesized from n-6 linoleic acid (LA, 18:2 (n-6), and n-3 alpha-linoleic acid (ALA, 18:3 (n-3) by the sequential action of desaturase and elongase enzymes (Jones et al., 2015). Along this line therefore, WHO recommends that therapeutic formulations for SAM, contain LA and ALA at levels of 3 to 10% and 0.3 to 2.5% of total energy respectively (WHO, 2007). Both formulations not only had comparable amounts of these fatty acids, but also had levels within the WHO recommendations. The high LA could be attributed to peanuts (king et al., 2008 & Jones et al., 2015).

4.3 Anti-nutrients

Though generally present in lower levels; trypsin inhibitors, phytates and condensed tannins were higher in METU-2 compared to plumpy-nut. Literature reports considerably higher levels of these three in sorghum and peanuts (Gassem & Osman, 2003; Gibson, Bailey, Gibbs, & Ferguson, 2010; Léder, 2004). Thermal treatment reduces the levels and this mechanism could have substantially attenuated the levels in plumpynut (Hotz & Gibson, 2007). In the same line, peanut roasting could also have had a considerable effect on levels found in METU-2. Anti-nutrients have a number of nutritional effects which can jeopardize recovery from SAM. Trypsin inhibitor inhibits the activity of pancreatic proteolytic enzymes, mainly trypsin and chymotrypsin. High levels may consequently result in child growth faltering (Michael H Golden, 2009). Phytates and condensed tannins complex minerals and trace elements (Gibson et al., 2010). Condensed tannins also influence protein digestion through forming complexes with larger quantities of proteins (Knuckles, Kuzmicky, & Betschart, 1985). Presently, there are no prescribed limits for anti-nutrients; therefore, the principle is to reduce their presence as much as possible. Traditional processing methods like germination, soaking and fermentation have the capacity to reduce anti-nutrients and provide a cheaper solution at household level (Hotz & Gibson, 2007).

4.4 Alflatoxins

While aflatoxin contamination in METU-2, met the 5ppb maximum limits set for therapeutic diets by WHO, contamination levels in plumpynut were slightly above this limit. Groundnuts and cereals are highly affected with aflatoxins (Kaaya & Warren, 2005). Aflatoxin intake is associated with growth retardation (Khlangwiset, Shephard, & Wu, 2011; Smith, Stoltzfus, & Prendergast, 2012). Evidence from West Africa shows a dose-response relationship between serum aflatoxin and stunting (Gong et al., 2004). One way growth retardation is mediated is gut inflammation (Smith et al., 2012). Additionally, Aflatoxins being immune suppressing, they further expose acute malnourished children to incidences of infectious diseases. Aflatoxin contamination should therefore be kept as low as possible in therapeutic dietary formulations.

5. Conclusion

METU-2 has a proximate and fatty acid profile which is not only comparable to plumpy-nut, but also meets the

WHO recommendations for SAM diets. However, it is lacking in important zinc, potassium, and vitamin A. Present anti-nutrients further limit the bioavailability of zinc. Despite a few gaps, METU-2 provides a nutritional profile which can cure SAM and further studies could ensue its efficacy.

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Synergistic Effect of Polysaccharide Gums and Antimicrobial Agents on Susceptibility and Protein Expression of Select Pathogenic Microorganisms in Milk

Bernice Karlton-Senaye[1], Sarah Adjei-Fremah[2], Mulumebet Worku[2] & Leonard Williams[1]

[1]Center for Excellence in Post-Harvest Technologies, North Carolina Agricultural and Technical State University, Kannapolis, NC, USA

[2]Department of Animal Science, North Carolina Agricultural and Technical State University, Greensboro, NC, USA

Correspondence: Bernice Karlton-Senaye, Center for Excellence in Post-Harvest Technologies, North Carolina Agricultural and Technical State University, Kannapolis, NC 28081, USA. Tel: 1-704-250-5700. E-mail: bekarlton@gmai.com

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Abstract

The quest for the use of natural ingredients as preservatives and antimicrobial agents is rising. Polysaccharide gums are usually used as emulsifying agents and as preservatives. The objective of this study was to investigate the combined effect of five different polysaccharide gums and antimicrobial agents on growth, susceptibility and protein expression of select pathogenic microorganisms in milk. Antimicrobial susceptibility and protein concentration were determined by disc diffusion and Pierce BCA assay, respectively. The proteome pattern and the number of protein spots were determined by two-dimensional gel electrophoresis. The results showed that xanthan (6.68±0.02 Log CFU/mL) caused the most growth inhibition of *Salmonella enterica*, compared to the control. Inclusion of pectin led to a significant ($P<0.0001$) 2-log reduction of *Salmonella enterica* during a 2-day refrigerated storage (4 ^{o}C)**.** The highest inhibition zones (20.50±0.70) was observed in *E. coli* O157:H7 exposed to carrageenan-maltodextrin-cefixime. The proteome pattern was impacted by the gums with protein band of size 30kDa being the most prominent band. The highest number of protein spots (35) were obtained in locust bean treated samples. These findings indicated that tested gums affected microbial protein expression and were effective in inhibitory activity against tested pathogens specifically *Escherichia coli* O157:H7, thus gums hold great promise as some antimicrobial agents. Further characterization of protein targets is warranted.

Keywords: polysaccharide gums, antimicrobial, pathogens, and protein expression

1. Introduction

There are increasing concerns about issues of food safety and antimicrobial resistance. These concerns come from growing occurrence of new and emerging foodborne disease outbreaks caused by pathogenic microorganisms including *Salmonella* spp, *Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Bacillus* spp. amongst others (Tajkarimi, Ibrahim & Cliver, 2010). The food industries therefore rely heavily on the use of synthetic preservatives to inactivate or inhibit growth of spoilage and pathogenic microorganisms whereas infected individuals often resort to the use of artificial antimicrobials for recovery from such infections (Demirci, Guven, Demirci, Dadandi and Baser, 2008). Due to the rising incidences of resistance to antimicrobials by microbes natural antimicrobials have gained preference for use as control agents. The benefits of natural antimicrobials include controlling microbial contamination in food, inhibiting pathogenic bacteria, extending shelf life and reducing antibiotic resistance by pathogenic microorganisms (Nazef, Belguesmia, Tani, Pre- vost, & Drider, 2008; Abou-taleb & Kawai, 2008).

Antimicrobial resistance remains a critical food safety issue globally as demonstrated by reports on the clinical and public health consequences of drug resistance in *E. coli* and other foodborne microorganisms. Antimicrobial resistance is acquired through frequent intake of prescription antibiotics because of food contaminations or infections, and antibiotics are often misused in food animals although their primary aim is for disease prevention and growth promotion (Hao et al., 2014). Polysaccharides gums, also referred to hydrocolloids, produced from

plant, animal, and microbial fermentation have been studied extensively (Jafari et al., 2012). Apart from serving as stabilizers, gums have been used to improve growth and viability in Lactobacillus strains (Hernandez-Hernandez et al., 2012; 2012; Karlton-Senaye, Tahergorabi, Giddings, and Ibrahim, 2014). Certain plant polysaccharide gums have exhibited antimicrobial activity (Yamashita et al., 2001; Ali et al., 2009; Daoud and Roula, 2013). In our previous studies, we demonstrated the combined effect of polysaccharide gums and antimicrobial agents on the growth and antibiotic susceptibility of pathogens in medium (Karlton-Senaye, Ayad, Davis, Khatiwada, and Williams 2016). The physiological response of bacterial cells to antimicrobials is being studied using gene-expression profiling technologies such as proteomic technologies (Pérez-Llarena, and Bou, 2016). Currently, there are few studies performed on the antimicrobial activity but no studies on protein expression of *E. coli* exposed to polysaccharide gums in milk. Therefore, the aim of this study was to investigate the effect of gums on the growth and susceptibility of pathogenic microorganisms and protein expression.

2. Materials and Methods

2.1 Culture Activation

Stock culture of *Escherichia coli* O157:H7 (ATCC 700927), *Salmonella enterica* (ATCC 4345111), *Listeria monocytogenes* (ATCC19116), and *Staphylococcus aureus* (ATCC 49775) strains were obtained from -80°C stock storage collections in the microbiology laboratory at the Center for Excellence in Post-Harvest Technologies (Kannapolis, USA). Bacterial cultures were transferred to fresh tryptic soy broth (TSB) then incubation at 37°C for 16h. Activated cultures were streak plated on tryptic soy agar (TSA) and incubated at 37°C for 24 h. Single colony of each strain was used for growth study.

2.2 Treatment Preparation with Different Gums

Five different gums including agar (AG), carrageenan-maltodextrin (CM), locust bean (LB), pectin (PE), and xanthan (XA) were individually dissolved into 200 mL batches of 1% fat liquid milk at 0.5 % (w/v) and pasteurized at 110 ºC for 10 minutes then cooled to 50 ºC before use (Karlton-Senaye et al., 2014).

2.3 Inoculation Procedure and Determination of Bacterial Population

Individual active bacterial culture was serially diluted in 0.1% peptone water. One milliliter (~4 log CFU/mL) from appropriate serial dilution and was inoculated into each 200-mL batch of milk and mixed thoroughly. Initial bacterial populations were determined using a color QCount® Colony Counter (Advanced Instruments Inc., MA, USA). Samples without gum were considered as negative control. Inoculated samples were incubated at 37 ºC for 16 h. After incubation samples were then serially diluted and appropriate diluent was spiral plated onto TSA, and then incubated at 37 ºC for 24 h. The initial and final bacterial population was determined using a color QCount® Colony Counter (Advanced Instruments Inc., MA, USA) (Karlton-Senaye et al., 2016).

2.4 Storage Study

After determination of bacterial population samples inoculated with *Salmonella enterica* were stored at 4 ^{o}C for 21 days. Aliquots from refrigerated samples were serially diluted and spiral plated weekly for 21 days to determine the inhibitory effect of the gum on *Salmonella enterica* during the refrigerated storage period.

2.5 Antimicrobial Activity of Bacterial Pre-treated with Gums in Milk

Antimicrobial activities were detected by the method according to Karlton-Senaye et al., 2016 with a slight modification. Two hundred milliliter (200mL) batches of 1% fat liquid milk (Maola, NC, USA) containing 0.5 % each of the five different gums were inoculated with each of the following pathogenic bacterial strains *Escherichia coli* O157:H7 (ATCC 700927), *Salmonella enterica* (ATCC 4345111), *Listeria monocytogenes* (ATCC 19116), and *Staphylococcus aureus* (ATCC 49775) and incubated at 37 ºC for 16 h. After incubation milk samples were spirally plated onto Mueller Hinton II Agar (MHA, BBL, Sparks, MD, USA) at a final concentration of 10^9 cfu/ml. Antibiotic disks (BBL, Sparks, MD, USA) impregnated with the following standard amounts of the active compound were placed in duplicates in appropriate distance on the MHA plates: Tetracycline (TET) 30 µg; Doripenem (DOR) 10 µg; Imipenem (IMP) 10 µg; Cefixime (CFM) 5 µg; Cipropoxacin (CIP) 5 µg; Ceftazidime (CAZ) 30 µg; Kanamycin (KAN) 30 µg; and Meropenem (MEM) 10 µg (BBL, Sparks, MD, USA). The control samples without gums were also treated with antimicrobial agents. Plates were then incubated aerobically at 37°C for 24 h. The diameters of inhibition zones were measured and interpreted according to the guidelines provided by the Clinical and Laboratory Standards Institute (2014). The experiments were performed in duplicates and replicated three times.

2.6 Protein Profile Studies

2.6.1 Endotoxin Assay

Endotoxin assay was done on all solution and diluents used in this study following the procedure used by Adjei-Fremah et al. (2016). The ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ) was used following the manufacturer's protocol.

2.6.2 Sample Preparation

Ten militers (10ml) of each of treated sample was collected and centrifuged at 3200g for 15 minutes. The supernatant was discarded and the cell pellet was resuspended in 5ml of Phosphate buffered saline (PBS) solution. Protein isolation was done using the B-PER® direct Bacterial Protein Extraction Reagent (Thermo Scientific) following manufacturer's protocol.

2.6.3 Quantification of Protein Concentration

The total protein concentration was determined using the Pierce Bicinchoninic assay kit (Thermo-Scientific, Waltham, MA) following manufacturer's protocol. Bovine serum albumin (BSA) with known concentration was used as standard.

2.6.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A volume of the protein sample (50 µg) was added to an equal volume of 2X Laemmli buffer (Bio-rad) and the samples were boiled for 10 min to denature the proteins. The proteins were then separated on a 4-12% miniprotean precast gel (Biorad). The SDS-PAGE electrophoresis conditions included 200 V, 4000 mA, for 1 hour (Obanla et al., 2016). The proteins were stained using Bio-safe coomassie blue, following manufacturer's procedure (Bio rad). Visualization of proteins was done using Image Lab^{TM} software version 5.2.1 (Bio-rad). The Precision Plus ^{TM}Protein Dual color (Bio-rad) was used as protein ladder (250kDa-10 kDa).

2.6.5 Sample Preparation for 2-D Electrophoresis

The extracted protein samples were further prepped with a ProteoExtract^{TM} protein precipitation kit (CALBIOCHEM) following the manufacturer's procedure as previously described by Adjei-Fremah et al., (2016). Protein concentration was determined by the Pierce BCA assay using bovine serum albumin as standard.

2.6.6 2D Electrophoresis

Two-dimension electrophoresis (2-DE) was performed using the ReadyPrep^{TM} 2-D Starter kit (Bio-Rad) following the manufacturer's manual. Treated and control samples containing 169 µg of total proteins were reconstituted with 125 ul of rehydration buffer (10 ml of 8M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 0.2% (W/V) Bio-Lyte 3/10 ampholytes and Bromophenol Blue (trace). The reconstituted samples were loaded onto precast IPG ReadyStrips (7cm, pH 3-10, Bio-Rad), and were rehydrated on a level bench for 16 hrs. Each of the strips was overlayed with 3 ml of mineral oil to prevent evaporation during the rehydration process. Isoelectric focusing of the proteins was done using a protean isoelectric focusing (IEF) cell (Bio-Rad) at 20^{o}C with an initial low voltage (250 V), 4000 V for 2 hrs, followed by a voltage gradient from 10000 Vh to 14, 000 Vh, with a limiting current of 50 µA/strip. Prior to SDS-PAGE, the IEF strips were with equilibration buffer I (6M urea, 2% SDS, 2% DTT, 0.375M Tris-HCl, pH 8.8, 20% glycerol) and equilibration buffer II (6M urea, 2% SDS, 2% DTT, 0.375M Tris-HCl, pH 8.8, 20% glycerol, 0.5 g iodoacetamide) for 10 mins each. The equilibrated strips were placed on a gradient polyacrylamide gel (4-15%), and were sealed with melted agarose gel as an overlay to ensure contact between the strip and gel. SDS-PAGE was carried out using a protean mini apparatus (Bio-Rad) and the electrophoresis was performed at 30A for first 1 hour and at 50 V, 100 A/ gel until the dye reached the bottom of the gel. The gels were stained with bio-safe Coomassie blue following manufacturer's protocol (Bio-Rad) and further destained in distilled water. After staining, the gels were scanned using a Bio-Rad Image Lab^{TM} software version 5.2.1 (Bio-Rad).

2.6.7 Protein Spot Detection and Quantification

Protein peak spots analysis was done on the 2D-gel images using the compound fitting algorithm method by Brauner et al. (2014). The compound fitting method uses two dimensional fitting Gaussian function curves to extract data from 2D images. The algorithm used was scripted in MATLAB and is made available as additional file by Brauner et al (2014). The 2DE images were analysed to detect the number of proteins spots, their isoelectric point (pH) and their molecular sizes. Comparative spot analysis was done for presence or absence of protein spot using the *E. coli* O157:H7 Control gel as standard.

2.7 Data Analysis

The mean and standard deviation values were calculated from tested samples from three replicates. The experimental data was analyzed as one-way ANOVA using the GLM procedure of SAS software version 9.4 (SAS, INST., Cary, NC). Statistical significance was considered at $P<0.0001$. The protein bands were analyzed for percent bands, lane percent, volume (intensity) and relative front using the Image Lab™ software version 5.2.1 (Bio-rad).

3. Results

3.1 Growth Inhibitory Activities of Gums

Figure 1 shows effect of gums on the growth of *Salmonella enterica* (ATCC 4345111), *Escherichia coli* O157:H7 (ATCC 700927), *Staphylococcus aureus* (ATTC 49775) and *Listeria monocytogenes* (ATCC 19116) in 1% fat fluid milk during 16 h incubation at 37 °C. The extend of growth inhibition or growth promotion were both strain dependent and on the gums used.

Apart from pectin, all tested gum slightly inhibited the growth of *S. enterica*, with the addition of xanthan (6.68±0.02 Log CFU/mL, $P<0.0001$) causing most growth inhibition compared to the control. The inclusion of pectin led to the least inhibition and most growth of *S. enterica* (8.09±0.59 Log CFU/mL) compared the control (7.56±0.13 Log CFU/mL).

Compare to the control, the inclusion of xanthan, carrageenan-maltodextrin, and pectin led to a slight growth promotion in *E. coli* O157:H7. In contrast, compared to the control (7.81±0.27 Log CFU/mL), the addition of locust bean and agar led to growth inhibition of *Escherichia coli* O157:H7 with agar gum (7.56±0.41 Log CFU/mL) causing the least inhibition and carrageenan-maltodextrin the most growth (8.19±0.18 Log CFU/mL).

Compare to the control, the inclusion of locust bean, agar, xanthan, carrageenan-maltodextrin and pectin resulted in a slight growth inhibition of *S. aureus* and *L. monocytogenes* (Figure 1). The addition of xanthan and carrageenan-maltodextrin led to the most inhibition of *S. aureus* (8.08 log CFU/mL) and *L. monocytogenes*, (7.89 log CFU/mL) respectively (Figure 1.).

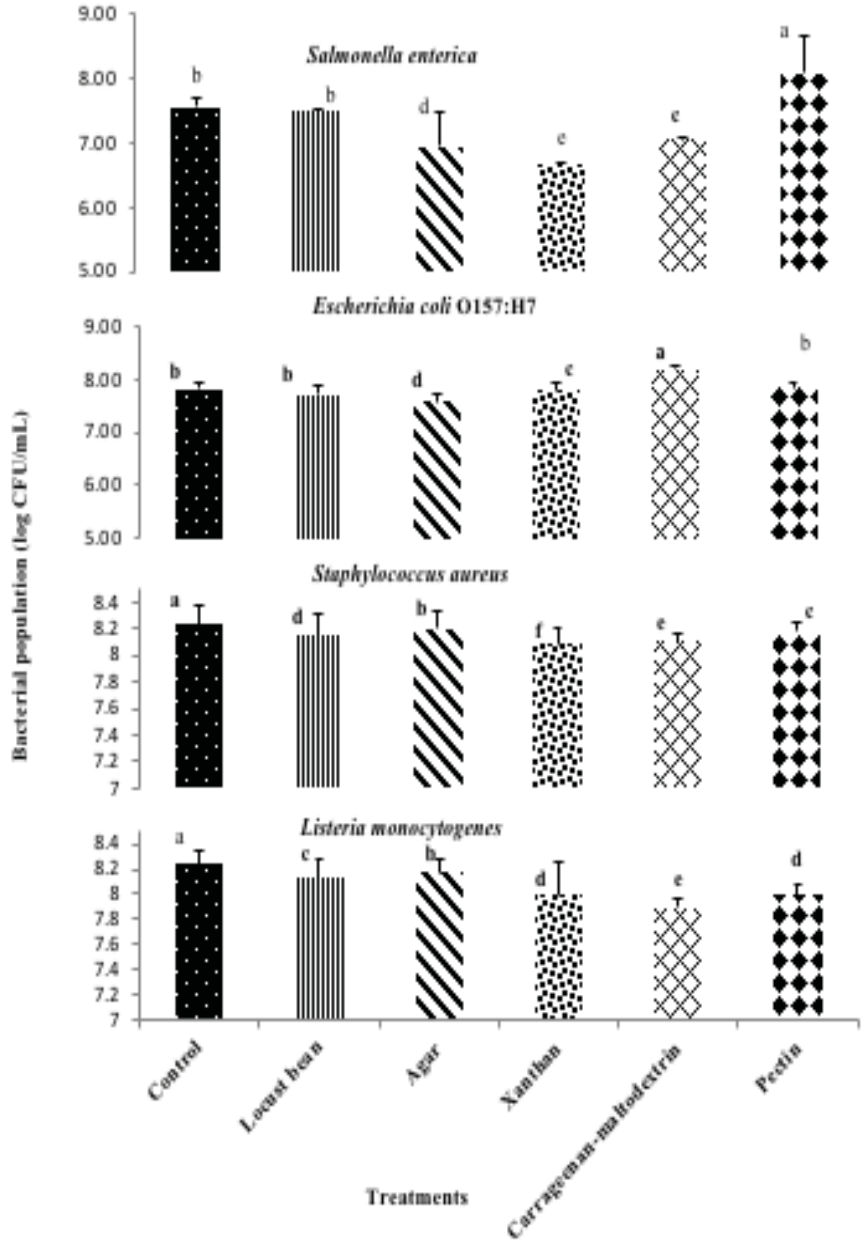

Figure 1. The growth of foodborne pathogens in 1% liquid milk containing different gums (0.5%) incubated at 37 ^{o}C for 16 h. Mean ± SD of three independent measurements. Graphs from top to down-*Salmonella enterica, Escherichia coli* O157:H7*, Staphylococcus aureus* and *Listeria monocytogenes*; Samples: 1-control (milk without gum); 2-treatment (milk +locust bean); 3-treatment (milk +Agar gum); 4-treatment (milk+xanthan); 5-treatment (milk+carrageenan-maltodextrin); 6-treatment (milk+pectin)

3.2 Effect of Gums on Bacterial Growth During Storage at 4 ^{o}C for 21 Days

Table 1. shows the combined effect of gums and milk on the growth of *S. enterica* during refrigerated storage at 4 ^{o}C. The results showed about a 1-log cfu reduction in both the control and treated samples. However, compared to the control, inclusion of pectin in milk samples led to a 2-log reduction ($P < 0.0001$) of *S. enterica* on day 21.

Table 1. Effect of gums on survival of *Salmonella enterica* during refrigerated storage at 4 °C for 3 weeks

Gums	Bacterial population (log CFU/mL)
Week 1	
Control	8.04±0.67[b]
Locust bean	7.83±0.86[d]
Agar	7.82±0.81[d]
Xanthan	7.95±0.85[c]
Carrageenan-maltodextrin	7.54±1.30[e]
Pectin	8.53±0.31[a]
Week 3	
Control	7.26±0.01[a]
Locust bean	6.83±0.06[b]
Agar	6.44±0.04[e]
Xanthan	6.65±0.05[c]
Carrageenan-maltodextrin	6.12±0.04[f]
Pectin	6.56±0.16[d]

Mean ± SD of three independent antimicrobial testing.

Means with different letters are significantly different ($P < 0.0001$)

3.3 Gums and the Antimicrobial Susceptibility of Pathogens

The synergistic effect of gums and milk on antimicrobial susceptibility of the tested foodborne pathogens was presented in Tables 2-6. With a few exceptions, the results showed, polysaccharide gums increased the susceptibility of tested pathogens to all antimicrobial agents compared to the control. The addition of all tested gums in milk rendered *S. enterica* susceptible to TET and DOR, although the control sample remained resistant. Inclusion of xanthan (46.5±0.57mm) and carrageenan-maltodextrin (46.25±0.57mm) increased the susceptibility of *Salmonella enterica* by more than two-fold (Table 2.). However, *S. enterica* remained resistant to CFM in both the control and the treatments. Notably, treatment of *S. enterica* with pectin resulted in resistance of *S. enterica* to CIP, CAZ, KAN and CFM.

Table 2. Combine effect of gums on antimicrobial agents on susceptibility of *Salmonella enterica* (ATCC 4345111) incubated at 37 ºC for 16h

Gums/Antimicrobial agents	Zone of inhibition (mm)			
	TET	DOR	IMP	CFM
Control	- (R)	- (R)	20.00±0.81(S)	- (R)
Locust bean	28.25±0.5(S)	30.25±0.5(S)	29.25±0.95(S)	- (R)
Agar	26.75±0.9(S)	23.25±0.5(S)	43.50±0.57(S)	- (R)
Xanthan	26.5±0.9(S)	25.25±0.5(S)	46.50±0.57(S)	- (R)
Carrageenan-maltodextrin	38.00±0.81(S)	37.25±0.5(S)	46.25±0.50(S)	- (R)
Pectin	23.75±0.5(S)	14.50±0.57(S)	22.50±0.57(S)	- (R)
	CIP	CAZ	KAN	MEM
Control	- (R)	- (R)	- (R)	- (R)
Locust bean	- (R)	- (R)	- (R)	12.50±0.57 (S)
Agar	32.00±2.31 (S)	- (R)	24.75±1.26 (S)	13.50±0.57 (S)
Xanthan	32.75±2.06 (S)	14.75±0.5(S)	27.25±0.5 (S)	15.50±0.57 (S)
Carrageenan-maltodextrin	32.50±0.57 (S)	- (R)	26.50±0.58 (S)	14.75±0.50 (S)
Pectin	- (R)	- (R)	- (R)	- (R)

Tetracycline (TET-30 µg); Doripenem (DOR-10 µg); Imipenem (IMP-10 µg); Cefixime (CFM-5 µg); Cipropoxacin (CIP-5 µg); Ceftazidime (CAZ-30 µg); Kanamycin (KAN-30 µg); and Meropenem (MEM-10 µg). Mean of three independent antimicrobial testing. Zone of inhibition was measured in nearest millimeter (mm) and interpreted based on NCLS for breakpoints. S: Susceptible; R: Resistance; (-): no zone of inhibition.

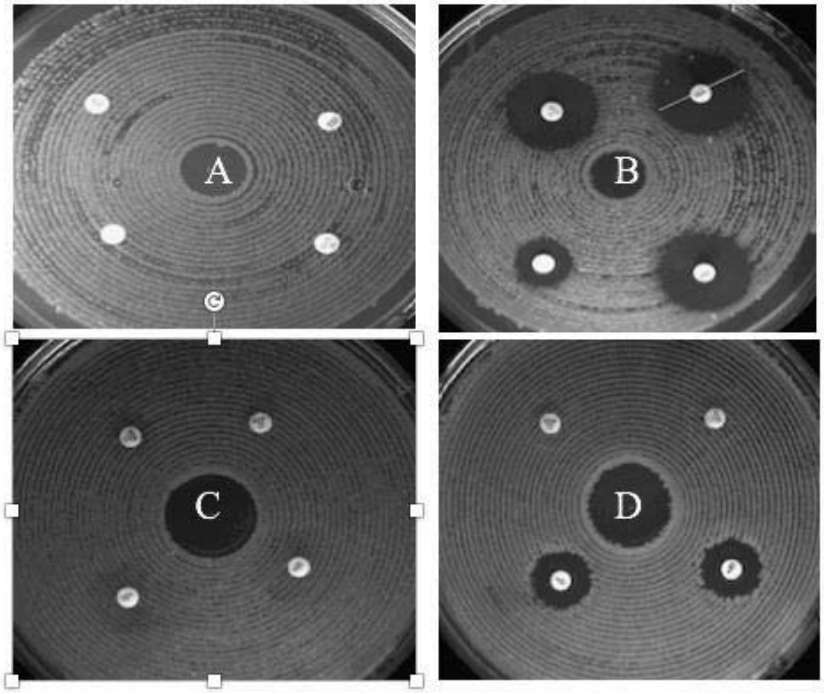

Figure 2. Comparing the effect of gums on antimicrobial susceptibility of *Salmonella enterica* in 1% liquid milk. Pre-treatment of *Salmonella enterica* with locust bean (top 2) and xanthan (bottom 2) followed by exposure to DOR and KAN resulted in making *Salmonella enterica* susceptible

Similar trends were observed in Table 3. that showed the effect of gums on susceptibility of *E. coli* O157:H7. Except for locust bean, the presence of all polysaccharide gums improved the susceptibility of *E. coli* O157:H7 to Ceftazidime (CFM) with up to 20.5±0.70mm inhibition zone. However, pretreatment with locust bean either decreased or maintained the susceptibility of *E. coli* O157:H7 to all tested antimicrobial agents. The zone of inhibition increased by 9.5mm, 9mm, 8.5mm in agar-Tetracycline, agar-doripenem, and agar-impinem, respectively, compared to their control.

Table 3. Combine effect of gums and antimicrobials on susceptibility of *Escherichia coli* O157:H7 (ATCC 700927) incubated at 37 ºC for 16h

Gums/Antimicrobial agents	Zone of inhibition (mm)			
	TET	DOR	IMP	CFM
Control	27.50±0.71(S)	37.50±2.12(S)	42.00±2.8 (S)	- (R)
Locust bean	24.00±1.41(S)	34.00±1.41(S)	39.50±0.71(S)	- (R)
Agar	36.50±0.71(S)	46.50±0.71(S)	50.50±2.12 (S)	16.50±0.71 (S)
Xanthan	33.50±0.71(S)	42.50±0.71(S)	48.50±0.71(S)	16.50±0.71 (S)
Carrageenan-maltodextrin	23.50±2.12(S)	45.50±0.70(S)	47.00±2.83 (S)	20.50±0.70 (S)
Pectin	24.50±3.53(S)	41.50±2.12(S)	45.00±1.41 (S)	12.50±0.70 (S)
	CIP	CAZ	KAN	MEM
Control	32.50±0.71 (S)	24.00±1.41 (S)	25.50±0.70(S)	38.00±2.83(S)
Locust bean	32.50±0.70 (S)	24.00±1.41(S)	24.50±0.71(S)	32.00±1.41(S)
Agar	34.50±0.71(S)	25.00±2.82 (S)	32.00±1.41(S)	43.00±1.41(S)
Xanthan	33.00±1.41(S)	24.50±0.71 (S)	28.50±0.71(S)	40.50±0.71(S)
Carrageenan-maltodextrin	39.50±0.71 (S)	28.50±2.12 (S)	36.00±1.41(S)	40.50±0.71(S)
Pectin	33.50±2.12 (S)	24.50±0.70 (S)	30.50±0.70(S)	39.50±0.70(S)

Tetracycline (TET-30 µg); Doripenem (DOR-10 µg); Imipenem (IMP-10 µg); Cefixime (CFM-5 µg); Cipropoxacin (CIP-5 µg); Ceftazidime (CAZ-30 µg); Kanamycin (KAN-30 µg); and Meropenem (MEM-10 µg). Mean of three independent antimicrobial testing. Zone of inhibition was measured in nearest millimeter (mm) and interpreted based on NCLS for breakpoints. S: Susceptible; R: Resistance; (-): no zone of inhibition.

Contrastingly, tested gums showed very little or no effect on the susceptibility of *S. aureus* (Table 4). Whereas *S. aureus* showed resistance to TET, CFM, CAZ and KAN even with treatment with gums, a minimal or no effect on susceptibility to DOR, IMP, CIP and MEM was observed in treatments (Table 4.).

Table 4. Combine effect of gums and antimicrobials on susceptibility of *Staphylococcus aureus* (ATCC 49775) incubated at 37 ºC for 16h

Gums/Antimicrobial gents	Zone of inhibition (mm)			
	TET	DOR	IMP	CFM
Control	- (R)	17.50± 0.70 (S)	18.75±0.35 (S)	- (R)
Locust bean	- (R)	22.50±0.70 (S)	19.25±1.06 (S)	- (R)
Agar	- (R)	21.50±2.12 (S)	21.50±0.71 (S)	- (R)
Xanthan	- (R)	19.50±0.70 (S)	23.00±1.41(S)	- (R)
Carrageenan-maltodextrin	- (R)	19.00± 1.41(S)	21.50±0.71 (S)	- (R)
Pectin	- (R)	21.00±1.41 (S)	23.00±1.41 (S)	- (R)
	CIP	CAZ	KAN	MEM
Control	11.00±1.41 (S)	- (R)	- (R)	15.50±0.70 (S)
Locust bean	11.00±1.41 (S)	- (R)	- (R)	17.50±0.71 (S)
Agar	11.00±1.41 (S)	- (R)	- (R)	16.50± 0.71(S)
Xanthan	18.50±0.71 (S)	- (R)	- (R)	18.00±1.41 (S)
Carrageenan-maltodextrin	17.75±0.35 (S)	- (R)	- (R)	17.50±0.71 (S)
Pectin	11.00±1.41 (S)	- (R)	- (R)	15.50±0.71 (S)

Tetracycline (TET-30 µg); Doripenem (DOR-10 µg); Imipenem (IMP-10 µg); Cefixime (CFM-5 µg); Cipropoxacin (CIP-5 µg); Ceftazidime (CAZ-30 µg); Kanamycin (KAN-30 µg); and Meropenem (MEM-10 µg). Mean of three independent antimicrobial testing. Zone of inhibition was measured in nearest millimeter (mm) and interpreted based on NCLS for breakpoints. S: Susceptible; R: Resistance; (-): no zone of inhibition.

Similarly, *Listeria monocytogenes* was resistant against TET, CFM, CAZ and KAN in both the treatments and the control. However, a minimal inhibitory activity to DOR, IMP, CIP and MEM was observed in treated samples. Interestingly, pre-treatment of *L. monocytogenes* with either locust bean or agar in milk resulted little or no different in zone size compared to the control (18.25±0.35 mm). The highest zone size (23.5± 0.71mm) was exhibited in samples that were pretreated with xanthan and exposed to IMP (Table 5).

Table 5. Combine effect of gums and antimicrobials on susceptibility of *Listeria monocytogenes* (ATCC 19116) incubated at 37 ºC for 16h

Gums/Antimicrobial gents	Zone of inhibition (mm)			
	TET	DOR	IMP	CFM
Control	- (R)	18.25±0.35 (S)	18.25±0.35 (S)	- (R)
Locust bean	- (R)	18 .25±0.35 (S)	18.25± 0.35 (S)	- (R)
Agar	- (R)	18.25±0.35 (S)	18.25±0.35 (S)	- (R)
Xanthan	- (R)	18.25±0.35 (S)	23.5± 0.71(S)	- (R)
Carrageenan-maltodextrin	- (R)	19.50± 0.70 (S)	22.5±0.71 (S)	- (R)
Pectin	- (R)	21.00±1.41 (S)	21.00 ±0.70 (S)	- (R)
	CIP	CAZ	KAN	MEM
Control	11.15±0.21 (S)	- (R)	- (R)	17.65±0.49 (S)
Locust bean	12.15±0.21 (S)	- (R)	- (R)	19.35±0.47 (S)
Agar	12.75±0.35 (S)	- (R)	- (R)	17.50±0.70 (S)
Xanthan	11.50 ±0.71 (S)	- (R)	- (R)	17.67±0.70 (S)
Carrageenan-maltodextrin	12.50±0.71 (S)	- (R)	- (R)	21.50±0.94 (S)
Pectin	12.50±0.70 (S)	- (R)	- (R)	21.17±0.23 (S)

Tetracycline (TET-30 µg); Doripenem (DOR-10 µg); Imipenem (IMP-10 µg); Cefixime (CFM-5 µg); Cipropoxacin (CIP-5 µg); Ceftazidime (CAZ-30 µg); Kanamycin (KAN-30 µg); and Meropenem (MEM-10 µg). Mean of three independent antimicrobial testing. Zone of inhibition was measured in nearest millimeter (mm) and interpreted based on NCLS for breakpoints. S: Susceptible; R: Resistance; (-): no zone of inhibition.

3.4 Effect of Polysaccharide Gums on Protein Expression

3.4.1 Total Protein Concentration

The effect of the gums on protein expression in *Salmonella enterica* was summarized in Figure 3. Total protein expressed has increased in all treatment in comparison to the control. Treatment with agar gum (110,000 µg/ml) resulted in the highest concentration of protein expression whereas treatment with xanthan (5000 µg/ml) depicted the lowest total protein concentration compared to the other treatments. Expression profiles monitored by gel electrophoresis (SDS-PAGE) showed some variation between treatments, control and the blank (milk only). There were 10 bands in blank, 8 bands in the control and 7 bands in sample treated with locust bean. On the other hand, 6 protein bands were seen in the expression profile for samples containing agar, xanthan, carrageenan-maltodextrin and pectin. About 7 to 12 protein bands of size 250 -10kDa were detected among all treatment groups with variable band volume intensity. Protein band of size 30kDa was the most prominent band among all treatments. However, the band intensity of this prominent protein decreased in treatment with xanthan, and highest band intensity in samples treated with locust bean and pectin, compared to the others. A protein band of size 12.5kDA was present in control and in samples treated with locust bean, agar gum and xanthan, but was absent in carrageenan and pectin treated samples.

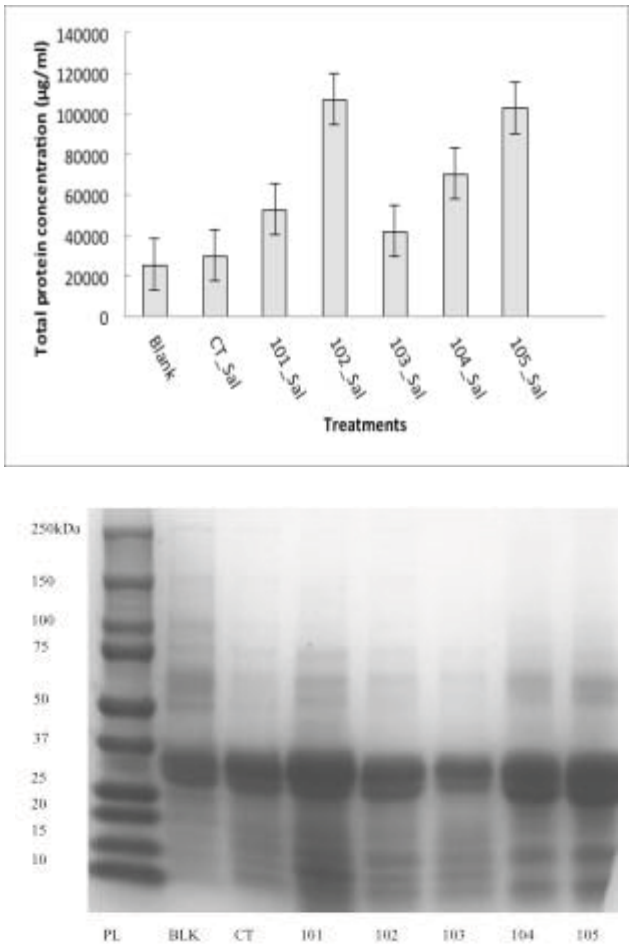

Figure 3. Effect of gums on total protein expression of *Salmonella enterica* in milk. CT: control; 101: locust bean; 102: agar gum; 103: xanthan; 104: carrageenan-maltodextrin; 105: pectin. BLK: 12 bands; CT: 11; 101: 9; 102: 10; 103: 7; 104: 8; 105: 8

Figure 4. shows effect of gums on total protein expression and gel intensity of *Escherichia coli* O157:H7 in milk, respectively. All treatments except locust bean treated sample had decreased total protein concentration compared to the control. Treatment with pectin resulted in the least protein levels. Four protein bands were common between blank, control, and all the treated samples at 65kDA, 37kDA, 18kDA and 12kDA. The protein

bands of size 30kDA were of strongest intensity in control, locust bean and agar treated samples, however lower band intensity band was observed in sample treated with xanthan and carrageenan-maltodextrin treated samples. A 20kDA protein band was distinctively present in samples treated with xanthan and carrageenan-maltodextrin. It was also observed that, a protein of size 60kDA was absent in samples treated with xanthan and carrageenan-maltodextrin.

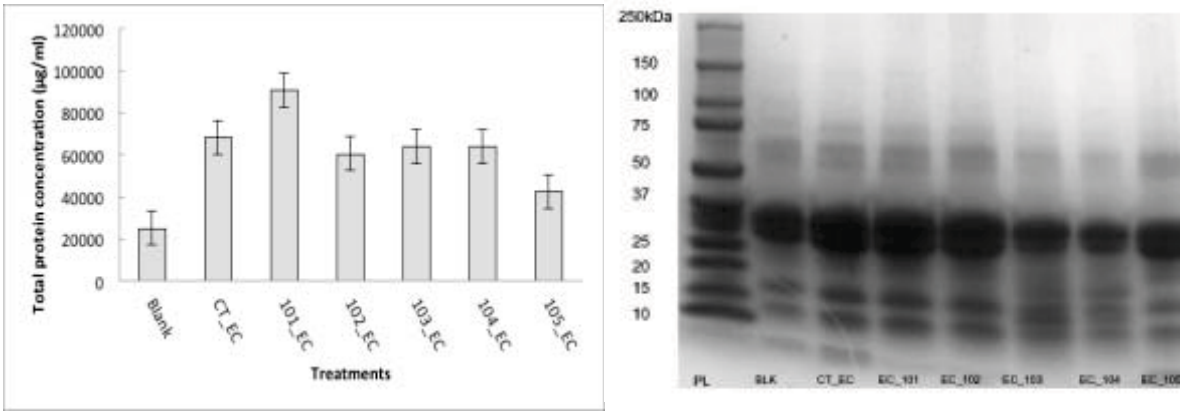

Figure 4. Effect of gums on total protein expression of *Escherichia coli* in milk. CT: (*E. coli* only); 101: (*E. coli* and locust bean); 102: (*E. coli* and agar gum; 103: (*E. coli* and xanthan); 104: (*E. coli* and carrageenan-maltodextrin); 105: (*E. coli* and pectin). SDS page result (right): BLK: Blank (milk only): 10 bands; CT-EC: 8 bands; EC_101: 7 bands; EC_102: 6; EC_103: 6; EC_104: 6 and EC_105: 6 bands

Protein expression in *Staphylococcus aureus* is summarized in Figure 5. The results showed that with the exception locust bean, all treatments inhibited the activity of *Staphylococcus aureus* and hence the reduced total protein concentration observed. Six protein bands of size 150-10kDa were detected among all treatment groups with variable band volume intensity. Protein band of size 30kDa was the most prominent band among all treatments, however, the band intensity of this protein decreased in treatment containing locust bean and carrageenan, and highest band intensity in agar and pectin treated samples, compared to control. Protein band of sizes 15kDA and 100kDA were present in treatments except samples with carrageenan-maltodextrin.

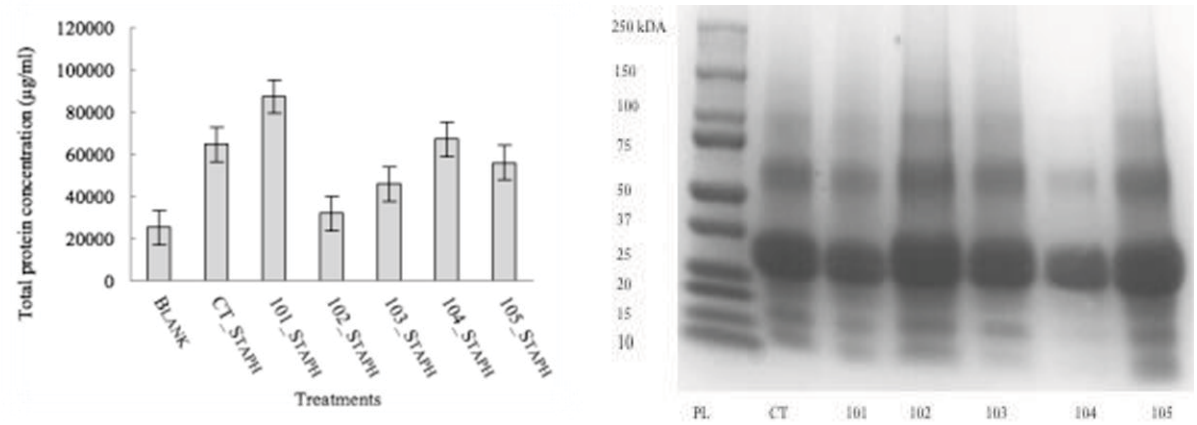

Figure 5. Effect of gums on total protein expression of *Staphylococcus aureus* in milk. PL: Protein ladder (250 kDa); Blank (without gum and *S. aureus*) CT: control (*S. aureus* only); 101: (*S. aureus* and locust bean); 102: (*S. aureus* and agar gum); 103: (*S. aureus* and xanthan); 104: (*S. aureus* and carrageenan-maltodextrin); 105: (*S. aureus* and pectin)

Figure 6. shows the effect of gums on protein expression in *Listeria monocytogenes*. Compared to the control, treatment with all the different gums increased total protein concentration. Treatment with pectin led to the highest total expression. It was also observed that the total protein expressed in both blank and control showed no difference. Six protein bands of size 150 -10kDa were detected among all treatment groups with variable band volume intensity. Protein band of size 30kDa was the most prominent band among all treatments. However,

the band intensity of this protein was found to be highest in control compared all the samples treated with gum.

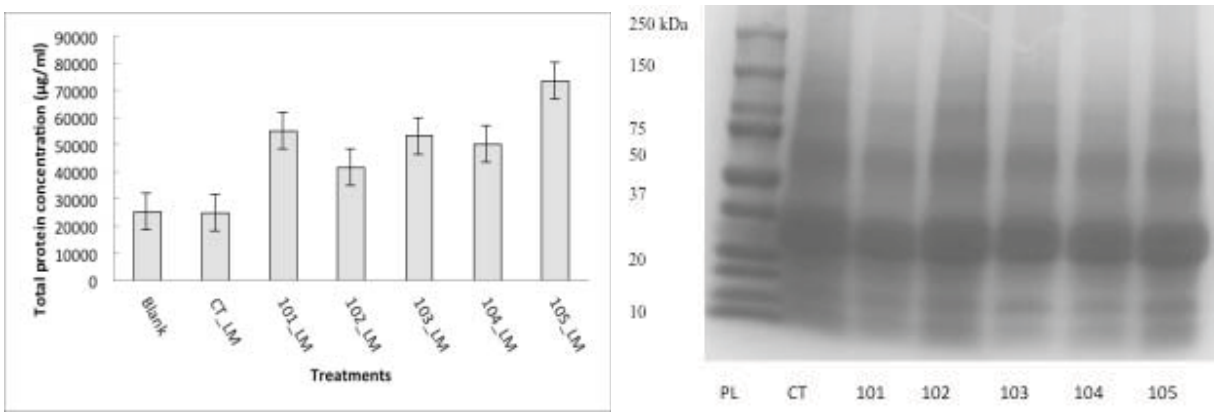

Figure 6. Effect of gums on total protein expression of *Listeria monocytogenes* in milk. PL: Protein ladder (250 kDa); Blank (without gum and *L. monocytogenes*) CT: (*L. monocytogenes* only) 101: (*L. monocytogenes* and locust bean); 102: (*L. monocytogenes* and agar gum); 103: (*L. monocytogenes* and xanthan); 104: (*L. monocytogenes* and carrageenan-maltodextrin); 105: (*L. monocytogenes* and pectin)

further studies were carried out to determine the effect of the tested gums on the number of protein spots expressed in *E. coli* O157:H7 using 2DE. Figures 7 and 8 depict a standard 2-DE gel image of *E. coli* proteome using pH 3-10, and 4-7 IPG strips. The 2DE gel images showing the effect of different gum treatments on *E. coli* is shown in Figure 9 to 14. Variations in entire proteome profile of *E. coli* O157:H7 in response to gums treatment were observed by comparing the 2-DE image of control to treated groups. Compound fitting algorithm (Brauner et al., 2014) analysis of the 2DE gels (Figures15) were performed to determine the number of protein spots in each treatment group. About twenty-three protein peak spots were identified in the control sample (Figure 9) usually within the pH ranges 4-6 and 8-10 (Table 6).

Highest number of protein spots (35) with greater intensity were obtained in the samples treated with locust bean (Figure 10) compared to the other treatment groups and the control. Additionally, about four protein spots at the isoelectric point pH 3 and molecular size ranging 10-50 kDa were distinctively present in locust bean treated groups but absent in the control (Figure 10). Also, a few protein spots with less intensity were detected in xanthan and carrageenan-maltodextrin treatment groups. The detected protein spots in the pH range of 8-10 had a horizontal train aspect, and this is typical of glycosylated/ phosphorylated proteins.

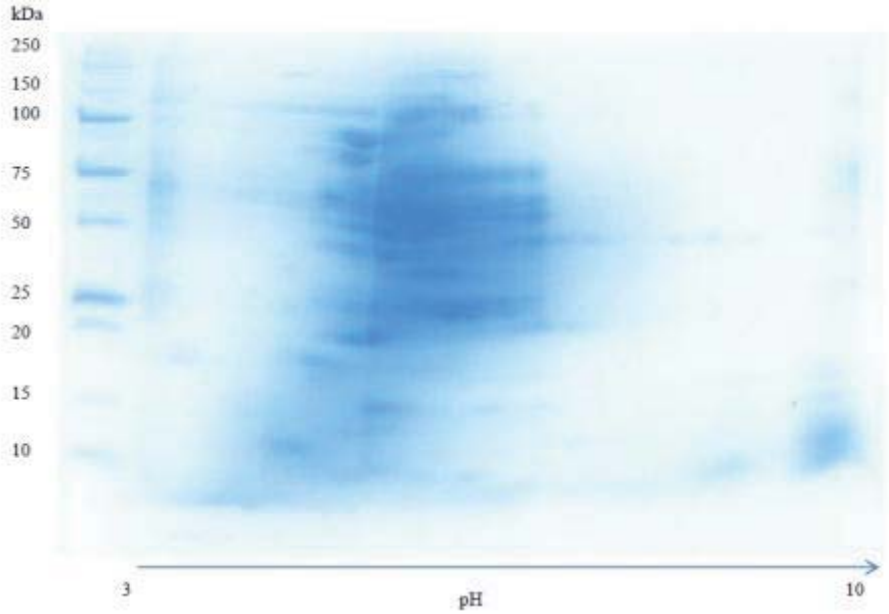

Figure 7. Expression of protein in *E. coli* O157:H7 at 3-10 pH

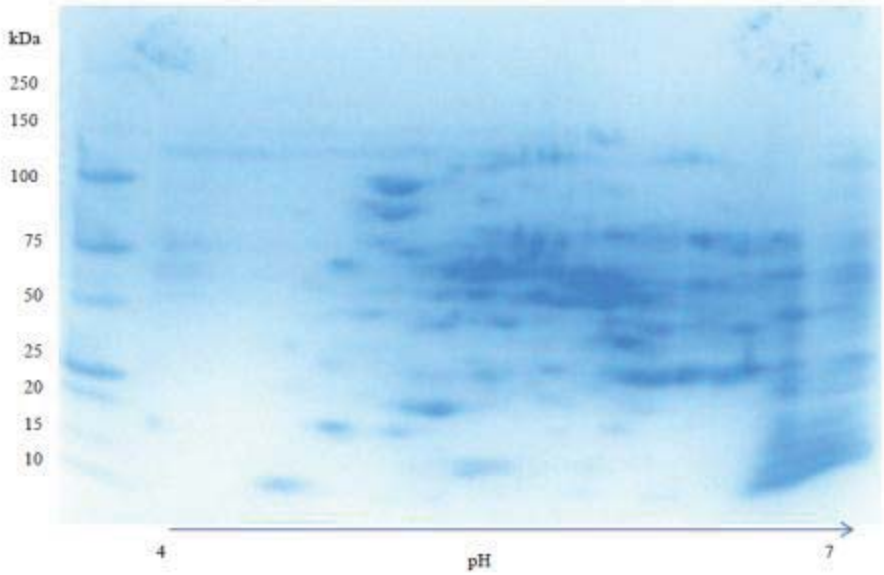

Figure 8. Expression of protein in *E. coli* O157:H7 at 4-7 pH

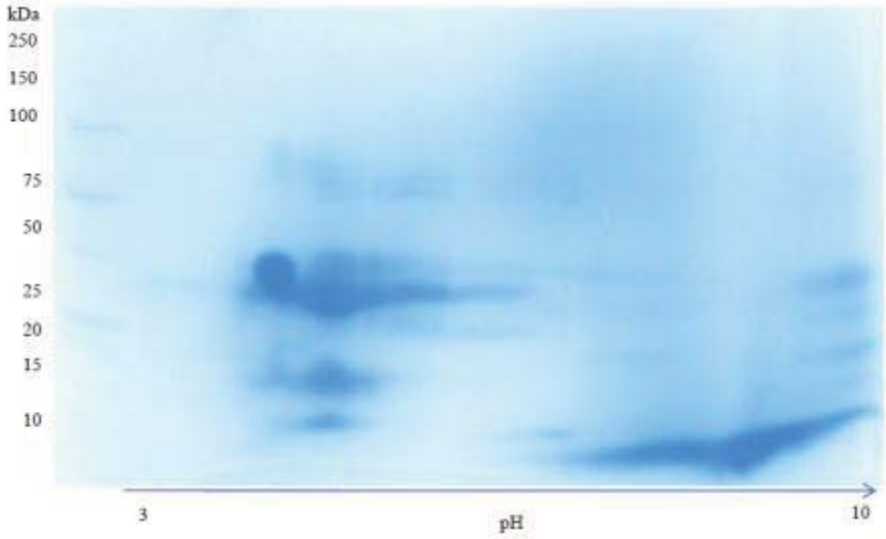

Figure 9. Number of protein spots expressed by in *E. coli* O157:H7 in milk at 3-10 pH

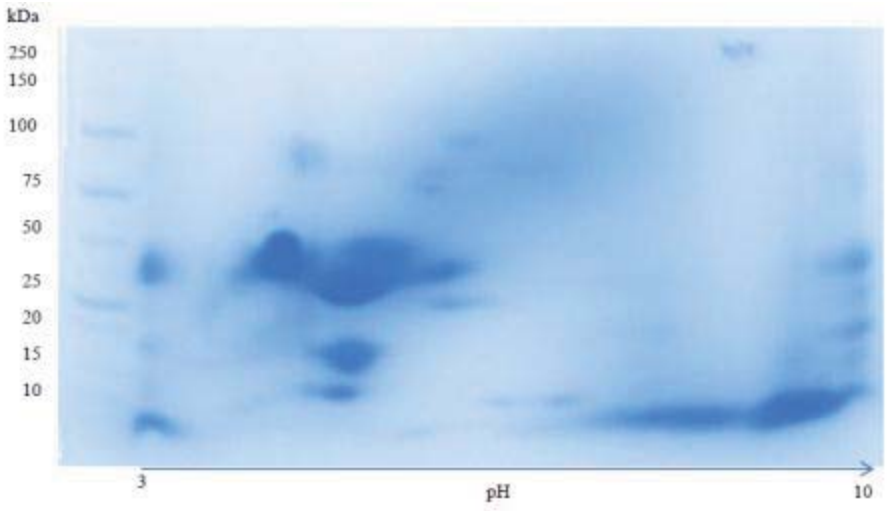

Figure 10. Effect of locust bean gum on number of protein expressed by *E. coli* O157:H7 in milk at 3-10 pH

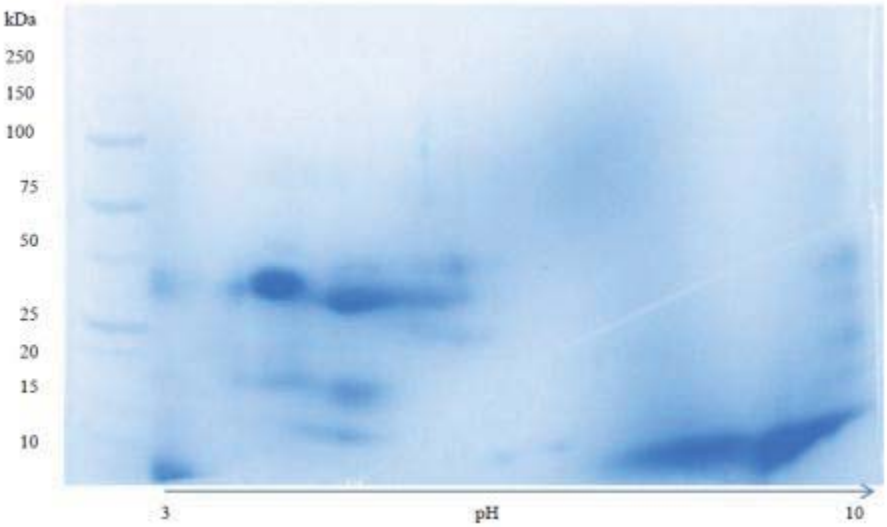

Figure 11. Effect of agar gum on number of protein expressed by *E. coli* O157:H7 in milk at 3-10 pH

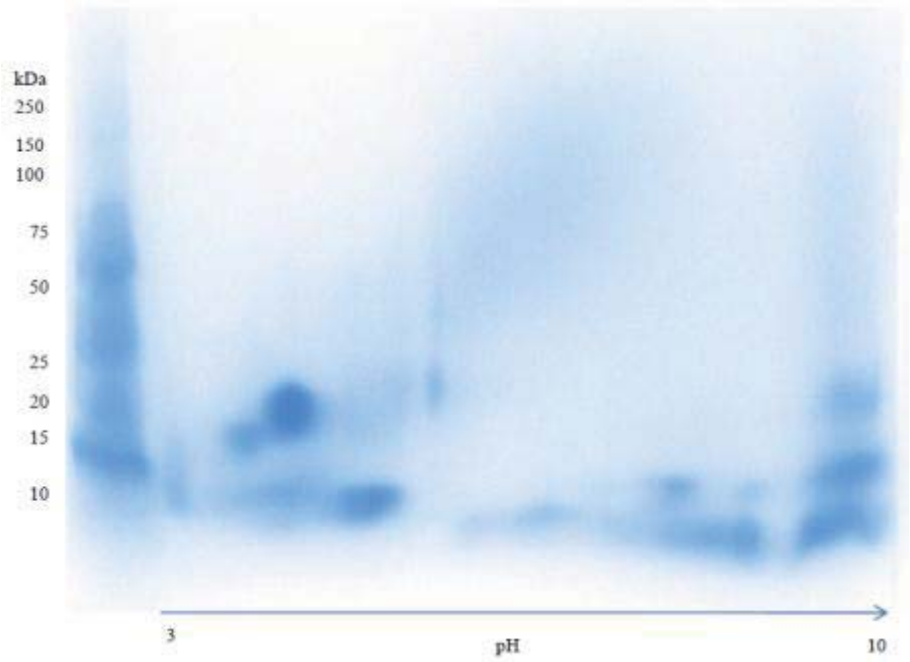

Figure 12. Effect of xanthan gum on number of protein expressed by *E. coli* O157:H7 in milk at 3-10 pH

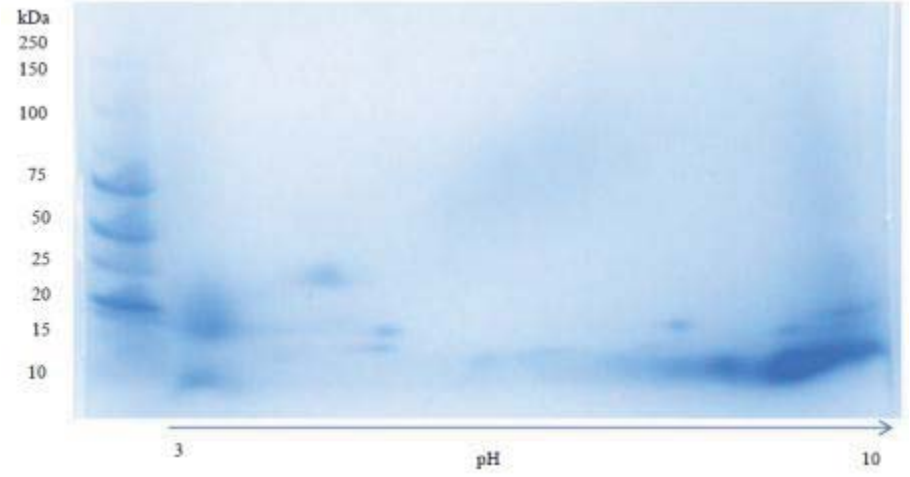

Figure 13. Effect of carrageenan-maltodextrin gum on number of protein expressed by *E. coli* O157: H7 in milk at 3-10 pH

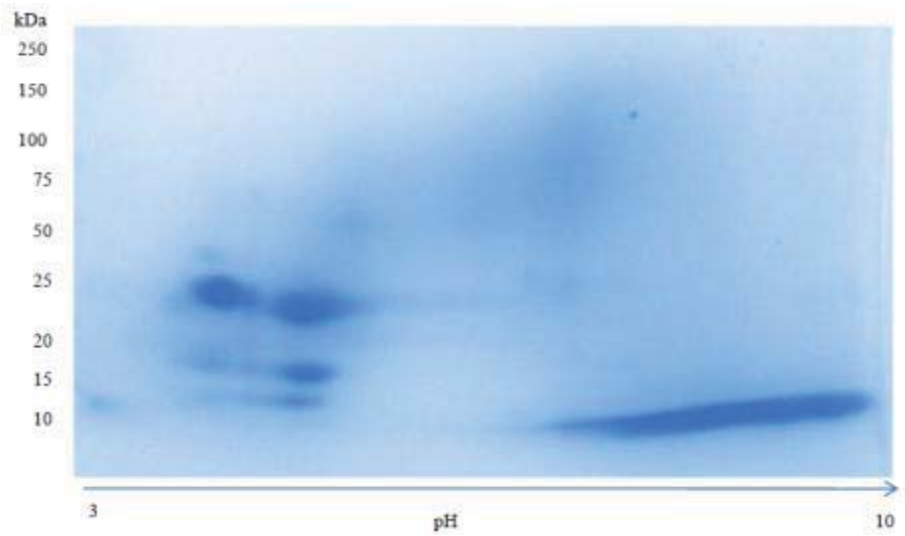

Figure 14. Effect of pectin gum on number of protein expressed by *E. coli* O157: H7 in milk at 3-10 pH

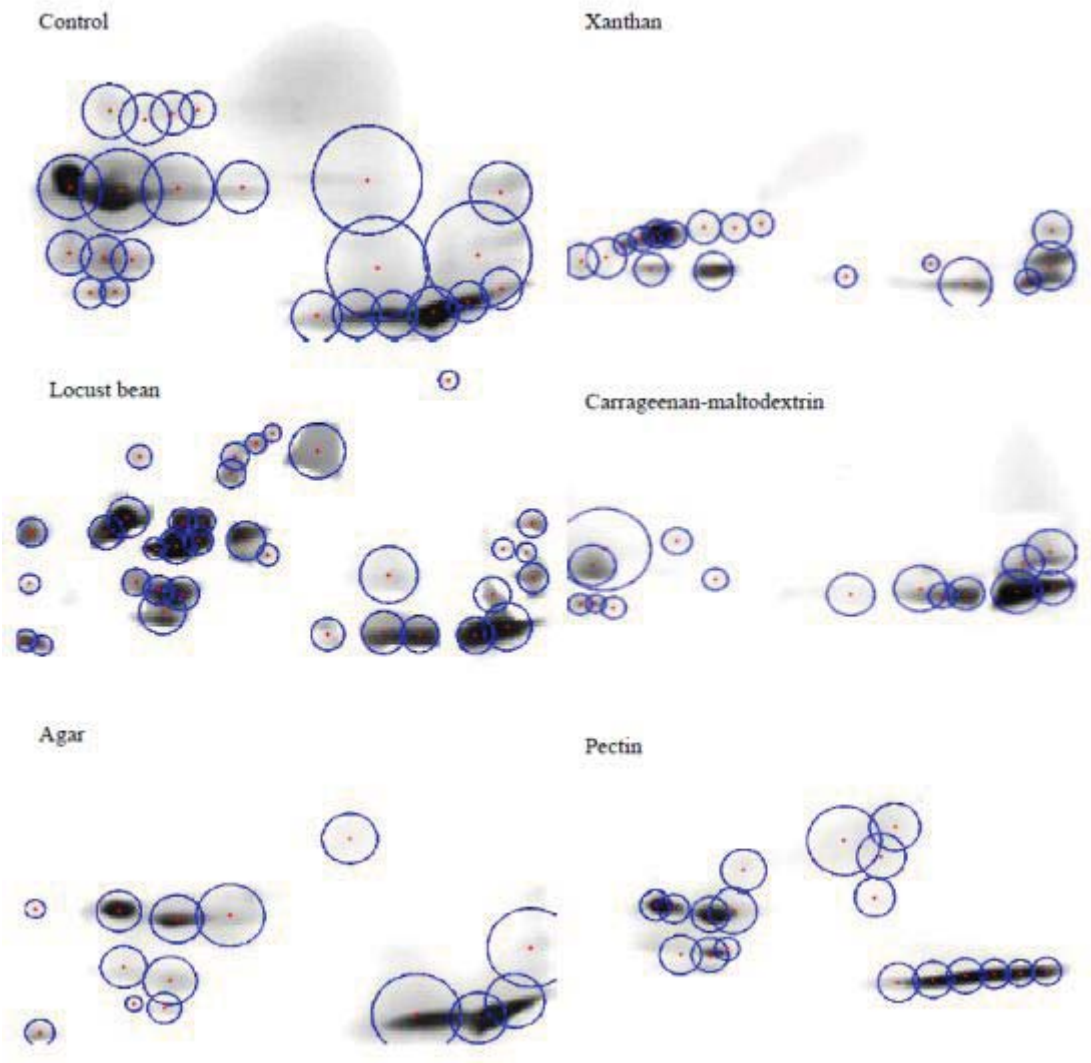

Figure 15. Protein peak spots detected in 2D image of *E. coli* O157:H7 in response to treatment with different gums

Table 6. Effect of gums on number of protein spots at different pH

Treatment	Number of peak spots	pH
CT	23	4-6, 8-10
EC_101	35	3-5, 8-10
EC_102	14	3-5, 8-10
EC_103	17	3-5, 8-10
EC_104	15	3-5, 8-10
EC_105	18	3-5, 8-10

Note: CT (Control**);** EC_101 (*E. coli* O157:H7 and locust bean); EC_102 *(E. coli* O157:H7and agar gum); EC_103 *(E. coli* O157:H7 and xanthan); EC_104 *(E. coli* O157:H7and carrageenan-maltodextrin); EC_105 (*E. coli* O157:H7 and pectin).

4. Discussion

This study investigated effects of polysaccharides gums on the growth of four different pathogenic bacteria and the microbial cellular response to different polysaccharide gums and antimicrobial agents' treatment at the proteome level.

4.1 Growth Inhibitory Activity of Gums

The two-log growth reduction of *S. enterica* grown in milk containing xanthan and pectin during refrigerated storage period could be due to poor interactions of *S. enterica* with xanthan and pectin in milk. A study conducted by Peter et al., (1989) revealed the accumulation of xanthan gum around cells grown on agar, whereas Contreras et al., (2005) have observed interactions during pathogenesis.

4.2 Antimicrobial Activity of Gums

Some select gums including agar (AG), carrageenan-maltodextrin (CM), locust bean (LB), pectin (PE), and xanthan (XA) were tested in combination with certain common antimicrobial agents including Tetracycline (TET) 30 µg; Doripenem (DOR) 10 µg; Imipenem (IMP) 10 µg; Cefixime (CFM) 5 µg; Cipropoxacin (CIP) 5 µg; Ceftazidime (CAZ) 30 µg; Kanamycin (KAN) 30 µg; and Meropenem (MEM) 10 µg (Tables 2-5). Some of these common antimicrobial agents have been proven to be effective against certain pathogens in media whereas other showed resistance as observed in our previous study (Karlton-Senaye et al., 2016). In this current study, we investigated the effect of pathogens that have been pretreated with different gums in milk with further exposure to certain antimicrobial agents. The ability of the tested polysaccharide gums to increase the susceptibility of *S. enterica* to TET, DOR, CIP, CAZ, KAN and CFM (Fig. 2) and *Escherichia coli* O157:H7 to CFM whilst showing resistance in non-pretreated samples was expected (Fig 3). This is because plants including polysaccharides are capable of synthesizing secondary metabolites such as phenols, flavonoids and essential oils that have antimicrobial properties against pathogens. Carrageenan and pectin have been shown to be have antimicrobial activity against Listeria monocytogenes (Yamashita et al. 2003). Therefore, the ability of the gums to increase the susceptibility of the studied pathogens could be due to the presence of hydroxyl groups. Increase hydroxyl groups may lead to increased hydroxylation, which results in increased antimicrobial activity (Cowan et al., 2017). The number and site of hydroxyl group are found to be linked to their toxicity to microorganisms (Fullerton et al., 2011).

4.3 Protein Expression

Results from our study demonstrated changes in the total protein concentration and proteome profile of *E. coli, S. aureus, L. monocytogenes* and *S. enterica* in response to all the treatments tested. Previous studies have shown proteomic differences in *L. monocytogenes* (Huang et al., 2014), *S. aureus* (Liu et al., 2013;) and E. *coli* (Schimdt et al., 2016). In this study, we used 2-DE approach to investigate the relative protein expression dynamics in certain food borne pathogens in response to different polysaccharide gums and antimicrobial agents. Liu et al., 2013, showed proteomic changes in *S. aureus* in response to the antibiotic oxacillin. In the current study, the different foodborne pathogens tested showed antimicrobial susceptibility to the polysaccharide gum treatment and was possibly demonstrated at the molecular level observed in changes in total protein concentration and 2-D gel patterns.

In response to stress (growth conditions and antibiotics), bacteria undergo many changes at the physiological level. These changes include an increase in membrane fluidity and a decrease in translation level (Ingram, 1990). Various transcriptomic and proteomic studies have been done to demonstrate the physiological implication of

cell wall stress in response to antibiotics and other treatments (Schimdt et al., 2016). This study reports the first comprehensive analysis of protein changes in foodborne pathogens specifically *E. coli* O157:H7 in response to different polysaccharide gums.

Microbes especially *E. coli* have been monitored to make changes at the molecular level in response to different stresses and growth conditions (Nystrom, 2004; Soufi et al., 2015). Previous studies have monitored these molecular adjustments at the protein level using 2-DE and quantitative mass spectrometry tools. For examples, system-wide protein changes have been studied in *E. coli* in response to ethanol stress (Soufi et al., 2015). Also, a study by Schmidt et al, 2016) identified protein allocation, expression regulation and post-translational adaptations in *E. coli* in response to 22 different experimental conditions. Similar proteome changes were observed in the current study; however, the specific proteins are yet to be identified and characterized.

Further studies are required to identify and characterize the biological function of these proteins using mass spectrometry. Furthermore, in *E. coli* O157:H7, small stress-induced proteins are missed using classical proteomic tools (Hemm et al., 2010). In addition to protein purification and mass spectrometry, identification and characterization of small proteins in response to gum treatment is warranted.

5. Conclusion

The results of this study showed that xanthan caused the most growth inhibition of *Salmonella enterica*, compared to the control. Inclusion of pectin in milk samples led to a 2-log reduction of *Salmonella enterica* during 21day refrigerated storage at 4 ^{o}C. The addition of all tested gums in milk rendered *Salmonella enterica* susceptible to TET and DOR, whereas the control remained resistant. The highest inhibition zones were observed in *E. coli* O157:H7 exposed to carrageenan-maltodextrin-cefixime. Protein band of size 30kDa was the most prominent band among all treatments. The most protein spots with greater intensity were obtained in the samples treated with locust bean. Additionally, about four protein spots at the isoelectric point pH 3 and molecular size ranging 10-50 kDa were distinctively present in locust bean treated groups but absent in the control. These findings indicated that tested gums were effective in inhibiting the growth of tested pathogens specifically *Escherichia coli* O157:H7, thus they possess antimicrobial activity and have as antimicrobial agents. There was some correlation between the antimicrobial activity of the tested gums and the protein expression of the pathogens. Therefore, further studies are necessary to identify the specific proteins responsible for antimicrobial properties of gums. Our study confirms that gums possess some antimicrobial tendencies against select pathogenic microorganisms and impact gene expression. Thus, the antimicrobial properties of these gums make them possible candidate for food preservation. Gums could potentially be used in nutraceuticals to enhance recovery from pathogenic infections. Further studies are required to identify and characterize the biological function of these proteins and possible regulation at the level of transcription.

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The Identity Crisis of Hard Cider

Nicolas Fabien-Ouellet[1] & David Scott Conner[2]

[1]Food Systems Graduate Program, University of Vermont, USA

[2]Department of Community Development and Applied Economics, University of Vermont, USA

Correspondence: Nicolas Fabien-Ouellet, Food Systems Graduate Program, University of Vermont, USA. E-mail: nfabieno@uvm.edu

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Abstract

In the past 5 years, the hard cider industry in the U.S. has undergone a sudden and dramatic growth period. This boom initially revealed challenges on the cider-specific apple supply side, but issues on the hard cider demand side have also emerged. This mixed methods study conducted in Vermont, a crucial player of the U.S. hard cider industry, addresses the gaps in the literature both on the apple supply side, and on the hard cider demand side. On the apple supply side, fourteen semi-structured interviews demonstrated that neither a long-term formalized contract nor a cooperative model (the two strategic partnership mechanisms used by world's leading industries to manage cider-specific apple production) are appropriate for the current Vermont industry context. On the hard cider demand side, cider makers expressed high interest in working under a geographical indication (GI) label to develop consumers' hard cider literacy and increase demand. This research further indicates that GIs can act as a powerful economic development tool. Introducing hard cider GIs could address current hard cider industry issues on both the supply side and the demand side.

Keywords: hard cider, geographical indications, strategic partnership, cider-specific apples, taste

1. Introduction

After years of exponential growth (Petrillo, 2016), the hard cider industry finds itself at a critical juncture. While the more mature European cider industries offer well-defined products, such as the "Herefordshire cider," the "cidre de Bretagne," or the "sidra de Asturias," the U.S. hard cider industry still offers products that oscillate between the borrowed identities of beer and wine, and lack clear and cohesive definitions. This malleable character prevents the U.S. hard cider industry from maturing, and makes it difficult for the industry to retain customers gained in the last few years, as well as attract new customers. The hard cider industry is aware of the issue. The number one goal in the 2017-2020 Strategic Plan for the United States Association of Cider Makers (2017) is to, "…grow demand for all styles of cider in the U.S. market." Additionally, the association would like to, "Establish a nationally-recognized consumer-focused cider lexicon with the explicit goal of helping consumers of differing cider knowledge identify cider styles and products they are most likely to enjoy." There are two layers of differentiation at play in this goal: (1) affirming American hard cider as a drink with its own identity, distinct from European cider; and (2) differentiating between the diverse hard cider styles within the U.S. industry.

According to Petrillo (2016), from 2011 and 2016, the hard cider industry has grown at an annualized rate of 27.3%. This sudden and rapid growth has inspired researchers across producing states to study the challenges faced by the industry. So far, the literature on the topic has focused on the lack of supply of cider-specific apples in the U.S. domestic market. Virtually no research has been undertaken on the demand side of hard cider, with the exception of Tozer, Galinato, Ross, Miles, & McCluskey (2015), who looked at the willingness to pay of consumers for specific profiles of hard cider.

This paper uses mixed methods to fill the gap in the literature on both the apple supply side and the hard cider demand side. On the apple supply side, this study uses semi-structured interviews conducted with industry stakeholders, primarily apple growers and cider makers, to explore the possibility of long-term formalized contracts or cooperatives to stimulate the production of cider-specific apples. On the hard cider demand side, a survey is used to gauge interest of cider makers in a geographical indication (GI) labeling system to differentiate between the different hard cider styles, potentially increasing demand. Overall, respondents emphasized that

efforts surrounding the planting of additional cider-specific apple trees must be preceded by an increase in demand for hard ciders made with cider-specific apples. Vermont cider makers expressed the desire to source more cider-specific apples from their area, so this study focuses on the Vermont cider industry (Becot et al., 2016a). In the following literature review, we summarize the current state of the hard cider industry in the U.S. and Vermont. We also describe how the world's leading cider-specific apple industries manage their production, and explore how ciders are differentiated through GI certifications.

2. Literature Review

2.1 What is Hard Cider?

In the United States, cider is categorized as sweet – made of unfiltered apple juice – or hard – made from the fermentation of unfiltered apple juice (Petrillo, 2016). The apples used by U.S. cider makers today can be sourced from an array of apple cultivars, including lower-grade dessert cultivars (e.g. 'McIntosh', 'Cortland'), dual-purpose cultivars that can be grown for both the fresh or cider market (e.g. 'Idared', 'Northern Spy'), cider-specific apple cultivars like bittersharp (e.g. 'Kingston Black'), and bittersweet cultivars (e.g. 'Dabinett', Fillbarrel'). Cider-specific apples are apples with the unique flavors, high-acid qualities, and astringent tannin characteristics suited only to hard cider production (McGee, 2004).

In 2017, the Alcohol and Tobacco Tax and Trade Bureau (TBB), which regulates alcohol sales in the United States, classifies hard cider as a wine, and defines three tax classes of hard cider (Alcohol and Tobacco Tax and Trade Bureau [TBB], 2017a). An "artificially carbonated hard cider" is a hard cider that is artificially injected with carbon dioxide, and contains between 0.392 and 0.64 grams of carbon dioxide per 100 milliliters. A "still hard cider" is a hard cider that contains less than 0.392 grams of carbon dioxide per 100 milliliters. "Sparkling hard cider" refers to a hard cider that contains between 0.392 and 0.64 grams of carbon dioxide per 100 milliliters, and is the result of the secondary fermentation of the cider within a closed container. The TBB does not consider whether a hard cider is made with cider-specific fruits, dual-purpose cultivars, or with dessert cultivars.

2.2 The Rise of the Hard Cider Industry

While many areas of Europe have their own version of the drink that draws on heritages that are thousands of years old, the apple was only brought to the Americas a few centuries ago by European settlers. Watson (2013) documents how hard cider shaped the lives of American colonists in the Northeast from the 17th to the 19th century and quickly became the U.S. "national drink," as both adults and children, the elite and working-class people all consumed the fermented apple beverage.

As seen in Figure 1, in 1899, 55 million gallons (2 million hectolitres) of hard cider were produced in the United States. However, following the passage of the 18th Amendment, production of hard cider decreased to 13 million gallons (492,000 hectolitres) in 1919. The drink was slowly abandoned in the 20th century; in 1990, only 271,000 gallons (10,000 hectolitres) were produced. Hard cider began to make a comeback in the 1990s, and in 1996, the production increased to 5.3 million gallons (200,600 hectolitres) (Watson, 2013).

By 2015, the TBB reported 55 million gallons (2 million hectolitres) produced in the United States (TBB, 2017b). This hard cider boom was in part attributed to the "craft beer" movement, which inspired consumers to experiment with new "craft beverages" (Petrillo, 2016). Watson (2013) further indicates that the hard cider comeback was largely driven by "…large national or multinational brands, whose owners have the capital and the distribution channels to get bottles (and now cans) of their product onto store shelves." In 2016, the production of hard cider went down to 47 million gallons (1.8 million hectolitres), and sales in the United States totaled $209.7 million in revenue (TBB, 2017b; Petrillo, 2016). IBISWorld projects that the recent U.S. decline in hard cider production will level off toward long-term stability, with a 1.2% annualized growth rate in revenue over the next five years (Petrillo, 2016). Even after the dramatic increase in production seen in recent decades, Petrillo (2016) suggests that the hard cider industry is not mature yet; rather, it has passed the stage of quantity growth, and has entered the stage of quality growth.

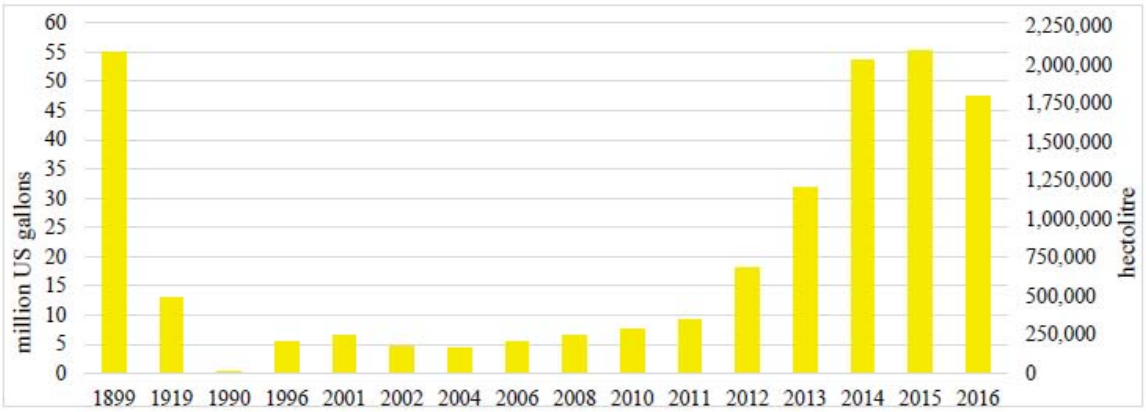

Figure 1. Hard cider production in the United States. Data prior of 2000s are from Watson (2013); data starting in the 2000s are from the TBB and account for the production of bottled hard cider for which taxes were paid (TBB, 2017b)

The hard cider industry in the United States now confronts challenges on both the apple supply side and the hard cider demand side. With the dramatic increase in hard cider production, some U.S. cider makers have expressed concern over the limited supply of cider-specific apples in the domestic market. This gap between the supply of and the demand for cider-specific apples has been researched in several apple-producing states, including Virginia (Farris, Peck, & Groover, 2013), Washington (Galinato, Gallardo, & Miles, 2014), Wisconsin (Baisden, 2015), and Vermont (Becot, et al., 2016a). On the hard cider demand side, Watson (2013) notes that "[e]ven today, when interest in cider is high and sales of major commercial brands are soaring, many people don't quite know what to make of hard cider—what it should taste like, when to serve or drink it. [US-Americans], especially, are still in the process of reinventing a 'cider culture." However, little research has been undertaken on the interest of cider makers in differentiating their hard cider styles, and on the demand for hard cider made with cider-specific apples (Kline & Cole, 2017). In one of the first studies that looked at demand, Tozer et al. (2015), who studied the willingness-to-pay (WTP) of consumers for different hard cider taste profiles, noted, "Given that there are no well-defined standards to categorize [hard] cider styles, such as there are for wines, consumers are faced with a difficult task of making a [hard] cider purchase based on inconsistent information on the product label." Such confusion likely hinders the maturation of the hard cider industry.

2.2 Vermont Hard Cider Industry

In 2015 and 2016, the TBB ranked Vermont as the state with the highest production of bottled hard cider on which taxes were paid (with 9.8% and 8.3% of U.S. total hard cider production respectively) (TBB, 2017b). Cider makers in Vermont are diverse, ranging from independent cider makers who grow some of their apples (orchard-based cider makers), to independent cider makers who do not grow their apples (non-orchard-based cider makers), to the number two hard cider brand in the U.S. (now owned by the transnational company C&C Group plc), which has a market share of 23.3% (Petrillo, 2016). Vermont is also the state with the most ice cider producers in the country (Vermont Ice Cider Association, 2017). As such, Vermont is a crucial player of the U.S. hard cider industry. (Note 1)

On the apple supply side, Becot et al. (2016a) demonstrated that cider makers in Vermont highly value the sourcing of apples from their state or regional (New England) markets. Due to the limited volume currently grown in these areas, Vermont cider makers who want to source more domestically grown cider-specific apples need to either enter or expand cider-specific apple production themselves, or enter strategic partnerships with apple growers that will produce the desired apples for their cidery. However, for apple growers established as dessert apples producers, the diversification of their orchards towards cider-specific production involves managerial, technical and financial challenges. Becot et al. (2016a) further suggest that orchard diversification for hard cider will bring socioeconomic gains to Vermont, including an increase in entrepreneurial activity, the creation of jobs, the enhancement of investments, and the augmentation of the Vermont brand. To concretize these gains, the authors state the need for research regarding long-term formalized contracts or other inter-organizational strategic partnerships that promote orchard diversification, while protecting both the growers' and the cideries' interests. This research project focuses on the feasibility of contracts and cooperative strategies,

as these are the inter-organizational strategic partnerships used by world's leading industries of cider-specific apples.

On the hard cider demand side, little research has been done in Vermont or on a country-wide scale. Understanding the demand for hard cider is further complicated by the shifting identity of the beverage. For example, Citizen Cider presents their Wits Up Cider as one that, "drinks like an ale," and concurrently markets another hard cider named the bRosé as a "lovely summer rosé style cider" (Citizen Cider, 2016). Stowe Cider describes some of their hard cider as "Chardonnay [and] barrel aged cider," while Windfall Orchard declares that their Farmhouse Hard Cider is "…bottle condition for a natural bubble reminiscent of Champagne" (Stowe Cider, 2017; Windfall Orchard, 2017). Lacking a universal hard cider lexicon, cider producers use terms employed to describe other alcoholic beverages, such as beer and wine, to help guide consumers to a hard cider they will enjoy.

2.3 Cider-Specific Apple Management by World's Leading Industries

France, the United Kingdom, and Spain are the three principal producers of cider-specific apples in the world (FranceAgriMer, 2016a). The U.K. has the second largest area of cider-specific orchards, with 7,000 hectares that yield an average production of 200,000 tons of cider-specific apples per year (FranceAgriMer, 2016a). About 80% of cider-specific apples in the U.K. are managed under long-term contractual schemes that legally bind an apple grower and a cider maker. Such contracts can be established up to three years before the trees are planted, and they last on average 20 years (Becot et al., 2016b). These contracts frame discussions about expected yields, quality standards, delivery of fruits, rejection of fruit, pricing, price adjustment, and payment (Becot et al., 2016b). Long-term formalized contracts for cider-specific apple production is said to be a strategic partnership that is mutually beneficial for apple growers and cider makers (Macdonald, 2016).

France claims to have the largest area of cider-specific orchards with 8,700 hectares that yield an average production of 260,000 tons of cider-specific apples per year (FranceAgriMer, 2016b). The *filière cidricole* (French cider industry) is composed of 10,000 fruit producers, 1,500 of which grow strictly cider-specific apples. These producers are located mainly in Bretagne, Basse-Normandie and Haute-Normandie (FranceAgriMer, 2017). Two cooperatives, Agrial and *Les Celliers associés*, manage 85% of *cidre* production in France (FranceAgriMer, 2016a). Today, 80% of the cider-specific apples of the *filière cidricole* are produced under contracts (FranceAgriMer, 2016a). In 2004, Agrial acquired *Cidreries du Calvados la Fermière* (CCLF) and now owns France's largest *cidre* brands: Loïc Raison, Écusson and Kerisac. In 2016, Agrial acquired Seattle Cider Company. In their 2016 annual report, Agrial described this U.S. acquisition as a positioning strategy that would allow the cooperative to capitalize on the world market for the "Anglo-Saxon cider style" that is becoming increasingly popular (Agrial, 2017).

2.4 Geographical Indication Certifications

Fermented apple-based beverage differentiation in the global market is nothing new. It should be noted here that "cider," "*cidre*," "*sidra*," or "*apfelwein*" are not pure translations of terms used interchangeably to designate fermented apple beverages; they also connote different tasting experiences. For example, in the *filière cidricole*, *cidre* implies the "French-style" (a fermented beverage made exclusively from apple or pear juice, with a maximum of 50% concentrate and no added sugar), and *cider* implies the "Anglo-Saxon style" (a sparkling beverage, with no limit on the use of juice concentrate and added glucose syrup) and is described as a "beer alternative" (FranceAgriMer, 2016a).

A popular differentiation mechanism used by fermented apple-based beverage industries is geographical indication certifications (GIs) (see Table 2). GIs can protect both consumers and producers against fraud: because registered labels are regulated by a code of practices that has been verified by authority figures, GI products indicate authenticity to consumers and guarantee that the product is genuine. At the same time, GIs insure producers against free-riding "imitators" (Hopper, Costley, & Friend, 2015; Teuber, 2009). As such, GIs provide a competitive advantage to producers as they, "create[s] an image of 'exoticness,' or scarcity that enables them to obtain premium prices for products that would otherwise be ascribed commodity status. The main source of this exoticness comes from unique quality differences that may be attributed to production in a particular geographical area based on quality characteristics associated with that location" (Agarwal & Barone, 2005).

In their review of the pros and cons of GIs, Giovannucci, Josling, Kerr, O'Connor, & Yeung (2009) offer the following conclusion: "[GIs can] be a unique and powerful tool when adequately managed. GIs can offer a comprehensive framework for rural development since they can positively encompass issues of economic competitiveness, stakeholder equity, environmental stewardship, and socio-cultural value." Among the rural development potentials listed are: "better quality rural employment," "foster business clustering and rural

integration," and increased tourism (Giovannucci et al., 2009). The authors further indicate that the downsides of GIs are largely attributed to bad management: "badly managed GIs can be dominated by limited political interests or just a few enterprises. In some cases, GIs can exclude the poorest producers or even stimulate inappropriate outcomes such as the dissolution of traditional practices or the destruction of biodiversity" (Giovannucci et al., 2009). Giovannucci and his colleagues identify four essential components to the success of GIs: (1) strong organizational and institutional structures to maintain, market, and monitor the GI; (2) equitable participation among the producers and enterprises in a GI region; (3) strong market partners committed to promoting and commercializing over the long term; and (4) effective legal protection, including a strong domestic GI system.

Registered GIs are instruments to facilitate free trade, and as such are regulated by international organizations like the Court of Justice of the European Communities and the World Trade Organization (Broude, 2004). However, Giovannucci et al. (2009) indicate that in the United States, "even unregistered GIs may be recognized as common law marks and thus be enforceable if they rise to the level of a 'source identifier'," which they define as a "trademark term meaning the capacity of a sign to clearly distinguish the goods or services of one enterprise (including a collective group of producers) from those of another enterprise." Florida Oranges, Idaho Potatoes, Maine Lobster, Napa Valley Wine, and Washington State Apples are all examples of preeminent GI brands active in the United States (Agarwal & Barone, 2005). *There are no GIs for hard cider.*

Although GIs are largely based on places of production, they also regularly contain process-based regulations. For example, two categories of *sidra* are marketed under the Sidra de Asturias GI: *sidra* and *sidra natural*. Their differences are largely based on process. Official documentation indicates that "traditional" techniques must be used to market under the *sidra natural* GI. Traditional techniques include wild yeast fermenting, no-filtration bottling, no added CO_2, and no added sugar (Ministerio de Agricultura y Pesca, 2017). Overall, 93% of the Sidra de Asturias production is *sidra natural*, 90% of which is made by independent orchard-based cider makers (Muñoz de Escalona, 2011).

As shown on Table 1, GIs are largely used in Europe. In the United States, as already detailed above, hard cider is regulated as a wine by the TBB. The TBB regulation does contain some GI guidelines in its "Wine Appellations of Origin" section (TBB, 2017c), but the regulations would not be adapted to the hard cider context according to several cider makers that participated in this research project. States and counties are authorized to label under the wine appellation of origin regulations. Several counties in the same wine producing area can collectively register as an American Viticultural Area (AVA). As of November, 20th 2016, there were 239 established AVAs in the United States, 17 of which are multi-state AVAs (TBB, 2016).

Table 1. GI certifications for fermented apple-based beverages

Country	Protected Product	Registered Date
United Kingdom	Gloucestershire cider	1996
United Kingdom	Herefordshire cider	1996
United Kingdom	Worcestershire cider	1996
France	Cidre de Bretagne	2000
France	Cidre Cornouaille	2000
France	Cidre de Normandie	2000
France	Pays d'Auge ; Pays d'Auge-Cambreme	2000
Spain	Sidra de Asturias	2005
France	Calvados	2008
Germany	Hessischer Apfelwein	2010
Canada	Cidre de Glace du Québec	2014
United Kingdom	Traditional Welsh Cider	2017
Finland	Verlados	In review
Spain	Euskal Sagardoa / Sidra Natural del País Vasco	In review
France	Cidre Cotentin	In review

Note. Data from the European Commision database and the Conseil des appellations réservées et des termes valorisants.

In addition to highlighting the place of production or the process, there are other means of differentiating hard ciders. Since 2014, cider makers in Bretagne have held an annual blind tasting contest. In this year's competition, 130 samples spread across 15 different categories were assessed by 88 judges (La maison cidricole de Bretagne,

2017a). A point system based on pre-defined criteria determines which cidre will be honored a medal each year. La maison cidricole de Bretagne, an organization of cider makers from Bretagne, indicates that these distinctions act as true reference points for consumers (La maison cidricole de Bretagne, 2017b). In the United States, the Great Lakes International Cider and Perry Competition (GLINTCAP) organizes an annual competition that is not regional like the one in Bretagne, nor exclusive to U.S. hard ciders; it is an international competition. Such a competition is thus not designed to distinguish between hard styles in the United States per se. Finally, it is important to mention that some differentiation mechanisms, like the Sidra de Manzana Seleccionada certification (a certification that is supplemental to the Sidra de Asturias GI), incorporates place, process and taste regulations (Sidra de Manzana Seleccionada, 2017).

This paper addresses a gap in the literature regarding the coordination of the hard cider supply chain, through two research questions: (1) are long-term contracts and cooperative strategic partnerships suitable to the Vermont hard cider industry to stimulate cider-specific apple production, and (2) are cider makers in Vermont interested in working under a geographical indication certification (GI) to differentiate their hard cider styles and increase demand for hard ciders made with cider-specific apples.

3. Methods

This study was conducted using a mixed methods approach. Using fourteen semi-structured and open-ended interviews, the first part of the project explores the perspectives of apple growers, cider makers, and other key industry stakeholders on stimulating cider-specific apple production in Vermont. The second part of the project summarizes the results from an online survey that measured the interest of cider makers in Vermont in working under several types of GIs. Table 2 provides a summary of the methods used, associated research questions, and participants.

Table 2. Method used, the associated research question, and participants

Mixed Methods	Research Questions	Participants
Semi-structured interviews	Are long-term contracts and cooperative strategic partnerships suitable to the Vermont hard cider industry to stimulate cider-specific apple production?	5 apple growers 6 cider makers 3 other industry stakeholders
Online survey	Are cider makers in Vermont interested in working under a geographical indication certification (GI) to differentiate their hard cider styles?	9 out the 14 Vermont cider makers listed by the Vermont Tree Fruit Growers Association

3.1 Interviews

For the first part of the project, data were collected through fourteen semi-structured interviews conducted with Vermont hard cider industry stakeholders. The director of the UVM Horticulture and Research and Education Center acted as a key informant and provided the research team with contacts. Maximum variation sampling (Patton, 2015) was used to ensure a wide representation of stakeholders and to minimize biases that could arise by only interviewing one particular group of stakeholders. For apple growers, the variation criteria was based on orchard acreage to ensure that all types of apple production operations were represented in the study. Participants ranged from growers who manage fewer than 15 acres and focus mainly on the "pick-your-own" market, to growers that have 200 acres or more in production and focus primarily on the wholesale market, to growers who sell to both markets. For cider makers, the variation criteria were based on the type of operation (orchard-based cideries, and non-orchard-based cideries) to capture the perspectives of all types of operations and hard cider styles. In total, the project included five apple growers, six cider makers, and three other stakeholders (the former president of the Vermont Tree Fruit Growers Association, one of the owners of an apple storage facility, and the executive director of the Vermont Fresh Network). To ensure anonymity of participants evolving in this small and tightly-knit industry, further particularities or potential biases cannot be provided here. As such, the variation criteria listed above will serve to provide generic categorization profiles for participants.

Participants were interviewed between February and September 2016. One cider maker and one apple grower selected were located just outside Vermont borders, but were included in the project as they were identified by many stakeholders as highly connected to and influential in the Vermont hard cider industry. Long-term formalized contracts and cooperative models were the two main strategic partnership mechanisms presented, although the semi-structured interview format allowed for other strategies to be discussed. The interview guides were inspired by the strategic partnerships that are in place in the well-established cider-specific apple industries of the United Kingdom (long-term formalized contracts), and France (cooperative). The interview protocols were

approved by the University of Vermont Committees on Human Subjects (IRB#16-358) and were classified as "exempt."

Eleven interviews were conducted in person, two were conducted over the phone, and one over email. Interview recordings were 52 minutes on average; the shortest one is 20 minutes and the longest one is 90 minutes. Interviews were transcribed verbatim for open-coding analysis of emerging themes in HyperResearch 3.7.3. The codes created for the analysis were generated to link the data to the specific research objectives, namely to measure interest in establishing strategic partnerships within the Vermont hard cider industry to stimulate cider-specific apple production in the state. In addition, sub-codes related to diversification barriers and infrastructure needs were used to identify motives for interest in strategic partnerships.

3.2 Survey

In November 2016, an online survey was sent to all fourteen Vermont cider makers listed by the Vermont Tree Fruit Growers Association to measure interest in the introduction of a label that would differentiate and define hard cider styles. The survey was open from November 2016 to February 2017. Out of the fourteen cider makers contacted, nine participated. The survey was designed using elements of GI certification of the world's leading cider industries. Three types of labels were proposed: a place-based label, a process-based label, and a taste-based label. An open-ended question allowed participants to elaborate on their answers. The survey did not specify the process for establishing or managing the proposed labels. The intent of this research project is not to establish a specific labeling regime, but rather to foster further discussion and research regarding the demand in Vermont and the U.S for hard cider made with cider-specific apples.

The place-based label proposed by the survey identified the Lake Champlain watershed area as a hard cider AVA (see figure 3), and the Vermont State borders were suggested to serve as an appellation of origin. The Champlain Valley and the state of Vermont were referred to by several cider makers as their perceived apple growing regions during the interview process. Testing a label that specified the Lake Champlain watershed area—which encompasses the Champlain Valley— was decided upon by the research team as a way to make the largest number of cider makers in Vermont feel included in GI conversations, while staying within a single growing region. In addition, some of the place-based labels tested also included process-based standards, which served to differentiate hard cider styles within a defined place. The survey also tested a label that indicated only process-based standards that could be applied regardless of production area. Finally, the survey asked Vermont cider makers to indicate their interest in working under a taste-based label that would be built around blind tasting procedures designed to differentiate between hard cider styles.

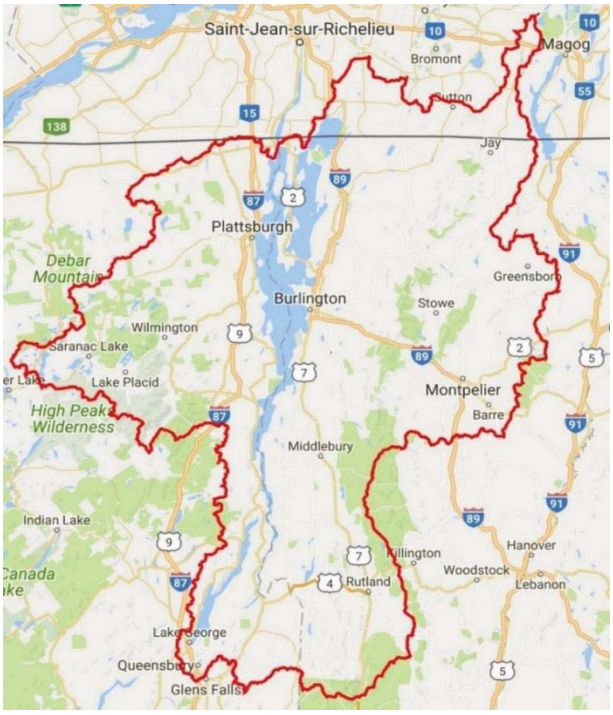

Figure 2. One of the hard cider AVA proposed: The Lake Champlain watershed area. Map from Google Earth4. Results

4.1 Interviews' Results

4.1.1 Cooperative

For the most part, interviewees were wary of the idea of using a cooperative model for coordinating cider-specific apple production. About a decade ago, an apple growers cooperative was shut down. The Shoreham Coop (1946-2002) was a storing, packaging and marketing hub that handled at its peak (in 1986) about half of Vermont apple production. Interviewees listed mismanagement and the apple industry crash of the 1990s as the primary factors that forced the coop to shut down. As a result of the relatively recent Shoreham Coop collapse, interviewees were not enthusiastic about the strategy of forming a new cooperative, even though it might facilitate cider-specific apple production by allowing growers to share infrastructure. Here is how an apple grower verbalized the matter: "Short term I think a lot of growers would be pretty apprehensive about entering in any kind of coop (…) there is still some bad feelings about the coop in Shoreham that felt apart, and guys might not be willing to... for some guys it is still very fresh to their memories so they are maybe feeling bitter about what happened there" (Apple Grower 1). Discussions regarding the cooperative model felt short with both apple growers and cider makers, and expressed interest was low.

4.1.2 Contracts

In the present-day Vermont cider industry, most of the apple sales between apple growers and cider makers are made through what interviewees referred to as "handshake agreements." Handshake contracts are embedded in the current apple agricultural practices of Vermont; they are very appealing to growers who have a desire for independence and flexibility. Although several interviewees indicated that discussions have taken place regarding making handshake agreements more formal by making them written, the strong cultural ties of current apple growers to handshake agreements is a key factor preventing contract formalization in Vermont. An orchard-based cider maker reported: "[apple growers] prefer to remain independent … so our partnerships are built the good old fashion way, on relationship and a handshake, and on honoring of these commitments year over year" (Cider Maker 6). As detailed hereafter, orchard scale and cider-specific apple prices are, with the handshake culture, the other two main elements preventing the formalization of contracts.

4.1.3 Contract Scale

Regarding scale, an orchard-based cider maker asserted: "Every time we sit down and try and think about structuring an agreement, the issues are: the scale is too small, what happens if there's a hailstorm? A big contract makes sense when you have like a Bulmers in Ireland that can guarantee a market for 2 million bushels of fruit" (Cider Maker 3). The way orchards operate in Vermont is vastly different as compared to the orchards from whom Bulmers sources their cider-specific apples. Apple growers expressed that growing cider-specific apples is an endeavor that requires means of production that are very different from how their orchards are currently organized for the production of dessert apples: "I think that is the problem, right, it is a different culture for growing cider apples than the culture of growing conventional apples…an orchard with hard cider in mind, it has to be separate, it has to be its own entity. Either the cider block is like a detriment to your conventional orchard, or you are putting the same amount of money and you are cutting your margins down" (Apple Grower 5). Some growers expressed fear of contamination of dessert blocks from cider blocks: "Replanting just cider-specific is not going to happen. Because I do not know any varieties that would not be obliterated by fire-blight" (Apple Grower 4). This is because cider blocks (which aim at producing juice) require less pesticide applications and overall maintenance than dessert apple blocks (which aim at producing apples with high cosmetic standards).

4.1.4 Contract Pricing

Regarding price within long-term formalized contracts, a non-orchard-based cider maker indicated that the same issue preventing cull (a word that refers to low-grade dessert apples) contracting would likely arise with contracts for cider-specific apples: "It's something to look towards in the future, but right now there is really no value for a grower to be in a contract for cull pricing, nor is there for cider makers to agree to a price for culls, because they may swing widely" (Cider Maker 4). Apple growers stated that prices for cider-specific apples are uncertain over the long-term, especially compared to the prices for dessert apples, on which they have relied for years. Many growers were skeptical that the current high value for cider-specific apples will persist as more and more growers start producing them. Cider makers are also reluctant to engage in long-term formalized contracts for cider-specific apples because of the low perceived demand for hard cider made with cider-specific apples: "The problem is...this would all be easy if we could sell cider for $25 a bottle. This would go away. There is only 1% of cider makers who are selling hard cider for a price that can justify the contract, so this is completely theoretical in the U.S. market" (Cider Maker 4). To balance financial risk and encourage orchard diversification, several apple growers and cider makers expressed the need for financial and technical support from university

extension to create a cider-specific growing program.

Table 3. Summary of the interest of interviewees regarding the use of long-term formalized contracts in stimulating cider-specific apple production

Participant	Long-term formalized contract interest
Apple Grower 1	for dual-purpose
Apple Grower 2	none
Apple Grower 3	none
Apple Grower 4	none
Apple Grower 5	for dual-purpose
Cider Maker 1 (orchard-based)	none
Cider Maker 2 (orchard-based)	none
Cider Maker 3 (orchard-based)	none
Cider Maker 4 (not orchard-based)	eventually
Cider Maker 5 (not orchard-based)	interested
Cider Maker 6 (not orchard-based)	none
Stakeholder 1	eventually
Stakeholder 2	eventually
Stakeholder 3	no opinion

4.1.5 Demand for Hard Cider Made with Cider-Specific Fruits

The emerging theme from these interviews regarding long-term formalized contracts is that the core issue preventing cider-specific apple production in Vermont is on the hard cider demand side, rather than on the apple supply side. One non-orchard-based cider maker indicated: “Currently fruit-flavored ciders made from dessert apples are the dominant growth area for cider, along with the addition of hops and ginger” (Cider Maker 4). An orchard-based cider maker expressed how, “there's nothing special about the apples in relationship to that cider and where the growth right now is in these ciders that are made from leftover dessert fruits, and boy those are apples that people know how to grow, they are already doing it (…) Where is the demand for super unique expensive fruit?” (Cider Maker 3).

4.1.6 Establishing Completely New Cider-specific Orchards

The semi-structured interview format allowed for another strategy (other than long-term formalized contracts and cooperatives) to be discussed: the establishment of completely new orchards, operated strictly for hard cider production. This solution generated positive feedback across all interviewee profiles, from orchard-based cider maker: “That would be a totally new orchard, and I think there is a lot to be said for that” (Cider Maker 1), to apple grower “To me that seems like the most clear-cut way to do it” (Apple Grower 5). In regards to establishing new orchards, a non-orchard-based cider maker said, “We would have to partner or hire whole bunch of expertise that we don't currently have to do that, but you know, things change” (Cider Maker 4). One apple grower indicated that some newcomers have been testing the idea of partnerships for establishing cider-specific orchards: “there were some people that wanted to be partners and plant 100 acres of that hard cider apples (…) all mechanically harvested, so that they have virtually no labor. They would have a big press running 24 hours a day and machines picking the apples (…) they had 2 million dollars to start with” (Apple Grower 2).

4.1.7 Differentiating Hard Cider

Many cider makers were adopting a differentiation rationale. This differentiation narrative is essential to the development of convincing GI labeling. The affirmation of their distinctiveness (from European ciders) is clearly articulated in the following quote taken from an interview conducted with a pioneer of the hard cider revival in the American Northeast:

“But there was a time in the late 90s or about 2000 when people were tasting and we were just beginning to learn how to actually do sensory analysis, we have not got really far down that road, but we started to say to each other: we are encountering aromas and flavors and feels in this cider that we are making from this fruit grown here that we never encountered in England, France, or anywhere else. And then it was really just why the fuck are we trying to do something imitative. Why don't we just say: right, we are apple growers, we found some really good stuff that grows that we think make really good cider, and now we are going to make something that is delicious by our likes, and that we think that is reflective of our fruits, and the land on which is grown and what we do there” (Cider Maker 2).

In other words, there are cider makers in the American Northeast who have developed hard cider with unique characteristics, and which differ from European ones. Such unique characteristics may provide a competitive advantage as consumers become aware of them.

4.1.8 Apple Supply Summary

In short, the interviews established that the current culture of handshake agreements, the relatively low volume of apples produced by apple growers, the price point for cider-specific apples, and the lack of cider-specific growing experience in the state make the notion of long-term formalized contracts ill-adapted to today's Vermont hard cider industry. The interviews also indicated that the recent collapse of the Shoreham Coop makes current hard cider actors distrustful of using this strategy to stimulate cider-specific apple production. A potential solution that has emerged from the interviews is the establishment of completely new orchard enterprises dedicated only to cider-specific apple production.

4.2 Survey's Results

Participating cider makers unanimously agreed that helping consumers differentiate between hard cider styles would benefit the Vermont hard cider industry. When asked which of the three proposed labels they would prefer to work under, five cider makers indicated place-based label, two indicated the taste-based label, and one indicated the process-based label. One cider maker did not specify any preference, and in fact rejected any place-based label. However, by analyzing the rest of the survey's data—which measures interest in each label instead of preference between the labels— a taste-based label generated more consensus, followed by a place-based label and a process-based label. Below is the breakdown of the expressed interest for each proposed label.

Table 4. GIs interests and preferred based

Cider Makers	Place-Based	Process-Based	Taste-Based	Preferred Based
A	Interested (Champlain Watershed)	Interested	Very interested	Place-Based
B	Not interested	Somewhat interested	Interested	Process-Based
C	Interested (Other Disjointed Areas)	Very interested	Very interested	(no answer)
D	Interested (Other Disjointed Areas)	Interested	Interested	Place-Based
E	Very interested (Vermont state border)	Interested	Very interested	Place-Based
F	Somewhat interested (Vermont state border)	Somewhat in Interested	Interested	Taste-Based
G	Very interested (Vermont state border)	Very interested	Very interested	Place-Based
H	Interested (Vermont state border)	Not at all interested	Not at all interested	Place-Based
I	Not interested	Somewhat in interested	Very interested	Taste-Based

4.2.1 Taste-Based

In the survey, a taste-based label was described as one that focuses exclusively on the final taste of a hard cider. Defined taste standards for different hard cider styles were not detailed, as these would have to be defined and certified by a panel of elected judges. All participating cider makers but one were either interested or very interested in submitting some of their hard ciders to an annual tasting evaluation with a panel of judges that would assess if their hard ciders qualified to be sold under a taste-based label. In the open-ended section of the survey, one of the cider makers interested in a taste-place label submitted an alternative labeling mechanism to an annual judging competition: "[a label that would have for visual] a multi-dimensional scale that would define standard levels represented by a label with a circle with four quadrants, where the quadrants have [a standard] indicator for sweetness, tartness, tannin, and fizz levels…such taste-based label is easiest to implement because it doesn't 'judge' quality of cider, and it does the most to help consumers identify a cider they will enjoy drinking…If we can all agree on a taste indicator, then it will have the full weight of all our cideries behind it" (Cider Maker I).

4.2.2 Place-based

A place-based label was described in the survey as one that differentiates a cider by putting forward a story of the unique characteristics of a place—or terroir. Within the place-based label, two process-based subcategories were proposed to further differentiate between the hard cider styles produced within a single area. Four cider makers expressed interest in working under a label based on the Vermont state borders, one under a label based on the Lake Champlain watershed area, two under a label based on more disjointed areas delineated by growing conditions, and two cider makers rejected the idea of working under a place-based label. In terms of process regulations contained within a place-based label, all participating cider makers (but one) agreed that under a place-based label, certified hard ciders should be made 100% from apples grown within the chosen area; the participant who did not agree suggested a threshold of 75%. In addition, all participating cider makers (but one) indicated that under a place-based label, certified hard cider would have to be made from 100% fresh-pressed apples. Six cider makers also indicated that to be certified, a ciderie would have to be located within the chosen area; two were unsure regarding this parameter, and one was opposed. Furthermore, five cider makers were in favor of having two subcategories in a place-based label that would categorize hard cider by style by imposing additional processing standards.

4.2.3 Process-based

A process-based label was described in the survey as one that does not focus on a particular area of production, but rather exclusively on the types of fruit and processes used, in a way that such a label could be used by any cider maker in the United States. The different sets of standards that would serve to differentiate between hard cider styles were not fully developed in the survey. Five cider makers indicated being either in favor or strongly in favor of working under a process-based label, three indicated being somewhat in favor, and one was not at all in favor.

5. Discussion

The goal of this research project was to gauge the interest of hard cider industry stakeholders in various strategies to stimulate the production of cider-specific apples domestically, as well as to explore stakeholder interest in GI labeling to differentiate between hard cider styles. Overall, the findings of this study indicate that before investing in planting cider-specific apple trees, there must be a plan in place to increase demand for hard ciders made with cider-specific apples. The recent decline of hard cider production in the U.S. reinforces the need for marketing strategies that aim at retaining and growing consumer demand.

Semi-structured interviews focused on the interest in and feasibility of strategic partnerships, specifically long-term formalized contracts and cooperatives, and established that both mechanisms are ill-adapted to the current Vermont hard cider industry context. In contrast to the cider industry in the U.K. and France, the current scale of the industry in Vermont is not conductive to using formalized long-term contracts. The Vermont hard cider industry is still emerging, and the value of cider-specific apples remains uncertain in the long-term, undermining the utility of long-term formalized contracts. Regarding cooperatives, the recent shutdown of the Shoreham Coop makes most stakeholders distrustful of a cooperative strategy. As such, the use of cooperative models involving experienced stakeholders is improbable in the short term—although follow-up research might prove otherwise as the Vermont hard cider industry reach maturity, or as new stakeholders establish themselves. Establishing completely new orchards dedicated to cider-specific apple production, which would increase the production of cider-specific apples domestically, was further identified as an alternative to long-term formalized contracts and cooperatives.

The surveys established that cider makers are ready to develop and introduce GI labels to differentiate between their hard cider styles, and increase consumer demand and literacy for hard cider made with cider-specific fruits. The cider makers surveyed were unanimously in favor of establishing some kind of differentiation mechanism, and both place-based and taste-based labels were identified as being of high interest. Process-based labels were also of interest, but more as a complement to a taste-based or place-based label rather than as a label that stands on its own. Although participants did not agree on a particular area for a place-based label, this research project provides useful data to inform and foster further discussions surrounding hard cider GI label development. Researching and interviewing cider makers for the present project, it became evident that the hard cider culture in Vermont does not stand as its own, but actually considers itself part of a broader hard cider culture that transcends Vermont state political borders.

As detailed in the literature review, GIs can serve as more than a differentiation tool; when properly managed, they can be a powerful economic development tool generating jobs and tourism, and can promote rural business clustering. The hard cider industry possesses all of the assets required by Giovannucci et al. to initiate the

development of successful hard cider GIs in the U.S. The first component, having "strong organizational and institutional structures to maintain, market, and monitor the GI," could be fulfilled by the United States Association of Cider Makers, or by a regional branch created specifically for regulating hard cider GIs in a particular region. In regards to the second and third assets listed by Giovannucci et al. ("equitable participation among the producers and enterprises in a GI region" and "strong market partners committed to promote and commercialize over the long term"), this research project indicates that cider makers from all types of profiles are willing to work under a GI in the area of study. This strong interest is a promising first step towards the inclusion of all actors in the development and management of a hard cider GI. In terms of the last component (effective legal protection including a strong domestic GI system), there are already other GI examples in place the U.S.

A GI for hard cider has the potential to succeed where long-term formalized contracts and cooperatives appear to be ill-adapted to the Vermont context. Agarwal & Barone (2005) indicate that when GIs are introduced, "new entrants will enter the geographical area to take advantage of the brand equity residing in the GI." The stimulation of cider-specific apple production could come with the introduction of a hard cider GI, which may attract new cider makers and related businesses to the area. This could lead to the development of a cluster of orchard-based cideries eager to capitalize on the competitive advantage provide by the GI, and may also bring enterprises with the capital to build completely new cider-specific orchards in the region specified by the GI.

In all cases, a marketing campaign is likely necessary if the hard cider industry goes forward with the introduction of GIs. Confronted with a decrease in demand for cidre, FranceAgriMer (2016a) prepared a report for the filière cidricole in France in which it suggested increasing the demand for cidre through a marketing campaign based on cidre distinguishing "authenticity," that would differentiate cidre from the increasingly popular "Anglo-Saxon cider style." A similar differentiating campaign would likely be necessary in the U.S. The socio-historical elements surrounding the revival of a beverage that was once labeled the "national drink" provide a compelling narrative for such a marketing campaign.

6. Conclusion

This research project has identified the establishment of a hard cider GI as the most promising strategy—compared to long-term formalized contracts and cooperatives—to tackle both cider-specific apple supply issues and hard cider demand challenges. The focus of the GI label introduced in this study was the American Northeast, more precisely Vermont and its surroundings, but the findings have implications for the whole U.S. industry. It should be noted here that although the research team made sure to study a wide variety of stakeholder profiles to limit biases, the content of the results section only reflects the views of those who participated—whom are likely stakeholders who had stronger opinions to voice and who enjoy taking surveys.

Although not all Vermont cideries answered the survey, the high response rate and the shared interest expressed by participants for the introduction of a GI indicate that the hard cider industry in the area of study is mature enough to initiate the development of a GI certification. The discussion section of this research paper has further established that the U.S. hard cider industry possesses all the assets required for the successful implementation and management of one—and potentially several—hard cider GI(s). This research project has also detailed how a GI can act as a powerful economic development tool. Measuring the interest of the rest of the U.S. hard cider industry would thus be valuable to locate other areas and additional cideries interested in hard cider GI discussions.

In addition to providing evidence that the right conditions exist for the establishment of a hard cider GI in the U.S., this research project has laid the groundwork for the development of a GI in the Northeast region. The discussions between the research team and the participants have jump-started the conversation regarding the appropriate basis for a Vermont GI (place-based, taste-based, or process-based). This study has detailed how such a GI could include more than one parameter; for example, a hard cider GI that is taste-based, with each taste further associated with a particular area and the result of a certain process. Ultimately, it is up to cider makers and their associations to further develop a hard cider GI regime and to delimit appropriate tastes, areas or processes.

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Notes

Note 1**:** Ice cider is a fermented apple-based beverage closely related to hard cider.

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Effect of Drying on Quality and Sensory Attributes of Lemongrass (*Cymbopogon citratus*) Tea

Phumudzo Mabai[1], Adewale Omolola[1] & Afam I. O. Jideani[1]

[1]Department of Food Science and Technology, University of Venda, South Africa

Correspondence: Afam I. O. Jideani, Department of Food Science and Technology, University of Venda, P.M.B X5050, Thohoyandou, 0950. South Africa. E-mail: Afam.Jideani@univen.ac.za

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Abstract

The aim of this study was to investigate the effect of drying on quality and sensory attributes of lemon grass (*Cymbopogon citratus*) tea. Lemongrass (*C. citratus*) leaves were dried using four different drying methods: sun, solar, oven (40, 50, and 60°C), and microwave (50 W). Teas made from the grass were analyzed for colour, pH and sensory attribute. Data obtained were statistically analyzed using SPSS Version 23 one way analysis of variance and means were compared using Duncan multiple comparison test ($p < 0.05$). Results obtained indicate that after drying the moisture content were significantly reduced. Ash content results showed no significant difference amongst lemongrass samples dried under difference drying methods. However, there was a significant difference ($p < 0.05$) in the pH of tea made from the lemongrass dried under different drying methods. Results indicate that drying temperature and time are the main factors affecting the colour of dried lemongrass leaves for tea. There was a significant difference ($p < 0.05$) in the colour profile of the dried leaves. Sensory evaluation results showed that the colour, aroma, taste, and overall acceptability scores of tea from lemongrass dried with oven at 40°C was highest. The study revealed that oven drying at 40°C for 15 hours was found to be most suitable for drying of lemongrass leaves for tea production in order to retain appreciable sensory attributes.

Keywords: lemongrass, drying methods, tea, organoleptic properties, chemical properties, sensory evaluation

1. Introduction

It is generally accepted that tea is one of the most popular beverages in the world. The demand for high-quality dried food products is permanently increasing all over the world. The main purpose of drying is to extend product shelf life, minimize packaging requirements and reduce shipping weights (Hamrouni-Sellami et al., 2012). Drying process increases the shelf life by slowing microbial growth and thus preventing certain biochemical reactions that might alter the organoleptic characteristics (Díaz-Maroto et al., 2003; Hamrouni-Sellami et al., 2012). Lemongrass (*Cymbopogon citratus*) is an herb that belongs to the genus *Cymbopogon* of aromatic grasses and contains essential oil with fine lemon flavour (Nur Ain et al., 2011). It is a tallperennial grass widely cultivated in Brazil for medicinal purposes, especially as tea and its essential oil (Martinazzo et al., 2009); grows up to 90 cm in height and 5 mm wide (Nur Ain et al., 2013); is highly sought after in the nutritional, pharmaceutical and flavouring industries (Lonkar et al., 2013). Cultivated on a large scale, especially in tropics and subtropics (Akhila, 2010), lemongrass is commonly used in folk medicine for treatment of nervous and gastrointestinal disturbances, and as an antispasmodic, analgesic, anti-inflammatory, anti-pyretic, a diuretic and a sedative (Brian & Ikhlas, 2002; Santin et al., 2009; Lodhi et al., 2014). It contains active ingredients like myrcene, an anti-bacterial and the pain relievers, citronella, and geraniol (Blanco et al., 2009). India is the largest producer of lemongrass of which about 80% is exported. Leite et al. (2000) also reported that lemongrass tea can be used to treat fevers, colds, coughs and stomach upset. With its wide use as an ingredient for cooking, Berry (2004), describes lemongrass as the rising star in the herbal world, having both floral and fragrant characteristics that add an instant exotic appeal to food and beverages.

Teas from various sources are available in popular flavours (spicy, smoky, cinnammon-like, sweet ether-like, weak sulfurous) and are consumed for amongst other benefits, their antioxidant properties (Hara, 1994; Hengel and Shibamoto 2013). Lemongrass tea has a flavour very true to the flavour of the herb itself, a characteristic lemon flavour due to its composition, rich in volatile oils that present chemical components of great importance to the industry (Barata et al 1998). This study investigated the effect of drying on quality and sensory attributes

of freshly harvested lemongrass (*Cymbopogon citratus*) tea.

2. Materials and Methods

2.1 Sample Collection and Preparation

Fresh leaves of lemongrass were collected with permission from a farmer in Matangari Village in Limpopo province, South Africa. The samples were selected on the basis of fresh green leaves. The leaves were washed with water and cut into small pieces with a clean scissors/stainless steel knife (Lonkar et al., 2013). All procedures including tests were performed in triplicate.

2.2 Drying Experiments

Fifty (50) g of fresh chopped green lemongrass were dried using sun, solar dryer (Janjai et al., 2002),microwave (model P70B17L-T8)at 50 W, and oven (Prolab Instrument - model OTE 80) at 40°C, 50°C, 60°C (Lonkar et al., 2013; Nur Ain et al., 2013). During the drying process, the weight of the sample were monitored at regular intervals (hourly) using weighing balance (ADAM AAA 300L/PW 254) and the process was stopped at a point where the weight of the sample remained constant (Lonkar et al. 2013). The dried lemongrass was stored in an airtight bag plastic bag until further analysis (Lonkar et al., 2013 & Nur Ain et al., 2013).

2.3 Quality Analysis

2.3.1 pH of Dried Lemongrass Leaves

A pH meter (Crison instrument, 042030, S.A) was used to assess the pH of each sample. Fresh standardization solutions of pH 4.01, 7.00, and 9.21 were used to standardize the instrument before using and after every five or six reading (Vargas et al., 2008).

2.3.2 Colour of Lemongrass Tea

A colorimeter (Hunter Lab s/n: cx2540) was used to measure the *L** (Lightness), *a** (Redness), and *b** (Yellowness) colour parameters of each tea sample. The colorimeter was calibrated with a standard white (L* = 93.71, a* = –0.84 and b* = 1.83) and black plate prior to each colour measurement.

2.3.3 Moisture Content of Dried Lemongrass Leaves

Dried lemongrass cuts (3 g) were dried in pre-weighed crucibles in an oven at 105°C for 3 hours, and cooled in desiccator for 30 min. Moisture content was calculated from difference in weight according to A0AC, 2007 method number 945.32. The following formula was used to calculate the moisture content (MC) of dried lemongrass:

$$MC\ \% = W_2 - W_3 / W_2 - W_1 * 100$$

where:

W_1 = Initial weight of empty crucible

W_2 = Weight of crucible + samples prior drying

W_3 = Final weight of crucible + sample after drying

2.3.4 Ash Content of Dried Lemongrass Leaves

Dried samples (3 g) in pre-weighed crucibles were transferred and kept in a muffle furnace at 550°C overnight and left until a light grey ash resulted, and cooled in a desiccator for 30 min before weighing (Aftab et al., 2011). The following formula was used to calculate ash content (AC) of lemongrass samples.

$$AC\ \% = W_3 – W_1 / W_2 - W_1 * 100$$

where:

W_1 = Weight of empty crucible

W_2 = Weight of crucible + samples prior drying

W_3 = Final weight of crucible + ash

2.4 Preparation of Tea from Dried Lemongrass Leaves

The unsweetened lemongrass teas were prepared using each of the dried samples as follows: sample (18 g) was weighed and put into a flask (1.8 L) to which boiling water (99.7^{o}C) was added. The teas were left to brew for 5 min and then passed through a 106 μm sieve. The tea was not sweetened.

2.5 Sensory Evaluation of Tea

Sensory evaluations of the teas brewed as described above were conducted in the Department of Food Science and Technology using 50 untrained panellists. The lemongrass tea were evaluated with respect to colour, aroma, taste, and overall acceptability using the 5-point hedonic scale where 1 represents dislike extremely and 5 like extremely respectively. Tea samples were coded and served randomly to panellists to avoid bias (Hashin et al., 2009). About 30 ml of each tea samples was served in a 120 ml paper cup. The tea samples were approximately 67.2°C at the time of tasting. The panellist were served and instructed to rinse their mouth with warm water (45.7°C) in between evaluating each tea sample to minimize the lingering tastes (Meilgaard et al., 1999).

2.6 Statistical Analysis

The statistical software package SPSS Version 23 program was used to analyse all experimental data collected. All comparisons were subjected to a one-way analysis of variance (ANOVA), and significant differences between treatments means were determined using Duncan's multiple range test (Duncan, 1955) at $p < 0.05$.

3. Results and Discussion

3.1 Moisture Content of Fresh Lemongrass Leaves

The initial moisture content of the freshly harvested lemongrass samples was found to be 73 %. It could be observed from Table 1 that the final moisture content of lemongrass samples varied with respect to the different drying methods.

Table 1. Effect of drying methods on moisture content of dried lemongrass

Drying methods	Temperature (°C)	Time (h)	Final moisture content (%)
Sun	34.7	10	3.33 ± 2.89
Solar	50.6	8	5.00 ± 0.00
Oven	40	15	5.00 ± 0.00
	50	8	5.00 ± 0.00
	60	6	2.10 ± 0.00
Microwave	**Power (W)**		
	50	0.9	3.33 ± 2.89

The final moisture contents of lemongrass samples ranged from 3.33 to 5.00%. The moisture content was in the range of 0% - 5% for different drying conditions. Products dried using sun and microwave at 50 W had similar (3.3%) amount of moisture content, however the highest (5%) amount of moisture were in solar, oven at 40°C, and 50°C while the lowest (0%) was oven at 60°C (Table 1). The shelf stability of a food product depends on the moisture content i.e. the higher the moisture content, the lower the shelf stability and vice versa (Fennema, 1996).The moisture content of dried lemongrass tea leaf samples were within the recommended moisture content range (3-12% (w/w) (Barbosa et al., 2008). This is an indication that all the dried samples are likely to stay longer before use or processing due to their low moisture content since the low moisture content of the leaves coupled with drying could hinder growth of microorganisms, hence storage life would be longer (Awogbemi & Ogunleye 2009).

3.2 Ash Content of Dried Lemongrass Leaves

The ash contents of lemongrass samples dried under the different drying conditions is as shown in Figure 1. The ash content of lemon grass samples varies with respect to the drying conditions. The ash contents of lemongrass samples ranged from 6.67 to 7.78, with oven dried at (60°C) samples having the highest ash content (7.78%) while sun, solar, oven (40°C, 50°C) and microwave (50 W) had the same (6.67%) ash content. Ash content is as a measure of the total amount of minerals present within a food. According to Kirk & Sawyer (1997), the range of the ash content of dried samples is 5.2% to 7.2% for teas. Product dried by sun, solar, oven at 40°C, 50°C, and microwave were within the recommended range of 5.2% to 7.2%. This is an indication that lemongrass leaves has a considerable amount of minerals. However, oven at 60°C exceeded the recommended range of 5.2% to 7.2% since it has the highest (7.78%) amount of ash, this could be attributed to the reduction in moisture content during drying that resulted in corresponding increases in dry mater content due to concentration of soluble solids (Tetteh, 2009). There was no significant difference amongst the ash content of lemongrass samples dried under difference drying methods (Table 2).

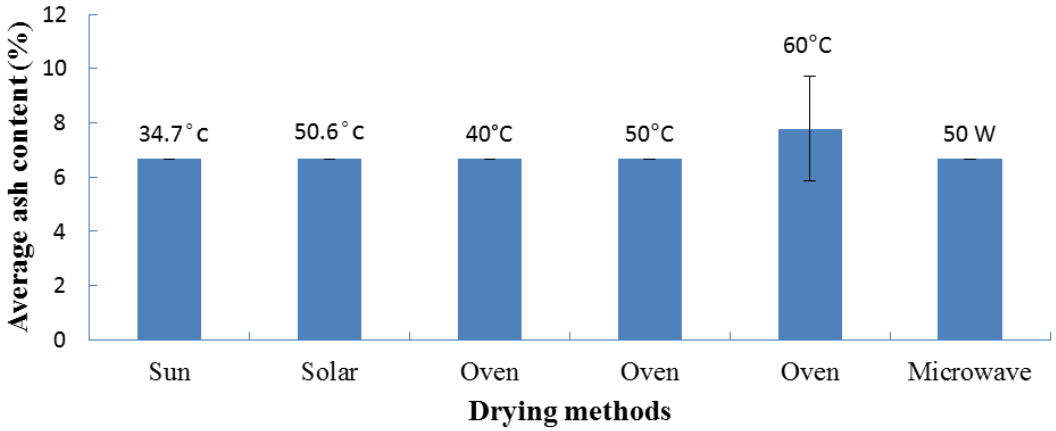

Figure 1. Average ash content of dried lemongrass under different drying methods

3.3 pH Measurement of Lemongrass Leaves

The lemongrass tea samples from different drying conditions gave pH readings as shown in Figure 2.It shows variation of ash contents of lemongrass samples with respect to the drying conditions. The pH contents of lemongrass samples ranged from 5.8 to 6.3.Variation in pH might be attributed due to delay in drying, wherein the leaves started to ferment even though they were stored in the cold room before analysis. The variation in pH values could further be explained by factors such as exposure times to drying air, drying air temperature, relative humidity in the drying site, nature of drying air flow as previously indicated by Franke et al., (2008). Almost similar pH readings were obtained from the analysis of lemongrass in Ghana (De-heer, 2011). There was a significant difference amongst the pH of the samples dried under different drying methods (Table 3). De-heer (2011) conducted a study in Ghana on formulation and sensory evaluation of herb tea from *Moringa oleifra, Hibscus scibdariffa* and *Cymbopogon citratrus* and obtained a pH of 4.53. The pH found in this study was higher than that reported by De-heer (2011). This could possibly be due to variation in climatic condition and the soil types.

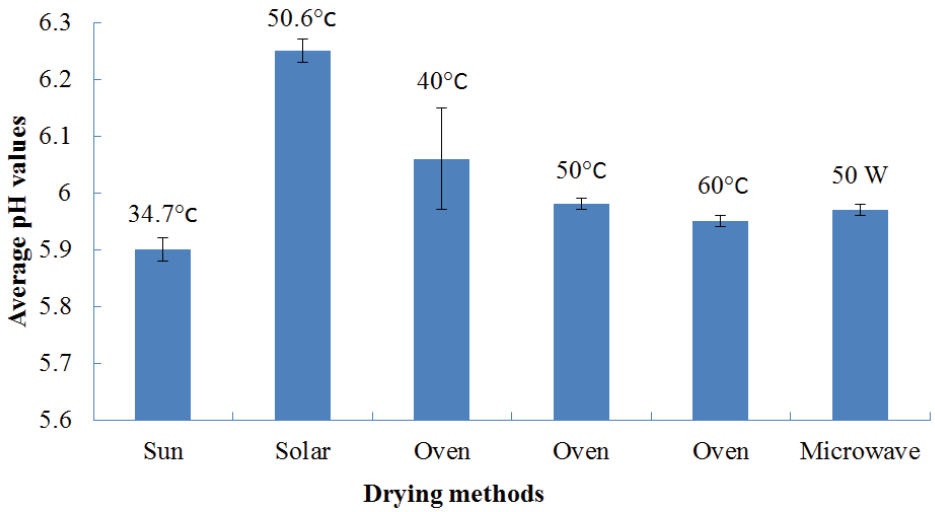

Figure 2. Average pH of dried lemongrass dried under different drying methods

3.4 Instrumental Colour Analysis of Lemongrass Tea

Colour is an important attribute of dried product from the consumer's acceptability view (Inchuen et al., 2010). It is also considered as an important quality indicator for acceptance of the final product in the market (Soysal, 2004).The results shows that drying temperature and time are the main factors affecting the colour of dried lemongrass leaves for tea. Similar observation was made by Somkiat et al., 2004. Oven at 40°C, 50°C, and 60°C (Table 2), shows the reduction in brightness value L* of the dried lemongrass leaves as the drying temperature increases. These results agreed with the observation of Hoque et al., 2013. They reported that lightness L*

decrease with an increase in drying temperature. These results again agreed with the observation of Sanmeema (2012). He reported that the colour parameters for L* values were high at 40°C when compared with 50°C and 60°C, this indicate that the dried products were darkened when the temperature increases. The highest L* value was obtained in products dried by sun and oven at 40°C, indicating the drying methods which are useful in maintaining the colour of the dried products. There was a significance difference ($p < 0.05$) between the L* values of lemongrass leaves dried under the difference drying methods. Negative a* values obtained for samples dried under sun, solar, oven at 40°C, 50°C, and 60°C, indicated that all lemongrass leaves dried under the different drying conditions were green. The lowest a* values were obtained from solar drying with decreased greenness followed by oven at 40°C, oven at 60°C, oven at 50°C and sun respectively. However, microwave at 50 W was having a positive a* value, indicating that the green colour was negatively affected and destroyed during drying. This was expected since the previous studies reported (Drouzas et al., 1999) that undesirable browning and reduced green colour of the microwave-dried product occurred in samples because of the high temperature generated by the microwaves. The b* values indicating the yellowness of the samples varied from 12.98 to 18.74. There was a significant difference ($p < 0.05$) of yellowness from all drying methods.

3.5 Sensory Evaluation of Lemongrass Tea Samples

3.5.1 Colour Acceptability

Colour of a food surface is the first quality parameter evaluated by consumers and is critical in product acceptance, even before it is tasted (Youssef and Mokhtar 2014). Consumers expect food to look appetizing and the colour of food is usually associated with expected the flavour of food (Downham & Collins 2000). Consumer responses on colour acceptability showed that, tea from microwave at 50 W, oven 60°C, and sun were least acceptable while the colour of oven 50°C, solar, and oven 40°C were the most acceptable. Similar observation was made by Cuervo-Andrade (2011). Colour acceptability of the tea samples increased in order as follow: microwave at 50 W < oven at 60°C < sun < oven at 50°C < solar < oven at 40°C (Table 3). This indicates that samples from microwave at 50 W, oven at 60°C and sun were negatively affected during drying. This agreed with the observation of Rahimmalek & Goli (2013). They reported that oven drying at higher temperature resulted in a considerable decrease in the colour quality of celak leaves. Shaw et al. (2007) reported a significant colour change in coriander foliage under microwave drying process. Ozkan et al. (2007) also reported that the colour of spinach was adversely affected under microwave drying process at very high microwave powers. Rahman et al. (2013) reported that direct exposure to sunlight reduces the quality of lemongrass such as colour, flavour leading to the production of low and variable quality of products. There was a significant difference amongst the colour acceptability of the samples dried under different drying methods (Table 3). The colour acceptability was in a range of 2.58 to 3.56. A possible explanation for this low colour acceptability range could possibly be that consumers in the study area expect the colour of the tea to be black. Lemongrass tea is not known or consumed in the study area, and assessors used for evaluation of the tea acceptability are not familiar with the lemongrass tea unlike other commercial tea found in shopping malls in the study area. These are possibly the reason for low colour acceptability range observed in the study.

3.5.2 Aroma Acceptability

Lemongrass tea is expected to have a strong aroma (Baratta et al., 1998; Kasali et al., 2001; Nur Ain et al., 2011). This is due to its high concentration of aromatic oils. Results show variation in aroma acceptability of tea samples obtained using different drying condition in the range of 2.66 to 3.52. A possible explanation for the variation in aroma (2.66 to 3.52) might be that aroma producing compounds are generally volatiles or they get combined with other biomolecules when grinding samples for preparation of powder (Lonkar et al., 2013). There was a significant difference amongst the aroma acceptability of the samples dried under different drying conditions (Table 3). Consumer responses on aroma acceptability showed that, tea from microwave at 50 W (2.66), oven at 60°C (2.74), and sun (2.98) were least acceptable while the aroma of oven at 50°C (3.20), solar (3.24), and oven at 40°C (3.52) were most acceptable.

3.5.3 Taste Acceptability

Lemongrass tea has a flavour very true to the flavour of the herb itself. As the name suggests, the tea tastes lemon (Leite et al., 2000). Taste is one of the sensory properties which form the component of flavour of a product (Meilgaard et al., 1999). The result shows variation in taste acceptability of lemongrass tea samples obtained from different drying condition. There was a significant difference amongst the taste acceptability of the samples dried under different drying conditions (Table 3).Consumer responses on taste acceptability showed that, tea from oven at 40°C, solar, and sun were the most acceptable while those dried by oven at 60°C, microwave 50 W, and oven at 50°C were least acceptable as shown in Table 3. Low taste acceptability scores

which were in a range of 2.62 to 3.40 could be due to the observed elements in the tea.

3.5.4 Overall Acceptability

Results show that product dried using oven at 40°C had the highest mean score in overall acceptability (3.50) as shown in Table 3. This was expected as it was the most preferred product in colour (3.56), aroma (3.52), and in taste (3.40). The overall acceptability of the tea is based on the organoleptic properties such as colour of tea, aroma of tea and taste of the tea (Lonkar et al., 2013). Low scores on colour, aroma and taste acceptability due to the fact that consumers were not familiar with lemongrass tea. Consumers expect the colour of the tea to be black. They also expect the taste of the tea to be sweetened. Exposure of aroma forming compounds to the natural environment due to the use of various treatments of cutting and powder making (Lonkar et al., 2013) could be possibly have caused a lesser overall acceptance by panellists which was in a range of 2.56 to 3.50 despite the fact that the highest score was 3.50.There was no significant difference amongst the overall acceptability of the samples with high scores which are dried by sun, solar, oven at 40°C, and oven at 50°C (Table 3). This indicate that these are the product which were most accepted by the panellist with respect to their colour, aroma, and taste. However, samples dried by oven at 60°C and microwave at 50 W were significantly different to samples dried by sun, solar, oven at 40°C, and at 50°C. This also indicate that products dried by oven at 60°C and microwave at 50 W were least accepted by the panellist with respect to their colour, aroma, and taste. These agreed with the observation of Rahimmalek & Goli, 2013. They reported that oven drying at higher temperature resulted in a considerable decrease in the colour quality of the leaves. Shaw et al. (2007), reported about the colour changes occurring during drying of most of the leaves using microwave drying process. There is increasing need for methods and tools for determining the characteristics of tea varieties (Buyukgoz et al., 2016).

Table 2. Physicochemical analysis for lemongrass dried under different conditions

			Oven (°C)			
Properties	**Sun**	**Solar**	**40**	**50**	**60**	**Microwave at 50 W**
L*	50.29 ± 1.15^{a}	47.13 ± 0.48^{b}	50.22 ± 1.96^{a}	43.74 ± 1.13^{c}	39.62 ± 1.21^{d}	40.09 ± 1.16^{d}
a*	-0.86 ± 0.49^{b}	-3.94 ± 0.55^{d}	-2.15 ± 0.19^{c}	-1.03 ± 0.13^{b}	-2.11 ± 0.58^{c}	1.44 ± 0.14^{a}
b*	12.98 ± 1.05^{d}	13.21 ± 0.76^{d}	15.23 ± 0.67^{c}	13.55 ± 0.70^{d}	18.74 ± 0.90^{a}	16.81 ± 0.33^{b}
Ash	6.67 ± 0.00^{a}	6.67 ± 0.01^{a}	6.67 ± 0.02^{a}	6.67 ± 0.03^{a}	7.78 ± 1.92^{a}	6.67 ± 0.03^{a}
pH	5.89 ± 0.02^{d}	6.25 ± 0.02^{a}	6.06 ± 0.09^{b}	5.98 ± 0.01^{c}	5.96 ± 0.03^{cd}	5.97 ± 0.01^{cd}

Mean scores in the same row with different superscripts are significantly different ($p < 0.05$). L* = Lightness, a* = Redness, b* = Yellowness.

Table 3. Sensory analysis for lemongrass tea dried under different drying conditions

			Oven (°C)			
Sensory properties	**Sun**	**Solar**	**40**	**50**	**60**	**Microwave at 50 W**
Colour	2.98 ± 1.27^{bc}	3.46 ± 1.27^{ab}	3.56 ± 1.23^{a}	3.24 ± 1.25^{ab}	2.94 ± 1.32^{bc}	2.58 ± 1.21^{c}
Aroma	2.98 ± 1.15^{bc}	3.24 ± 1.22^{ab}	3.52 ± 1.13^{a}	3.20 ± 1.14^{ab}	2.74 ± 1.34^{bc}	2.66 ± 1.32^{c}
Taste	3.02 ± 1.15^{abc}	3.30 ± 1.27^{ab}	3.40 ± 1.12^{a}	2.84 ± 1.29^{bc}	2.62 ± 1.24^{c}	2.74 ± 1.26^{c}
Overall acceptability	3.34 ± 0.98^{a}	3.40 ± 1.23^{a}	3.50 ± 0.91^{a}	3.16 ± 1.17^{ab}	2.78 ± 1.22^{bc}	2.56 ± 1.31^{c}

Mean scores in the same row with different superscripts are significantly different ($p < 0.05$).

4. Conclusion

The study investigated the effect of drying on quality and sensory attributes of lemon grass (*Cymbopogon citratus*) tea. Results showed that the sensory properties, moisture content, ash content, pH and colour of lemongrass samples differ with respect to the drying methods used. Lemongrass tea from samples dried by oven at 40°C was the most preferred in colour, aroma, taste and overall acceptability. Oven drying at 40°C was found to be most suitable for drying of lemongrass leaves for tea production in order to retain appreciable sensory attributes.

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Study on Consumers' Behavior on *Buffen* (Buffalo meat): Marketing Perspective

S. Islam[1], T. N. Nahar[2], J. Begum[1], G. K. Deb[2], M. Khatun[1] & A. Mustafa[3]

[1]Socioeconomic Research Division, BLRI, Savar, Dhaka-1341, Bangladesh

[2]Biotechnology Division, BLRI, Savar, Dhaka-1341, Bangladesh

[3]Buffalo Development Project, BLRI, Savar, Dhaka-1341, Bangladesh

Correspondence: S. Islam, Socioeconomic Research Division, BLRI, Savar, Dhaka-1341, Bangladesh. Tel: 880-171-959-9733. E-mail: s.islam@blri.gov.bd

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Abstract

The study was undertaken to examine the socioeconomic profile of buffalo farmers and to assess the marketing and consumers preference on *buffen* (buffalo meat) in the selected areas. Twelve districts namely: Mymensingh, Jamalpur, Moulovibazar, Bhola, Bagerhat, Feni, Potuakhali, Noakhali, Laxmipur, Chittagong, Tangail and Sirajgong were selected purposively. A total of 1400 buffalo farmers were interviewed following simple random sampling technique. Data were collected during June 2011 to April 2016 and analyzed data using SPSS software. Study revealed that the highest per cent of farmers were in age group 31-45 years indicating that farmers were mature enough to give more labour to their farming activities. On average, 88 per cent buffalo farmers were engaged purely in agriculture followed by business and service as primary occupation. The highest numbers of farmers were illiterate followed by primary education, SSC, HSC and Degree. About 49 per cent buffalo farmers had above 15 years of farming experience of rearing buffalo. Average farm size was estimated 0.95 hectare indicating small and medium category farm and average family size was calculated 6 persons per family which is higher than national average 4.9. Dependency ratio was also estimated to 0.94. The study showed that *buffen* contributes 7.16 per cent of total red meat production and 6.19 per cent of total meat production in Bangladesh and about 50 percent farmers reported that they did fattening before selling of buffalo. About 48 per cent consumers reported that they prefer *buffen* most among different kinds of meats. In view point of butcher, about 46 percent consumer preferred *buffen* than beef.

Keywords: Buffen, marketing, consumption and buffalo

1. Introduction

Bangladesh is an agrarian country having small territory and large number of population. Its economy is primarily depending on production of crop, livestock and fisheries. The livestock sub-sector contributes 1.78% to the GDP (DLS, 2015), contributes 16.71% to the agricultural GDP, generates 20% of country's employment directly and 40% indirectly, contributes 4.31% to country's total export earning, provides 25% of households energy supply, produces 125 MMT of organic manure utilized for crop production (AIS, 2014). In financial year 2015-16, the livestock population is 542.27 lakh (ruminant) whereas the buffalo is 14.69 lakh in Bangladesh (BER, 2016). The buffalo population has been gradually increasing. The global buffalo population is 194.29 million and buffalo in Asia dominate the world population, representing 92.52% (179.75 million) of the total buffalo population (FAO, 2012; Chakravarty, 2013).

Buffalo is a multipurpose domestic animal that helps the livelihood of people by providing high quality milk and meat, dung as fuel and organic fertilizer; mechanical or draft power and hides and skins as raw material for industry (Irshad, A., Tariq, M. M., Bajwa, M. A., Abbas, F., Isani, G. B., Soomro, G. H., Waheed, and Khan, K. U., 2011). As a result of observation on buffalo numbers of Bangladesh of last ten (10) years from 2003-04 to 2012-13, average growth rate reached at 1.0355 percent. Based on the growth rate, projected number of buffalo in 2019-20 is 18.51 lakh and in 2029-30 is 26.23 lakh. Indigenous buffaloes are three times heavier than cattle and produce two times more milk than cattle (Rahman, S. M. A., Begum, J., Sayeed, M. A., Hossain, M., and Alam, J., 2008). The average milk production per buffalo per lactation is estimated to be 504 kg against 157 kg

for cow. Buffalo milk is popular and has a high nutritive value and is excellent for the preparation of dairy products.

Buffalo meat called *buffen* is similar to beef in basic properties, structure, chemical composition, nutritive value and palatability. Compared to beef, *buffen* has a lighter flavor, tastes slightly sweet and is deliciously tender. It is naturally lower in fat and also has more protein, more iron and more polyunsaturated fatty acids (such as the healthy omega-3 and omega-6 fatty acids) than beef (Consumers detect little difference between cooked joints of buffalo meat and beef derived from animals kept under the same conditions and slaughtered at the same age and stage of fattening (Rahman, S. M. A. , Sayeed, M. A., Yasmin, F., Begum, J.,2006).

In Bangladesh, there are four main types of ruminants as Cattle, Buffalo, Goat and Sheep. Dressing percentage of Cattle, Buffalo, Goat and Sheep of our country is 48.99 ± 4.84 (Ali, M. M., Hossain, M. M., Akhter, S., Islam, M.S., Hashem, M. A., 2013), 44% (Hamid, M.A., Ahmed, S., Rahman, M.A. and Hossain, K.M., 2016), 37.22% and 39.85%, respectively. Total red meat production in Bangladesh is 3.912 Million MT where total meat production is 4.52 Million MT. Beef contributes 88.19 per cent of total red meat production and 76.32 per cent of total meat production. *Buffen* contributes 7.16 per cent of total red meat production and 6.19 per cent of total meat production. Chevon (Goat meat) and mutton (sheep meat) contributes 3.88 per cent and 0.77 per cent of total red meat production and 3.36 per cent and 0.66 per cent of total meat production respectively (Field survey 2017 and author's calculation). *Buffen* is the healthiest meat among red meats and economical also. *Buffen* is becoming more popular worldwide because of its some inherent properties over cattle meat with respect to attributes such as lower intra muscular fat, cholesterol and high calories and units of essential amino acids, biological value and iron content. Sound knowledge about the procedure of buffalo and *buffen* marketing is essential to increase the publicity of such kind of healthy meat. Increased consumption of *buffen* can fulfill the growing demand of protein of growing population.

Though the buffalo is an important part of livestock, there were hardly very few research accomplished so far that investigated marketing and consumers' preference on *buffen* in Bangladesh. So, the study had an attempt to examine the different socioeconomic factors related with buffalo production and to investigate the marketing system of *buffen*.

Objectives of the study

The overall objective of the study was to explore the existing socioeconomic status and consumers' preference on *buffen*. Moreover, the specific objectives of the study are as follows:

- To examine the socioeconomic profile of buffalo farmers; and
- To assess the market and consumers' preference on *buffen*.

2. Methodology

The methodology was followed for the study as: twelve districts namely: Mymensingh, Bagerhat, Moulvibazar, Potuakhali, Laxmipur, Jamalpur, Noakhali, Bhola, Feni, Chittangong, Tangail and Sirajgonj were selected purposively on the basis of buffalo population density. Field survey method was followed to collect primary data. A total of 1400 buffalo farmers were interviewed following simple random sampling technique (Table 1).

Table 1. Sample distribution in the study areas

Sl. No.	Districts	Upazilas	Sample size
1.	Mymensingh	Trishal, Fulbaria, Haluaghat	170
2.	Moulvi Bazar	Borolekha, Kamalganj, Rajnagar	170
3.	Potuakhali	Potuakhali sadar, Kalapara, Galachipa	170
4.	Laxmipur	Laxmipur sadar, Kamalnagar, Ramgati	170
5.	Bagerhat	Morolgong, Kachua, Rampal	180
6.	Jamalpur	Bakshiganj, Madargong	110
7.	Noakhali	Subarnachar, Hatia	110
8.	Bhola	Manpura, Charfashan	110
9.	Feni	Sonagaji	60
10	Chittagong	Swandip	50
11	Tangail	Shakhipur	50
12	Sirajgonj	Ullahpara	50
Total			1400

To collect the necessary data, an interview schedule was prepared in accordance with the objectives set for the study. The prepared interview schedule then pre-tested in the field among some buffalo keepers before final data collection. After pre-testing, the interview schedule was finalized after making required corrections, modifications and adjustment in the light of the experience gained from the field. The survey method was direct interview. Secondary data and information were also collected as required. Beside quantitative survey, qualitative tools such as Focus Group Discussion (FGD) and KII (Key Informant Interview) were carried out on buffalo farmers, veterinary technicians, vaccination service providers and Upazila livestock officers to have better understanding on existing constraints, challenges and opportunities of buffalo keeping. The data collected in the mentioned regions during the month of June 2011 to April 2016. In order to obtain reliable data, cross check was made. Data were analyzed using Statistical Package for Social Sciences (SPSS) software. Tabular analysis was used to find out simple statistical measures like average, percentage, ratios etc.

3. Result and Discussion

Socioeconomic Profile of the Buffalo Farmers

Socioeconomic parameters such as age, education, occupation, experiences of buffalo rearing, farm size, family size and dependency ratio were studied to know the buffalo farmers` socioeconomic condition.

Age: The classified age groups were up to 30 years, 31-45 years, 46-60 and above 60 years. The highest per cent (41%) of farmers were in age group of 31-45 years indicating that farmers were mature enough for taking household decisions properly & timely and also strong enough to give more labour to their farming activities followed by age group 46-60 years, up to 30 years, and above 60 years, respectively (Table 2).

Table 2. Farmers' age

Areas	Age range (Years)			
	Up to 30	31 to 45	46 to 60	Above 60
Mymensingh	33 (20)	75 (44)	45 (26)	17 (10)
Moulvibazar	41 (24)	71 (42)	43 (25)	15 (9)
Patuakhali	33 (19)	69 (41)	51 (30)	17 (10)
Laxmipur	43 (25)	68 (40)	47 (28)	12 (7)
Bagerhat	39 (22)	78 (43)	50 (28)	13 (7)
Jamalpur	37 (34)	41 (37)	23 (21)	9 (8)
Noakhali	25 (23)	43 (39)	35 (32)	7 (6)
Bhola	28 (25)	45 (41)	27 (25)	10 (9)
Feni	10 (17)	22 (37)	20 (33)	8 (13)
Chittagong	7 (14)	20 (40)	20 (40)	3 (6)
Tangail	8 (16)	17 (34)	19 (38)	6 (12)
Sirajgonj	6 (12)	29 (58)	13 (26)	2 (4)
All areas	**310 (22)**	**578 (41)**	**393 (28)**	**119 (9)**

Source: Field survey 2012, 2014, 2016. (Value in the parentheses indicates percentage).

Occupation: Most of the farmers were engaged with agriculture as income generating activity. On an average, 88 percent buffalo farmers were engaged full time on agriculture as primary occupation. Besides, 11 percent farmers had business and only 1 percent engaged with service (Table 3).

Table 3. Occupation

Areas	Occupation		
	Agriculture	Business	Service
Mymensingh	158 (93)	12 (7)	-
Moulvibazar	161 (95)	6 (3)	3 (2)
Patuakhali	139 (82)	28 (16)	3 (2)
Laxmipur	144 (85)	22 (13)	4 (2)
Bagerhat	162 (90)	18 (10)	-
Jamalpur	102 (93)	8 (7)	-
Noakhali	88 (80)	19 (17)	3 (3)
Bhola	83 (76)	20 (18)	7 (6)
Feni	51 (85)	9 (15)	-
Chittagong	47 (94)	3 (6)	-
Tangail	49 (98)	-	1 (2)
Sirajgonj	47 (94)	3 (6)	-
All average	**1231 (88)**	**148 (11)**	**21 (1)**

Source: Field survey 2012, 2014, 2016. (Value in the parentheses indicates percentage).

Education: Education contributes to economic, social, environmental and ethical development. It plays a pivotal and significant role in adoption of new and innovative technology and agricultural modernization especially in rearing buffalo. It makes a man more capable of managing scarce resources and maximizing profit. Table 4 showed the education level of the buffalo farmers in the study areas. It is evident that highest percent (48%) of buffalo farmers were illiterate and 38 percent farmers were in primary level education followed by SSC, HSC and Degree.

Table 4. Education

Areas	Education				
	Illiterate	Primary	SSC	HSC	Degree & Up
Mymensingh	70 (41)	78 (46)	13 (8)	8 (4)	1 (1)
Moulvibazar	78 (46)	54 (32)	24 (14)	10 (6)	4 (2)
Patuakhali	84 (49)	56 (33)	19 (11)	8 (5)	3 (2)
Laxmipur	86 (50)	63 (37)	10 (6)	10 (6)	1 (1)
Bagerhat	90 (50)	62 (34)	14 (8)	11 (6)	3 (2)
Jamalpur	64 (58)	32 (29)	9 (8)	4 (4)	1 (1)
Noakhali	53 (48)	38 (35)	10 (9)	6 (5)	3 (3)
Bhola	39 (35)	64 (58)	4 (4)	2 (2)	1 (1)
Feni	33 (55)	20 (33)	4 (7)	2 (3)	1 (2)
Chittagong	10 (20)	36 (72)	3 (6)	1 (2)	-
Tangail	27 (54)	20 (40)	2 (4)	1 (2)	-
Sirajgonj	38 (76)	12 (24)	-	-	-
All areas	**672 (48)**	**535 (38)**	**112 (8)**	**63 (5)**	**18 (1)**

Source: Field survey 2012, 2014, 2016. (Value in the parentheses indicates percentage).

Experience: It was found that 49 per cent buffalo farmers had above 15 years of experience followed by 18 per cent 6-10 years, 22 per cent 11-15 years and 11 per cent up to 5 years of rearing buffalo (Table 5).

Table 5. Experience

Areas	Range (Years)			
	Up to 5	6 to 10	11 to 15	Above 15
Mymensingh	7 (4)	29 (17)	47 (28)	87 (51)
Moulvibazar	10 (6)	35 (21)	43 (25)	82 (48)
Patuakhali	20 (12)	47 (28)	50 (29)	53 (31)
Laxmipur	11 (6)	26 (15)	35 (21)	98 (58)
Bagerhat	15 (8)	30 (17)	35 (19)	100 (56)
Jamalpur	11 (10)	17 (15)	24 (22)	58 (53)
Noakhali	6 (6)	24 (21)	34 (31)	46 (42)
Bhola	17 (15)	8 (7)	14 (13)	71 (65)
Feni	20 (33)	12 (20)	8 (14)	20 (33)
Chittagong	6 (12)	4 (8)	9 (18)	31 (62)
Tangail	18 (36)	10 (20)	3 (6)	19 (38)
Sirajgonj	13 (26)	15 (30)	4 (8)	18 (36)
All areas	**154 (11)**	**257 (18)**	**306 (22)**	**683 (49)**

Source: Field survey 2012, 2014, 2016. (Value in the parentheses indicates percentage).

Family size: It is also revealed that average farm size was 0.95 hectare. The average family size in the study area was 6 persons per family which was slightly higher than the national average. Average Active members and dependency ratio were calculated 3.18 persons per family and 0.94 respectively (Table 6).

Table 6. Family size

Areas	Farm size (ha)	Family size	Active members	Dependency ratio
Mymensingh	1.14	5.77	3.52	0.64
Moulvibazar	1.06	6.61	4.12	0.60
Patuakhali	1.04	6.16	2.92	1.11
Laxmipur	0.96	5.71	2.46	1.32
Bagerhat	0.98	6.26	3.12	1.00
Jamalpur	0.11	5.59	2.20	1.54
Noakhali	1.37	6.62	3.86	0.71
Bhola	1.61	5.84	2.92	1.00
Feni	0.79	6.35	3.25	0.95
Chittagong	0.54	6.06	3.18	0.95
Tangail	1.25	4.82	3.46	0.46
Sirajgonj	0.52	6.10	3.14	1.00
All areas	**0.95**	**6.00**	**3.18**	**0.94**

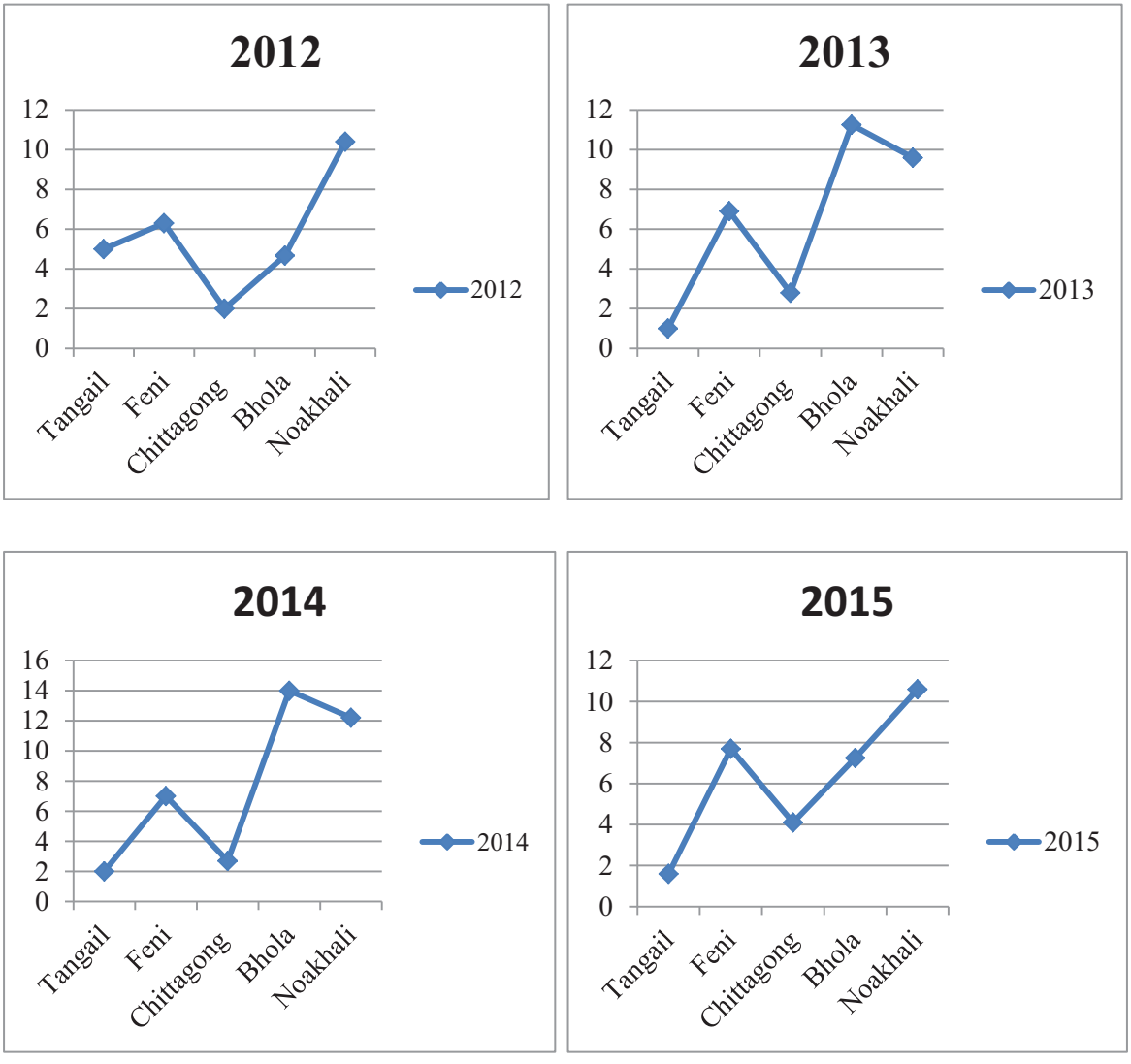
Source: Field survey 2012, 2014, 2016. (Value in the parentheses indicates percentage).

Buffalo Marketing: Number of buffalo sold by farmer per farm in last five years in specific locations was observed to understand the marketing pattern of buffalo. In 2012, highest (10.4) number of buffalo were sold in Noakhali and lowest (2.0) in Chittagong, in 2013 highest (11.25) was in Bhola and lowest (1.0) in Tangail. In 2016, highest (13.3) number was found in Noakhali and lowest (1.25) in Tangail. Highest Average (11.22) number of buffalo sold in Noakhali and lowest average (2.17) was found in Tangail (Table 7 and Figure 1).

Table 7. Number of buffalo sold by the farmer per farm in different Location & years

Year	Tangail	Feni	Chittagong	Bhola	Noakhali
2012	5	6.3	2	4.67	10.4
2013	1	6.9	2.8	11.25	9.6
2014	2	7	2.7	14	12.2
2015	1.6	7.7	4.1	7.25	10.6
2016	1.25	8.1	2.28	10.8	13.3
Average	**2.17**	**7.2**	**2.76**	**9.46**	**11.22**

Source: Field survey 2017 and author's calculation.



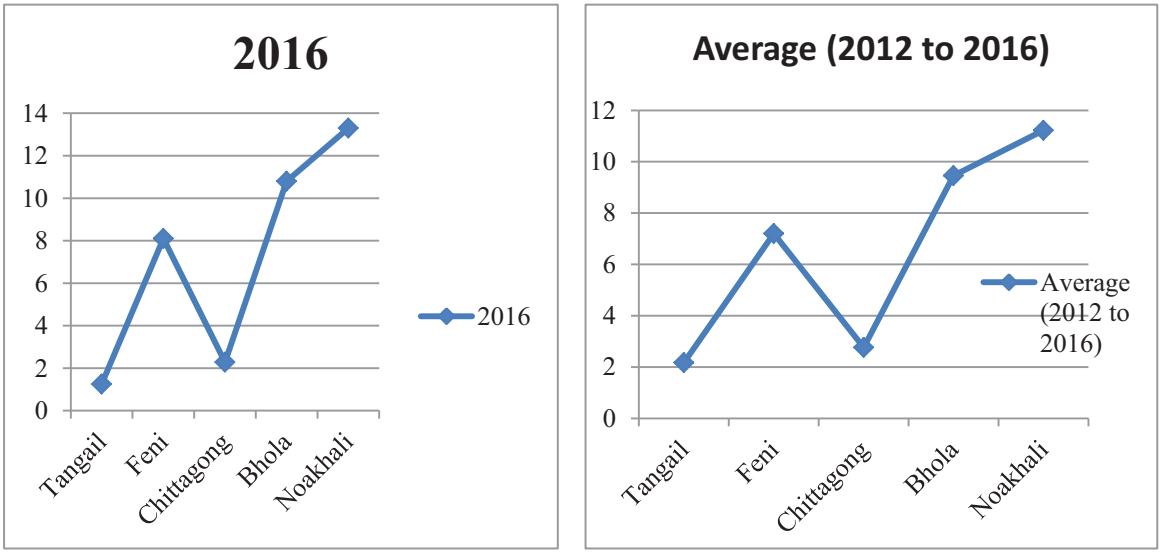


Figure 1. Per Household Buffalo Selling Trend in the selected areas in different years

Buffalo Fattening: According to market concepts, four categories were selected to whom the farmers sell their buffalo i.e. General buyer means another farmer who buy buffalo for further rearing, Wholesaler who buy buffalo from the farmer and rears one or two months and then sell to butcher. Some farmers reported that they sell their buffalo to both general buyer and wholesaler included in other category. The study revealed that 46.67 percent farmers sold their buffalo to general buyer and 62.5 percent to wholesaler. About 15 percent farmers sold their buffalo to butcher. Almost 44 percent farmer sold their buffalo from their own farm and about 13 percent taking their buffalo at village *hut* (market). Sixty percent farmers sold both from own farm and at *hut*. About 50 percent farmers reported that they do fattening before selling (Table 8).

Table 8. Buffalo sale & Fattening

Location (n=10)	Sell to whom (%)				Where to sell (%)			Buffalo fattening
	General Buyer	Wholesaler	Butcher	Others	Own farm	Market (*hut*)	Both	
Tangail	70	30	-	-	80	20	-	40
Feni	-	90	10	-	10	-	-	50
Chittagong	30	-	20	50	30	10	60	100
Bhola	40	40	-	20	90	10	-	-
Noakhali	-	90	-	10	100	-	-	10
All areas	**46.67**	**62.5**	**15**	**26.67**	**44**	**13.33**	**60**	**50**

Source: Field survey 2017 and author's calculation.

Meat preference & Consumption: Forty eight percent consumer reported that they preferred *buffen* most among red meats and 65 percent preferred beef indicating that still *buffen* is not as much acceptable as beef in our country. It was showed that average number of days of meat consumption per month was about 5 days. In average, they consumed *buffen* 1.67 days/month, 1.86 days/month consumed beef, 1.49 days/month consumed chicken and 1.0 day/month consumed mutton. Average consumption amount of *buffen*, beef, chicken and mutton was 2 kg/month, 2.28 kg/month, 2.37 kg/month and 1 kg/month respectively (Table 9).

Table 9. Meat preference & Consumption

Areas (n=10)	*Buffen* pref. (%)	Beef pref. (%)	Average days per month	*Buffen*		Beef		Chicken		Mutton	
				Avg. days	Am.(kg)	Avg. days	Am. (kg)	Avg. days	Am.(kg)	Avg. days	Amt. (kg)
Tangail	10	90	5.3	1.33	1.33	2.0	2.78	1.11	2.22	-	-
Feni	30	70	4.8	1.67	1.67	2.6	2.8	2.2	3.4	-	-
C.gong	80	20	4.4	2.1	2.4	1.0	1.12	1.0	1.1	1.0	1.0
Bhola	20	80	5.3	1.23	1.23	2.5	3.5	1.67	2.83	-	-
Noakhali	100	-	4.6	2.0	3.37	1.21	1.22	1.48	2.3	1.0	1.0
All areas	**48**	**65**	**4.88**	**1.67**	**2.0**	**1.86**	**2.28**	**1.49**	**2.37**	**1.0**	**1.0**

Source: Field survey 2017 and author's calculation.

Consumer's preference on meat in view point of butcher: In view point of butcher, about 46 percent consumer preferred *buffen* as they buy more *buffen* than beef and 54 percent liked beef. Average price of *buffen* was Tk. 430 in the selected regions (Table 10).

Table 10. Consumer's preference on meat in view point of butcher

Areas (n=10)	Meat preference		Buffalo meat price (average)
	Buffen (%)	Beef (%)	
Tangail	-	100	387
Feni	10	90	434
Chittagong	100	-	450
Bhola	20	80	430
Noakhali	100	-	450
All areas	**46**	**54**	**430**

Source: Field survey 2017 and author's calculation.

4. Conclusion and Recommendations

The study was undertaken to identify the socioeconomic profile of buffalo keeping farmers, to determine financial profitability of buffalo rearing and to estimate the marketing procedure of *buffen*. The study revealed that the benefit cost ratio (BCR) from buffalo rearing was 1.31 which indicated that buffalo rearing is a profitable venture. The study exposed that 31% annual household income generated from buffalo, 5% from livestock except buffalo, 12% from crop, 23% from business and 27% from service sector. The study Researchers found some important issues as recommendations for the improvement of the buffalo farmers from this study, such as:

- Government should provide feed support to the flood affected areas also should provide subsidies for feed and forage production.
- Good medical facilities should be provided by the veterinary workers.
- Government should provide good quality seed and develop public-private partnerships in diversified vaccine production.
- Security of the buffaloes should be ensured by the ULO Officers.
- Government should provide financial support to buffalo keeping farmers as they can afford treatment of their buffaloes & also can afford high quality breed.

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The Risk of Dietary Exposure to Pesticide Residues and Its Association with Pesticide Application Practices among Vegetable Farmers in Arusha, Tanzania

Purificator Andrew Kiwango[1], Neema Kassim[1] & Martin Epafras Kimanya[1, 2]

[1]Department of Food Biotechnology and Nutritional Sciences, School of Life Sciences and Bioengineering, The Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Arusha. Tanzania

[2]Department of Rural Economy and Agriculture, African Union Commission, P.O. Box 3243, Addis Ababa, Ethiopia

Correspondence: Purificator Andrew Kiwango, Department of Food Biotechnology and Nutritional Sciences, School of Life Sciences and Bioengineering, The Nelson Mandela African Institution of Science and Technology, P.O. Box 447, Arusha. Tanzania. Tel: 255-7634-07361. E-mail: puriandrew@gmail.com; puriand@yahoo.com

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Abstract

This study was conducted to assess dietary exposure to pesticide residues and pesticide application practices leading to the presence of these residues among vegetable farmers in Arusha, Tanzania. Face-to-face interviews using semi-structured questionnaires (including 24-hour recall and food frequency questionnaire techniques) were conducted to collect information on pesticide application practices and vegetable consumption, from 76 farmers. A sample of ready-to-eat vegetables was collected from each farmer's household to determine the level of pesticide residues. Pesticide residues were analyzed by gas chromatography-mass spectroscopy

A deterministic approach was used to assess dietary exposure to pesticide residues. Among the analyzed samples, 31.4% contained detectable levels of organophosphate residues. The detected organophosphates were dimethoate (mean, 8.56 mg kg^{-1}), acephate (mean, 2.9 mg kg^{-1}), profenofos (mean, 8.44 mg kg^{-1}), dichlorvos (mean, 20.8 mg kg^{-1}) and malathion (mean, 5.47 mg kg^{-1}). The mean exposure for dimethoate (0.0021 mg kg^{-1} body weight (wt) day^{-1} was higher than its corresponding acceptable daily intakes of 0.002 mg $kg^{-1}bwd^{-1}$ resulting in hazard quotient of 1.044 with a consequent hazard index of 1.19 for organophosphates. Pyrethroid pesticides (permethrin, cypermethrin, and lambda-cyhalothrin) were also detected but at a lower frequency (17.1%) and hazard index (0.029). The exposure to pesticide residues was significantly associated with limited access to expert advice on pesticide application (p=0.031, adjusted odds ratio=6.56) and over-dosage (p=0.038, adjusted odds ratio=3.751).The risk may be minimized by increasing access to support by extension service providing guidance on good practices and ensuring application of appropriate doses for pesticides.

Keywords; pesticide residue, exposure, ready-to-eat, application practices, vegetable farmers, organophosphates, pyrethroids, agricultural extension officers, over-dosage

1. Introduction

Malpractices in pesticides application result to unacceptable levels of pesticide residues in foods, and consequently increase the risk of unsafe dietary pesticide exposures in humans. Dietary exposure to unacceptable levels of pesticide residues has been associated with risks of developing cancer, genetic and immune system defects and neurological system disorders (Hashmi, Imran, & Dilshad, 2004; Keifer., 2008; Thatheyus & Gnana Selvam, 2013). Parkinson's and Alzheimer's diseases are the most common neurodegenerative disorders which are associated with exposure to pesticides (Campdelacreu, 2012; Sanchez-Santed, 2015). Pesticides possess estrogenic activity and therefore are associated with breast cancers in women and low sperm count in males (Laffin, Chavez, & Pine, 2010; Toft, Hagmar, Giwercman, & Peter, 2004). To ensure the pesticide safety of vegetables and other foods, Codex Alimentarius Commission in collaboration with Environmental Protection Agency (EPA) has set maximum tolerable residual levels (MRLs) for particular pesticides in food including vegetables (European Food Safety Agency [EFSA], 2012; Food and Agriculture Organization [FAO]/World Health Organization [WHO], 1997).

Vegetables form an important part of human diet, however, are food crops with a very high likelihood of containing pesticides. Surveys in developing countries indicate that in vegetables there is an indiscriminate use of pesticides for pest and disease control, combined with non adherence to pesticides' pre-harvest intervals, and lack of knowledge of the correct use of pesticides, all of which could likely result in excessive levels of pesticide residues in vegetables (Amera & Abate, 2008; Banjo, Aina, & Rije, 2010; Lozowicka et al., 2015; Zyoud et al., 2010). A study done in Chile revealed that 27% of 118 leafy vegetable samples analyzed were contaminated with pesticide residues above MRLs and 65% of them had multiple pesticide residues (Elgueta, Moyano, Sepúlveda, & Quiroz, 2017). A study by Sheikh, Nizamani, Panhwar & Miran (2013) in Pakistan which analyzed pesticide residues in vegetable samples from markets found that okra, bitter gourd, brinjal, tomato, onion, cauliflower, and chilies were highly contaminated with chlorpyrifos, profenofos, endosulfan, imidacloprid, benzoate, lufenuron, bifenthrin, diafenthiuron, and cypermethrin. Another study analyzed dichlorvos residue levels in vegetables sold in Lusaka, Zambia and found that the average dichlorvos residue levels were significantly higher than the country's set maximum limits (1 mg kg^{-1}) (Sinyangwe, Mbewe, and Sijumbila, 2016). High pesticide residue levels in vegetables imply that those consuming vegetables might be at risk of exposure to unacceptable levels of pesticides. In order to ensure that dietary exposures to pesticide residues are within safe limits, the FAO/WHO Joint Meeting of Pesticide Residues (JMPR) establishes acceptable daily intakes (ADI) of pesticides (FAO/WHO, 1997). For instance, the ADI for dimethoate is 0.002 mg kg^{-1} body weight and that of dichlorvos is 0.004 mg kg^{-1} body weight. Malathion which is relatively less toxic has an ADI of 0.3 mg kg^{-1} body weight.

Tanzania as a developing country is affected by the problem of malpractices in pesticide application. Evidence from surveys conducted in Southern highlands (Iringa), Central (Morogoro) and Northern zones (Arumeru and Karatu) confirms this (Lekei, Ngowi, and London, 2014a; Manyilizu & Mdegela, 2015; Ngowi, Mbise, Ijani, London & Ajayi, 2007; Nonga, Mdagela, Sandvik & Skaare, 2011). These studies suggest that vegetables from these areas may be highly contaminated with pesticide residues, posing a risk of exposure to pesticide residues. However, only limited studies have been done in Tanzania to estimate pesticide residues or exposure in vegetables. A study by Ndengario-Ndossi and Cram, (2005) which analysed 33 samples of spinach found that 72.7% of the samples were contaminated with gamma-hexachlorocyclohexane (g-HCH) (0.08 µg kg^{-1}), 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene (pp-DDE) (0.74 µg kg^{-1}), dichlorodiphenyltrichloroethane (pp-DDT) (2.15 µg kg^{-1}) and chlorpyrifos (0.02µg kg^{-1}). Mahugija, Khamis & Lugwisha (2017) analyzed 72 samples of cabbage, onion, and spinach for pesticide residues in which 72.2% of the vegetables were found to be contaminated with DDT and its metabolites, endosulfan, and cypermethrin. However, these two studies were done in Dar es Salaam, which is not a major vegetable producing area in Tanzania. In this country, vegetables are mainly produced in highlands of Morogoro, Iringa, and Arusha (Putter & Koesveld, 2007; Small & Medium Enterprise Competitiveness Facility [SCF], 2008), of which Arusha leads in pesticide trading and use (Agenda, 2006). The reported levels of pesticide residues in the vegetables sampled in markets in Dar es Salaam indicate that farmers in major vegetable producing areas such as Arusha might be exposed to high levels of pesticide residues. A study in Arumeru district in Arusha analyzed 50 tomato samples for pesticide residues and 12% of the samples contained permethrin and chlorpyrifos at mean concentrations of 5.2899 mg kg^{-1} and 7.5281 mg kg^{-1}, respectively (Kariathi, Kassim, & Kimanya, 2016). However, the results from this work are not adequate for drawing a conclusion on the dietary exposures through vegetables in Arusha as there are more varieties of vegetables consumed in the region. Furthermore, in Tanzania and other developing countries, there is no documented information on specific pesticide application practices that can be attributed to pesticide residues. The current study assessed pesticide residue exposures through vegetable consumption among vegetable farmers in Arusha and determined pesticide application practices attributable to the exposures.

2. Materials and Methods

2.1 Study Area

The study was conducted in one district of the Arusha region. The Arusha district in Arusha region was selected due to its high production of vegetables and known pesticide use (Agenda, 2006). The district covers an area of 1446.692 km^2 with a population of 290,041. It is characterized by two agricultural zones (green and low land belt zones). The main vegetable producing areas of the Arusha district are in the green belt (highlands), which covers the wards of Ilkiding'a, Kimnyaki, Kiranyi, Sambasha, and Olmotonyi. The main vegetable crop cultivated in Arusha is cabbage. Due to its high vulnerability to pest infestation, the crop requires frequent application of pesticides (Ngowi et al., 2007; United Republic of Tanzania [URT], 2012).

2.1.1 Study Design and Sample Size

A cross-sectional study design was adopted to survey pesticide residues, exposure, and application by 76 farmers

selected by simple random technique, from seven villages in four wards of the green belt zone of the Arusha district. At ward level, village(s) leading in vegetable farming were purposively selected as follows: Ilkiding'a (Ilkiding'a), Olimring'aring'a and Olevolous (Kimnyaki), Siwandeti (Kiranyi), Timbolo and Shiboro (Sambasha) and Emaoi (Olmotonyi). The wards were purposively identified, with the assistance of district agricultural extension officers, based on their potential for vegetable production.

The sample size was estimated at 90% confidence level, following the formula for calculating sample size for cross sectional studies (Charan & Biswas, 2013). Farmers participating in this study were selected using a set of criteria. Of the criteria used was the willingness of a farmer to participate in the research during the field survey and his/her availability during both the first and the second vegetable consumption surveys. Farmers were pre-informed of the objectives of the research and those who consented to participate in the study were recruited.

2.1.2 Data Collection

Data collection was done from June to November 2015 (a period that covers dry and rainy seasons) during face to face interviews. Semi-structured questionnaires were used in the interviews to obtain information on socio-demographic characteristics of the participants, the vegetable cropping system used, pesticide application practices and vegetable consumption. Detailed information on vegetable consumption was further collected using two-time point 24-hour dietary recall and food frequency questionnaires. Prior to actual data collection, the questionnaires were pre-tested in Seela village of Sing'isi ward which is in a similar geographical location and of the same socio-cultural characteristics to those of the study area.

2.2 Sampling and Quantification of Ready-to-eat Vegetables

Repeated 24 hours dietary recall and food frequency techniques were employed to estimate the amount of vegetables consumed by the farmers (Kimanya et al., 2009). Two home visits were conducted, on non-consecutive days. The respondent farmer was requested to recall what she/he ate during the past 24 hours. If vegetables were among what she/he consumed, she/he was requested to mention the type of vegetables consumed. The respondent was also requested to mention the source of the consumed vegetables, whether from her/his own farm, a neighbour's farm or a market. The respondent was further asked to mention the number of days in the previous week during which she/he ate the same type of vegetables.

The respondent was requested to explain how the vegetable was prepared and mention all ingredients in the vegetable recipe. She/he was also requested to estimate the amount of ready-to eat-vegetable consumed during the previous day, by using a bowl or any other utensil that is usually used for serving vegetables. Grains or pulses were used to aid in estimating the vegetable volumes of the bowl by filling into the bowl up to the usual level of the share per single serving. The left-over or shared amounts were deducted from the volume served per single serving and the actual estimate obtained and noted. The respondent was requested to prepare vegetables and provide a duplicate portion (per serving) of the ready-to-eat vegetables as reported in the interview. Arrangements were made for those who had no vegetables in their home at the time of survey so that the samples were collected on the next day. The sample of the ready-to-eat vegetables was then collected in a glass container and kept in a cool box with ice blocks and transported to the Tropical Pesticide Research Institute (TPRI) laboratory where its weight was measured using an electronic kitchen scale (CAMRY, model EK3131) and recorded before it was stored at -20^{o}C in a freezer until analysed for pesticide residues. The average weight of vegetable consumed by each respondent as collected during the two home visits was calculated and recorded.

Respondents who reported that they had not consumed vegetables on the previous day were requested to estimate the amount that they usually consume, and the duplicate sample was measured based on this amount. In order to be able to estimate per capita vegetable consumption per kg body weight per day, the weight of the respondent was taken using a weighing scale (Ashton Meyers' model 7757; maximum scale 130 kg) and recorded.

2.3 Analysis of Pesticide Residues in Ready-to-eat Vegetables

2.3.1 Chemicals and Reagents

All chemicals and reagents were of analytical grade. Pesticide standards (96% or more purity) were obtained from various suppliers, namely Ciba-Geigy Ltd. for profenofos and cypermethrin, Calliope rural Traders, Australia for lambda-cyhalothrin, Sapa chemicals industries Ltd Tanzania for malathion, Dow AgroSciences France for dimethoate, Baytrade Tanzania Ltd for acephate, Novartis S.A. for dichlorvos, Zeneca Agrochemicals for permethrin and Twiga Chemicals Ind. Ltd. Tanzania for heptachlor. Solvents (acetonitrile, acetic acid, and acetone), salts (sodium acetate, magnesium sulphate and sodium sulphate) Primary Secondary Amine (PSA), glassware, centrifuge tubes and GC-MS vials were obtained from a local dealer Smacco-Flo General Supplies,

Arusha. All glassware was washed with a detergent dissolved in water and rinsed with distilled water followed by acetone, before and after each use. Centrifuge tubes and GC-MS vials were non-recyclable.

2.3.2 Pesticide Residue Extraction and Analysis

Pesticide extraction and clean-up were done following the QuEChERS Protocol (AOAC, 2007) . Briefly, samples were removed from the freezer and brought to room temperature before homogenization. After homogenization using a motor and pestle, 15 g of a sample was weighed into a 50 ml polypropylene centrifuge tube and extracted using acetonitrile with 1% acetic acid (1:10 v/v ml). 15 ml of the solvent followed by 100 µl or 200 µl of 1 mg ml^{-1} or 0.1 mg ml^{-1} heptachlor as an internal standard were added to the sample followed by 6g of anhydrous magnesium sulphate and 1.5 g sodium acetate. The mixture was then centrifuged in a Universal 320 centrifuge from Andreas Hettick GmbH Co KG, Tuttlingen Germany, at 536.64 x*g* for 5 minutes. Three millilitres of the supernatant was transferred to a 15 ml polypropylene centrifuge tube containing 600 mg anhydrous magnesium sulphate, 150 mg primary secondary amine (PSA) and 150 mg graphitized carbon and homogenized on a vortex mixer (Vortex Genie-2 from Bohemia, USA). The mixture was centrifuged at 536.64 x*g* for 5 minutes, and 2 ml of the supernatant transferred to the GC-MS vial for analysis of pesticide residues.

Pesticide residues were analyzed by GC-MS (Agilent 7890A equipped with 7693 auto-sampler coupled to 7000B triple quadruple MS system). The column was a fused silica DB35 capillary column 30 mm long with a 0.25 mm internal diameter and a 0.25 µm film capable of operating at a range of 50 ^{o}C to 360 ^{o}C. The temperature was set at 50 ^{o}C for 1 minute, then raised to 150 ^{o}C at a rate of 50 ^{o}C per minute for 1 minute, followed by 280 ^{o}C at a heating rate of 5 ^{o}C per minute and held for four minutes. The injector temperature was 250 ^{o}C. The carrier gas was helium at a flow rate of 1.2 ml min^{-1} splitless injection. The injection volume was 1µl at a pressure of 43.193 Psi. The MS ion source temperature was 250 ^{o}C operated in full scan mode at a scan range of 50-550 ^{o}C atomic mass unit.

2.4 Method Performance and Quality Assurance

The method performance was validated according to the European Commission guidelines (SANCO, 2014) by performing analyses to determine recovery, limit of detection (LOD), limit of quantification (LOQ), precision and linearity. Recovery was performed by analyzing, in triplicate, a mixture of standard pesticides in blank vegetable samples at levels of 0.0050, 0.0100 and 0.0200 mg kg^{-1}. These levels are below or above the MRLs of most of the pesticides approved for use in horticultural crops in Tanzania, and therefore could provide information on performance of the method at a range of the concentrations below, at, and above the MRLs of the pesticide residue in the vegetables. LOD was determined as the lowest concentration of the pesticide that could be detected but not quantifiable. LOQ was determined as the lowest concentration that could be quantified at acceptable accuracy and linearity. LOD and LOQ were determined as 1:3 and 1:10 signal to noise ratio, respectively. Precision was determined by calculating relative standard deviation (rsd) of the lowest concentration that could show linearity (n=5) in blank vegetable sample, whereas linearity was assessed by analyzing a mixture of pesticide standards at 0.005, 0.0075, 0.01, 0.0125, 0.0150, 0.0175 and 0.0200 mg kg^{-1}. The routine quality control was done by adding heptachlor as an internal standard in each analytical sample and calculated percentage recovery. Blank reagents were analyzed at the beginning and end of each batch to check for interference from chemicals and equipment. The concentration of pesticides analyzed was quantified from their corresponding calibration curves.

2.5 Estimating Dietary Pesticide Residue Exposure

Dietary exposure [mg kg^{-1} body weight (bw) per day] of a pesticide residue in an adult vegetable farmer was determined following the deterministic approach as guided by WHO and FAO (FAO/WHO, 2009). The exposure was estimated by multiplying concentration of the pesticide residue (mg kg^{-1}) in the vegetable sample (from the farmer's household) with the estimated amount of vegetable consumption by the individual (kg day^{-1}) and dividing by bw (kg) of the individual as shown in equation 1

$$EDI = \frac{Q(kg/day)xC(mg/kg)}{bw(kg)} \quad (1)$$

Where EDI is the estimated daily dietary intake of the pesticide residue in milligram per kilogram body weight of the consumer, Q is the quantity of vegetable consumed per day (kg per day) and C is the concentration of the residue in the vegetable in mg kg^{-1}.

2.5.1 Estimating the Risk of Unacceptable Exposures

Risk of unacceptable exposure to a particular pesticide residue was determined by calculating the hazard quotient of such particular pesticide using the equation described by JMPR (2005) and USEPA (2005) (equation 2):

$$HQ = \frac{EDI}{ADI} \tag{2}$$

Where: HQ is the hazard quotient, EDI is the estimated daily intake (mg kg^{-1} bw day^{-1}) of a particular pesticide and ADI is the corresponding acceptable daily intake (mg kg^{-1} bw day^{-1}) for the pesticide.

For multiple exposures to pesticide residues falling under the same chemical group (same mechanism of toxicity) such as organophosphates or pyrethroids, the risk of exposure was calculated by adding the HQs of pesticide residues of the same chemical group to obtain the hazard index, using equation 3 (EFSA, 2008; FAO/WHO, 2005; USEPA, 2005).

$$HI = \frac{EDIa}{ADIa} + \frac{EDIb}{ADIb} + \ldots \frac{EDIn}{ADIn} \tag{3}$$

Where: HI is the hazard index, *a, b…n* represent different pesticides of the same mechanism of toxicity, EDI is the estimated daily intake of each pesticide and ADI is the corresponding acceptable daily intake. HQ or HI ≤ 1 indicates that adverse health effect(s) are not likely to occur and thus the amount of pesticide residue consumed can be considered tolerable. HQ or HI > 1 denotes that the exposure is greater than ADI and that there might be a risk from the residue consumed, a situation which calls for a risk management action to be taken (FAO/WHO, 2005; United States Environmental Protection Agency [USEPA], 2005). Exposure in farmers who consumed vegetables with undetectable pesticide residues was performed by assigning a default value of half the limit of detection for each pesticide (middle bound scenario), according to the US-EPA's Office of Pesticide Programs (USEPA, 2000).

2.6 Data Analysis

Data entry and clean-up for pesticide application practices were done using Epidata version 3.1, a free downloadable software owned by WHO which was obtained from The Tanzania National Institute for Medical Research (NIMR). The data were then exported to Microsoft Excel 2007 and SPSS version 21 for analysis. Data for pesticide residue content, vegetable consumption and body weight were used to calculate and estimate daily intakes and risk of exposure using equations *'1'* to *'3'* of this section. Descriptive statistics (frequency and percentage) were used to interpret information captured from questionnaires. Logistic regression was used to analyze the association between level of education, the source of vegetables (between home-grown and market or neighbour sourced), or pesticide application practices and exposures of pesticide residues to farmers. The significance level of association was set at $p \leq 0.05$.

3. Results and Discussion

3.1 Method Performance and Quality Assurance

Average recoveries of all pesticide standards in sample matrices ranged from 79% to 112% indicating that the results obtained are reproducible. Limits of detection ranged from 0.001 to 0.004 mg kg^{-1} whereas limits of quantification ranged from 0.002 to 0.015 mg kg^{-1} which shows that the sensitivity of the method is good enough for detection and quantification of pesticide residues in the vegetable samples below the set MRLs for most of the pesticides. The percent rsd ranged from 1.02% to 18.6 % and coefficient of correlation was between 0.955 and 0.999 (Table1) showing good repeatability of the method. Recovery for heptachlor (added to each analytical sample to check for the on-going performance of the method) ranged from 70% to 132% with an average of 95%. No corrections made to the concentration of residues in the samples as the recoveries were within the recommended range. It is recommended that for on-going method performance verification, recovery should range from 60%-140% (SANCO, 2014). No pesticide residues detected in the blank chemical reagents which indicate that there was good control of interferences from chemicals and instruments. These results indicate that the method was reliable for analysis of the pesticide residues of interest in the ready-to-eat leafy vegetables. For a method to be reliable, initial method validation recovery should be between 70 and 120%, percent rsd not higher than 20% and coefficient of correlation equal to or higher than 0.95 (Kofi Akomeah, Akuamoa, Frimpong & Buah-kwofie, 2016; SANCO, 2014).

Table 1. Results of QuEChERS multi-residues method validation in leafy vegetables

Analyte	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	r^2	Mean recovery (%)	rsd % (n=5)
Permethrin	0.001	0.005	0.997	88.01	13.9
Cypermethrin	0.002	0.006	0.999	92.52	9.60
Cyhalothrin	0.001	0.005	0.992	112.3	13.6
Dimethoate	0.004	0.015	0.955	89.98	9.93
Acephate	0.003	0.009	0.960	78.86	18.6
Profenofos	0.004	0.010	0.992	91.12	1.02
Malathion	0.001	0.002	0.995	83.15	12.2
Dichlorvos	0.004	0.010	0.995	100.2	1.70
Heptachlor	0.001	0.030	0.999	102.0	9.80

3.2 Socio-demographic Characteristics of the Participants

The results on socio-demographic characteristics of the surveyed vegetable farmers in Arusha district are as indicated in Table 2. The socio-demographic characteristics recorded were gender, age and level of education.

Most of the respondents in this study, (52 representing 74.3% of participants), were male aged from 25 to 65 years with a mean age of 42.3±13.6 years. It was reported that pesticide application in Arusha is done by men. In cases where farmers are women, they hired men to apply pesticides. As a consequence and considering exposure through inhalation or skin, the risks of exposure to pesticides for men can be higher than in women. The gender distribution is congruent to that made previously in the Manyara basin in Tanzania by Nonga et al. (2011), who reported 75% of farmers being male with a mean age of 47±14 years. In a similar work done in Muheza, Arumeru, Singida and Kongwa, it was found that 85% of all farmers involved in vegetable cultivation were men (Weinberger & Msuya, 2004). Studies done in other developing countries also report similar results (Amera & Abate, 2008; Banjo et al., 2010).

More than half (52.9%) of the vegetable farmers in Arusha district had a formal education of up to primary level. About one-fourth of the respondents had no formal education, and less than a quarter had secondary and college education. Illiteracy of farmers has been linked to poor pesticide application practices by farmers in previous surveys (Mengistie, Mol & Oosterveer, 2015; Nonga et al., 2011).

Table 2. Socio-demographic characteristics of vegetable farmers (n=70)

Variable	Category	Percentage (%)
Sex	Male	74.3
	Female	25.7
Age	15-35	37.1
	36-45	21.4
	46 and above	41.5
Level of education	No formal education	25.7
	Primary school	52.9
	Secondary and higher level	21.4

3.3 Pesticide Residue Contents in Ready-to-eat Vegetables

Ready-to-eat vegetable samples were available from 70 out of the 76 farmers as six farmers were not willing to provide samples. The seventy (70) ready-to-eat vegetable samples were analyzed for pesticide residues. They included 31 African nightshade (44.3%), 15 kale (21.4%), 10 cabbage (14.3%), three spinach (4.3 %), two Ethiopian mustard (2.9%), one Chinese cabbage (1.4%), two *Amaranthus spp*. (2.9%) and six vegetables prepared with combinations of nightshade with kale (4.3%), nightshade with kale and spinach (1.4%), nightshade with Ethiopian mustard (1.4%), or kale with spinach (1.4%). Overall, 40% of all the 70 samples contained detectable levels of pesticide residues. Individually, 60.0% of cabbage, 53.3% of kale, 35.5% of nightshade, 33.3% of spinach and 33.3% of the mixed vegetables contained pesticide residues. No pesticide was detected in *Amaranthus spp*, Chinese cabbage, and Ethiopian mustard.

Among the 70 samples, 58 (83%) were obtained from respondents' own grown vegetables whereas the remaining 12 (17%) samples were from vegetables purchased from outside homes as follows: three and two nightshade samples, respectively, from neighbours and market, two kale samples (one from market and the other from a neighbour), two *Amaranthus spp.* samples (both from neighbours), kale and nightshade and kale and

spinach for two mixed vegetable samples from the market and a neighbour, respectively. All the cabbage samples were obtained from respondents' own grown vegetables. Of the 12 samples from market or neighbours only two (17%) contained detectable levels of pesticide residues whereas among the 58 samples from farmers own farm vegetables, 26 (45%) contained detectable levels of residues. The farmers who obtained their vegetables from their neighbours disclosed that they preferred neighbours' vegetables because were grown without pesticides. This might be the reason why pesticide residues were not detectable in vegetables obtained from neighbours, except one nightshade sample which contained permethrin. It is also possible that the market vegetables had taken longer time, from harvest to consumption, as compared to home-grown vegetables until residues were measured. The longer time could allow reduction of pesticide residues to undetectable levels. This is concurrent with the statement of European Food Safety Authority that depending on the point along the distribution chain where vegetables are obtained, pesticide residues may have declined to levels not detectable at the time of consumption (EFSA, 2012).

There are published reports of higher prevalence of pesticide residues in vegetables than found in the current study. For instance, in Chile, pesticide analysis was done in 118 leafy vegetable samples and it was found that 72% of spinach samples contained detectable levels of pesticide residues (Elgueta, Moyano, Sepulveda & Quiroz, 2017). In Algeria, 120 vegetable samples were analyzed and pesticide residues, detected in 57.5% of the samples (Mebdoua, et al., 2017). Another study which analyzed pesticide residues in parsley, lettuce and spinach in Turkey found that all of the samples contained detectable levels for two or more pesticide residues, including dichlorvos which was quantified in every vegetable at a prevalence of 100% (Esturk, 2014). The detected pesticide residues in the current study were all above EU-MRL. Other studies also detected pesticide residues in vegetables at levels above MRL. For instance, the pesticide residues quantified by Esturk, 2014 were at levels above MRL in 28%, 20% and 40% of parsley, lettuce and spinach, respectively. High prevalence of pesticide residues at levels above MRL in ready-to-eat vegetables reflects the indiscriminate use and misuse of pesticides as reported in the literature (Ngowi et al., 2007; Nonga et al, 2011) and observed in the current study.

On the other hand, two studies in India found the lower prevalence of pesticide residues compared to the levels found in the current study. In those studies, 10% of 50 vegetable samples from Karnataka and 34% of 250 vegetable samples from the Andaman Islands contained detectable levels of pesticide residues of which all of the positive samples (10%) from Karnataka and 15.3% contained pesticide residues above MRL (Pujeri, Pujar, Hiremath, Pujari & Yadawe, 2015; Swarman & Velmurugan, 2012).

The detected pesticide residues were insecticides in the groups of organophosphates and pyrethroids which were, in 31.4% and 17.1% of the analysed vegetable samples, respectively. Organophosphate pesticides detected (with their prevalence in brackets) were dimethoate (14.3%), acephate (12.9%), profenofos (8.57%), malathion (2.86%) and dichlorvos (2.86%) and the pyrethroid pesticides were permethrin (17.1%), cypermethrin (1.43%) and lambda-cyhalothrin (1.43%). Representative chromatograms of the detected pesticides are presented in Figure 1 and 2. Range and mean concentration of pesticide residues in the ready-to-eat vegetables are presented in Table 3.

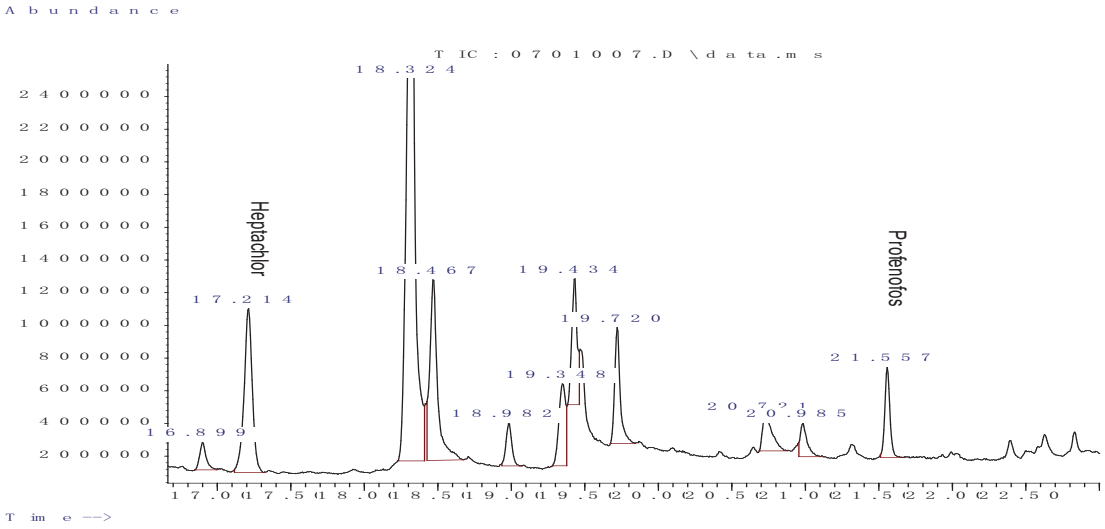

Figure 1. A chromatogram of Profenofos in kale

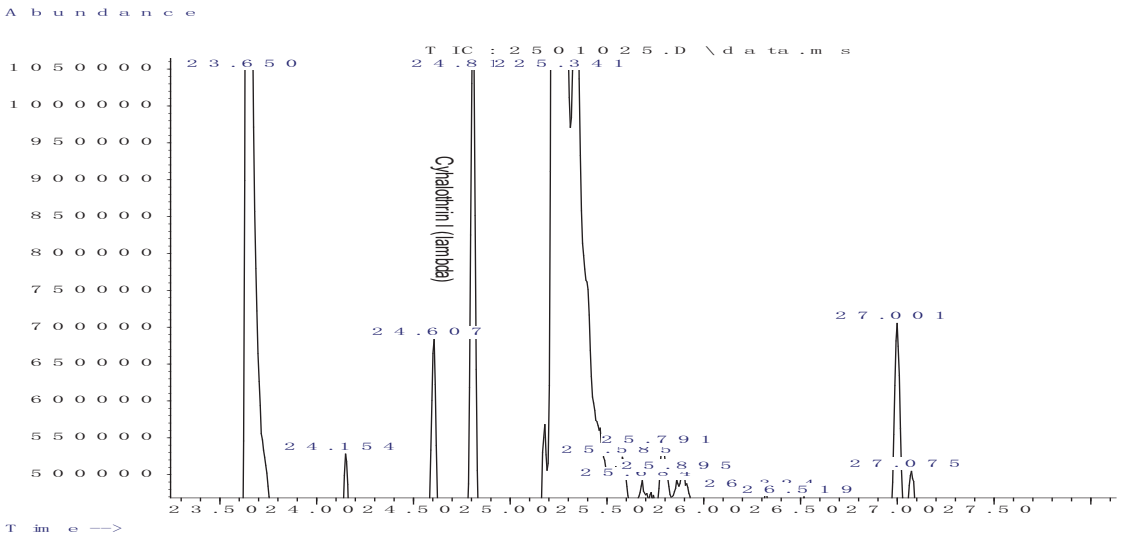

Figure 2. A chromatogram of Lambda cyhalothrin in kale

Organochlorine pesticides were not detected in the current study which is a good indication that there is a shift from the use of organochlorine pesticides to pyrethroid and organophosphorus pesticides and therefore a reduced risk of negative health effects associated with exposure to organochlorines. Organochlorines were detected in the studies done in Dar es Salaam (Mahugija, Khamis, & Lugwisha, 2017; Ndengerio-Ndossi & Cram, 2005). The organochlorine pesticides were found in foods as reported in 2005 despite the fact that they had been banned from use in agriculture in Tanzania since 1997 due to their long persistence in the environment and bioaccumulation properties (URT, 2005).

Table 3. Occurrence of pesticide residues in ready-to-eat vegetables (n=70)

Pesticide group (prevalence, %)	Pesticide	Prevalence (%)	Range (mg kg^{-1})	Mean±SD[1] (mg kg^{-1})
Organophosphates (31.4%)	Dimethoate	14.3	2.88-15.4	8.56±4.52
	Acephate	12.9	0.33-12.4	2.90±3.81
	Profenofos	8.57	6.53-16.6	8.44±3.98
	Dichlorvos	2.86	8.60-33.0	20.8±17.3
	Malathion	2.86	4.63-6.31	5.47±1.19
Pyrethroids (17.1%)	Permethrin	17.1	1.23-8.18	2.95±1.92
	Lambda cyhalothrin	1.43	<0.05-16.2	16.2±0.05
	Cypermethrin	1.43	<0.06-2.34	2.34±0.06

[1]Standard deviation

Distribution, range and mean concentration of pesticides in individual vegetables are presented in Table 4. The highest mean concentration for permethrin, dimethoate, and lambda-cyhalothrin (3.44±0.93 mg kg^{-1}, 10.8±6.90 mg kg^{-1}, and 16.2±0.05 mg kg^{-1}), respectively, were found in kale samples whereas that of cypermethrin, acephate, malathion, and dichlorvos (2.34±0.06 mg kg^{-1}, 4.19±5.55 mg kg^{-1}, 5.47±1.19 mg kg^{-1} and 33.0±0.01mg kg^{-1}), respectively, were found in nightshade. The highest mean concentration for profenofos (16.4±0.01 mg kg^{-1}) was found in mixed vegetables.

The lowest mean concentrations of permethrin, cypermethrin and malathion (1.7±0.05 mg kg^{-1}, 2.34±0.06 mg kg^{-1}, 5.47±1.19 mg kg^{-1}), respectively, were found in nightshade. The lowest concentration for dimethoate (6.48±2.68 mg kg^{-1}) was estimated in cabbage whereas that of acephate (0.36 mg kg^{-1}), was found in mixed vegetables. The lowest mean concentration for dichlorvos and profenofos were quantified in kale (8.60±0.01 mg kg^{-1}) and nightshade (6.64±0.01 mg kg^{-1}), respectively. It should be noted that lambda cyhalothrin and cypermethrin were detected in one sample only. For the samples in which pesticide residues were detected, the concentrations were above the respective EU MRLs (Table 4). Quantification of pesticide residues in ready-to-eat-vegetables at levels higher than MRLs indicates poor adherence to good agricultural practices by vegetable farmers.

Table 4. Variation of pesticide residues in individual types of ready-to-eat vegetables

Vegetable (n)	Source (n)	Group	Prevalence (%)	Pesticide	MRL-(EU)	Range (mg kg^{-1})	Mean±SD[1] (mg kg^{-1})	f^2>LoD (%)	f^3>MRL (%)
Cabbage(10)	own farm(10)	Organophosphate	6(60)	Dimethoate	0.05	4.58-8.37	6.48±2.68	(2)20.0	(2)20.0
				Profenofos	0.01	<0.01-7.07	7.07±0.01	(1)10.0	(1)10.0
				Acephate	0.01	<0.01-1.97	1.97±0.01	(1)10.0	(1)10.0
		Pyrethroids		Permethrin	0.05	1.44-3.91	2.37±1.34	(3)30.0	(3)30.0
Kale(15)	own farm(13)	Organophosphate	8(53)	Dimethoate	0.02	2.88-15.4	10.8±6.90	(3)20.0	(3)20.0
				Acephate	0.01	2.04-4.60	3.32±1.81	(2)13.3	(2)13.3
				Profenofos	0.01	<0.01-7.24	7.24±0.01	(1)6.70	(1)6.67
				Dichlorvos	0.01	<0.01-8.60	8.60±0.01	(1)6.67	(1)6.67
		Pyrethroids		Permethrin	0.05	2.62-4.45	3.44±0.93	(3)20.0	(3)20.0
				Cyhalothrin	0.05	<0.05-16.2	16.2±0.05	(1)6.67	(1)6.67
	purchased (2)	Organophosphate		Profenofos	0.01	<0.01-6.53	6.53±0.04	(1)6.67	(1)6.67
Nightshade(31)	own farm(26)	Organophosphate	11(35.5)	Dimethoate	0.02	4.25-12.0	8.05±3.74	(5)16.1	(5)16.1
				Acephate	0.01	0.33-12.4	4.19±5.55	(4)12.9	(4)12.9
				Malathion	0.02	4.63-6.31	5.47±1.19	(2)6.45	(2)6.45
				Profenofos	0.01	<0.01-6.64	6.64±0.01	(1)3.22	(1)3.22
				Dichlorvos	0.01	<0.01-33.0	33.0±0.01	(1)3.22	(1)3.22
		Pyrethroid		Permethrin	0.05	1.23-8.18	3.40±3.20	(4)12.9	(4)12.9
	purchased (5)	Pyrethroid		cypermethrin	0.05	<0.06-2.34	2.34±0.06	(1)3.22	(1)3.2
				Permethrin	0.05	<0.05-1.70	1.70±0.05	(1)3.22	(1)3.2
Mixed (6)	own farm (4)	Organophosphate	2(33.3)	Profenofos	0.01	<0.01-16.4	16.4±0.01	(1)16.7	(1)16.7
				Acephate	0.01	0.30-0.42	0.36±0.01	(2)33.3	(2)33.3
		Pyrethroid		Permethrin	0.05	<0.05-2.60	2.60±0.05	(1)16.7	(1)16.7
Spinach (3)	own farm (3)	Organophosphate	1(33.3)	Profenofos	0.01	<0.01-6.63	6.67±0.01	(1)33.3	(1)33.3

[1]Standard deviation; [2]detection frequency of the pesticide in the particular vegetable; [3]Frequency of detected pesticides that were above MRL; Source (MRLs): (European Comission, 2017)

Multiple pesticide residues were detected in 14.9% of the 70 samples. This prevalence is equivalent to 35.7% of the 28 samples which were positive for pesticide residues. Among the 31 nightshade and six mixed vegetable samples analyzed, 16.13% and 16.67%, respectively, had multiple residues whereas among 15 kale samples 20% had multiple residues. Samples of cabbage had the lowest occurrence of multiple residues (one out of six (10%)) (Table 5). Multiple occurrences of pesticide residues in vegetables have also been reported in literature: a study in Khazastan which analyzed 82 samples of tomato and cucumber found that 30% of the samples contained two to nine multiple pesticide residues in one sample (Lozowicka et al., 2015). Presence of multiple pesticide residues in one sample indicates that consumers are at higher risk of exposure and synergistic negative health effects of pesticides.

Table 5. Co-occurrence of multiple pesticide residues in ready-to-eat vegetables

Vegetable	Pesticide residues combination	Prevalence (%)
Kale	Acephate, permethrin	20.00
	Dimethoate, permethrin, cyhalothrin	
	Profenofos, dichlorvos,	
Overall prevalence in kale		
Nightshade	Dimethoate, dichlorvos, malathion	16.13
	Acephate, dimethoate, permethrin	
	Acephate, dimethoate	
	Dimethoate, malathion	
	Permethrin, cypermethrin	
Overall prevalence in nightshade		
Nightshade with kale and spinach mix	Acephate, profenofos, permethrin	16.67
Cabbage	Dimethoate, permethrin	10.00

The quantified concentrations of most pesticide residues in the current study were higher than those found in other studies. Elgueta et al., 2017 quantified low pesticide residue concentrations in vegetables whereby

lambda-cyhalothrin cypermethrin and permethrin were quantified at a range of 0.029-1.000 mg kg^{-1}, 0.00-1.61 mg kg^{-1} and 0.00-1.45 mg kg^{-1}, respectively, in chard, lettuce, and spinach. However, they quantified methamidophos (29.47 mg kg^{-1}) and chlorpyrifos (6.86 mg kg^{-1}) at higher concentrations than quantified in the current study. Also, a study in the Andaman Islands in India quantified profenofos, dimethoate and acephate in vegetables at a lower concentrations than that found in the current study whereby profenofos concentrations in the study done in the Andaman Islands ranged from 0.023-1.696 mg kg^{-1}, acephate 0.083-0.509 mg kg^{-1} and dimethoate at 0.345 mg kg^{-1} (Swarman & Velmurugan, 2012). However, a study in Egypt found a concentration of profenofos in green parsley (7.2 mg kg^{-1}) (Gad-Alla, Lontfy, Shendy & Ahmed, 2015) similar to that of the current study (8.44 mg kg^{-1}). In Ghana, lower concentrations of 0.120-0.143 mg kg^{-1} as compared to 4.6-6.3 mg kg^{-1} in the current study were found in vegetables. However, the prevalence of samples quantified with pesticide residues was higher in the Ghanian study than in this one (Darko & Akoto, 2008). In Turkey, analysis of pesticide residues in 120 samples of parsley, lettuce and spinach found dichlorvos at concentrations ranging from 0.002-0.071 mg kg^{-1}, levels that are lower than the 8.6-33.0 mg kg^{-1} levels found in this study. In Zambia, Sinyangwe et al. (2016) analysed dichlorvos residues in 14 lettuce, 15 cabbage and 9 rape samples and, by summing up the prevalence of the residues detected below and above MRL, found that 71%, 93% and 100% of lettuce, cabbage and rape plant samples, respectively, contained mean dichlorvos concentrations of 5.23 mg kg^{-1}, 6.35 mgkg^{-1} and 398.28 mg kg^{-1}. The reported overall prevalence (89%) is much higher than that obtained in the current study (2.86%) for dichlorvos. Also, the concentration of dichlorvos residues in the rape plant reported in the same study is considerably higher than that found in the current study (33 mg kg^{-1}).

WHO recommends classifying pesticides by acute risk to health whereby class Ia refers to pesticides that are extremely hazardous, class Ib are highly hazardous, class II are moderately hazardous, class III are slightly hazardous and class U are unlikely to cause acute health hazard (International Programme on Chemical Safety [IPCS], 2010). The pesticides residues found in the ready-to-eat vegetables analysed in this study are in class Ib, II and III. Most pesticides were found under Class II insecticides with exception of dichlorvos which is classified as class Ib and malathion classified as class III insecticides. These results indicate that vegetable farmers are shifting from using more to less hazardous pesticides and therefore exposed to reduced health effects. The class Ib pesticides are registered under restriction and therefore less accessible to vegetable farmers.

3.3.1 Presence of Unauthorized Pesticides in Ready-to-Eat Vegetables

In Tanzania, dichlorvos is restricted for the control of the larger grain borer in maize grain storage facilities. Pesticides registered for restricted use are those that are highly hazardous and intended for specific use or are technical materials for formulation purposes and must be used by specifically trained personnel or under close supervision of specifically trained personnel (URT, 2011). Dichlorvos, although less frequently detected (2.86%) as compared to other organophosphate pesticides, was detected at the highest mean concentration of 20.8 mg kg^{-1} with a range of 8.6-33.0 mg kg^{-1} (Table 4). Detection of dichlorvos in ready-to-eat vegetables indicates misuse of pesticides. It is recommended to provide continuous training to vegetable farmers on pesticide application, and undertake regular monitoring of pesticide residues in vegetables to ensure that restricted pesticides such as dichlorvos are not inappropriately used and to control pesticide residues (in general) to acceptable levels in vegetables.

3.4 Risk of Exposure above Acceptable Daily Intakes

3.4.1 Type, Frequency and Quantity of Consumed Vegetables

The vegetable consumers in Arusha district consume vegetables as side dishes to main dishes that include stiff porridge, rice and banana. Among the mainly consumed leafy vegetables, African nightshade was the one most consumed. It was consumed by 43% of the respondents. For the vegetables used as a minor ingredient in the recipe, onions and tomatoes were consumed by most respondents (76.3 and 70.4, respectively). The average daily vegetable consumption at the time of the survey was 119 g per person. The consumption rates ranged from 14 -302 g per person per day. In Sub Saharan Africa, per capita daily vegetable consumptions ranging from13 g (Malawi), through 70 g (Ethiopia) to 84 g (Guinea) and higher quantities ranging from 126 g (Rwanda) through 137 g (Ghana) and 142 g (Uganda) to 242 g (in Kenya) are reported (Minot and Smith, 2004). *Note that the values in the review work were reported consumption per year but were converted into consumption per day in the current work to enable comparison*. With the exemption of Kenya, average consumption of vegetables in developing countries is a half of the recommended amount of 200 g per person per day (Smith & Eyzaguirre, 2007). It is recommended to consume at least 200 g of vegetables per day (Agudo & Joint FAO, 2005; Keding, Weinberger, Swai, & Mndiga, 2007). When considering this recommendation, only 18.6% of vegetable farmers in the Arusha district met the required daily vegetable consumption. If the farmers in Arusha consumed

vegetables at the recommended intake of 200g per person per day the risk of unacceptable pesticide intakes would increase considerably. Assuming a vegetable farmer with a body weight of 67 kg (the average body weight of farmers in Arusha district), consumes 200g of vegetables, every day, containing pesticides at the mean concentrations determined in this study, mean exposures in mg kg^{-1} bwd^{-1} for this farmer, with the pesticide in bracket, would be 0.0036 (dimethoate), 0.0018 (dichlorvos), 0.0022 (profenofos) and 0.0011 (acephate), 0.0005 (malathion) for organophosphate pesticides. For pyrethroids, the mean exposures would be 0.0011 (permethrin), 0.0001 (cypermethrin) and 0.007 for lambda-cyhalothrin. These would lead to unacceptable hazard quotients of 1.829 for dimethoate, and a hazard index of 2.385 for organophosphates. The hazard index for organophosphates is more than twofold the hazard index of 1.19 determined in this study with the normal vegetable consumption pattern. This indicates that promotion for increased vegetable consumption should go hand in hand with training and awareness raising to vegetable farmers on the appropriate use of pesticides and continuous monitoring and control of pesticide residues in vegetables.

3.4.2 Risk of Chronic Pesticide Exposure

Overall assessment of chronic exposure to pesticide residues through vegetable consumption indicates potential health risks to vegetable farmers. Among the 70 farmers that participated in this study, 18.6% were at potential health risks of unacceptable exposure to pesticide residues. Exposure levels and hazard indices of organophosphate and pyrethroid pesticides to vegetable farmers in Arusha district are presented in Tables 6a and b and 7a and b, respectively.

The vegetable farmers were at higher health risk of unacceptable exposure of organophosphate pesticides. The hazard quotient of 7.5 was determined for dimethoate when considering positive detects only (Table 6a), and even after including non-detects assigned with the respective half limit of detection (0.5 LOD) in the mean exposure estimation was still above one (1.044) (Table 6b). The mean exposure level for this chemical was 0.015 mg kg^{-1} bwd^{-1} when considering positive detects only, and 0.0021 when 0.5 LOD of this residue was included in the exposure estimation. Both values were above the ADI of dimethoate (0.002 mg kg^{-1} bwd^{-1}). The HQ of dimethoate was above one for kale (2.57 and 12.8 with and without 0.5 LOD included in the exposure estimation, respectively) whereas in cabbage it was 0.928 and 4.75 with and without the 0.5 LOD included in the estimation, respectively. Mean exposure for dichlorvos was 0.011 mg kg^{-1} bwd^{-1}which was also above its corresponding ADI (0.004 mg $kg^{-1}bwd^{-1}$) yielding HQ of 2.75. After including 0.5 LOD in the exposure estimation for this residue, the mean exposure was reduced to 0.0003 and HQ of 0.075 was estimated, indicating a minimum potential health risk.

Mean exposure for other organophosphate (acephate, profenofos, and malathion) and pyrethroid (permethrin, cypermethrin, and lambda cyhalothrin) pesticide residues quantified in this study were below one in both scenarios indicating a minimum health risk. These results indicate that vegetable farmers in Arusha district are at risk of intolerable health effects associated with exposure to organophosphate pesticides and that the risk is mainly contributed to by intake of dimethoate through consumption of kale. Risk of cumulative exposures to the organophosphate pesticide residues is above one even after including the 0.5 LOD of the non-detects in the exposure estimation as shown by the Hazard index (HI) of 11 for positives only (Table 6a) and 1.19 after including the 0.5 LOD of the respective residues in the exposure (Table 8). The HI for pyrethroid pesticide residues was found to be below one in both scenarios (0.029 and 0.9 with and without the 0.5 LOD, included in the estimation, respectively) (Table 7a and 7b). These results show that the risk of exposure to the pesticide residues is overestimated when values for non-detects are not included in estimation of the risk. However, even after including these values it shows that there remains a risk of intolerable health effects and the risk is aggravated through multiple exposures to the organophosphate pesticide residues.

A study in Egypt reports cumulative hazard indices for organophosphates higher than those of pyrethroids but both of them below one (Gad-Alla et al., 2015; Thabet, Shendy, & Gadalla, 2016). Usually, in Arusha district, vegetables are prepared for consumption for the entire family including children and pregnant women who are reported to be more vulnerable to health risks associated with exposure to pesticide residues than other groups of the population (FAO/WHO, 2009). Exposure to organophosphate pesticides during pregnancy has been linked with autism spectrum disorders (ASD) characterized by problems in socio-communication and restricted repetitive behaviours and pregnancy miscarriage. Children are more adversely exposed to the pesticide residues due to their small body size and therefore might be at a higher risk than estimated in this study for adults (Arbuckle, & Lin, 2001; Eskenazi et al., 2004; Bouchard, et al., 2011). Furthermore, dietary exposure to pesticides is not limited to vegetables. The farmers may also be exposed to pesticides from other food types, water and air.

Table 6a. Risk of dietary pesticides exposures above ADIs for organophosphate (positives only)

Pesticide (Prevalence)	Code	Vegetable	Concentration (mg kg^{-1})	EDI (mg kg $bw^{-1}d^{-1}$)	ADI (mg kg^{-1} bw)	HQ/HI
Dimethoate (14.3%)	B26	Nightshade	4.25	0.011	0.002	5.500
	B41	Nightshade	12.0	0.013	0.002	6.500
	B1	Nightshade	11.7	0.005	0.002	2.500
	B74	Nightshade	7.75	0.016	0.002	8.000
	B4	Nightshade	4.54	0.005	0.002	2.500
		Nightshade (average)		0.010		5.000
	B71	Kale	15.4	0.052	0.002	26.00
	B36	Kale	14.1	0.023	0.002	11.50
	B5	Kale	2.88	0.002	0.002	1.000
		Kale (average)		0.026		12.80
	B62	Cabbage	4.58	0.003	0.002	1.500
	B6	Cabbage	8.37	0.016	0.002	8.000
		Cabbage (average)		0.010		4.750
				0.015		**7.500**
Dichlorvos (2.86%)	B73	Kale	8.60	0.007	0.004	1.750
	B1	Nightshade	33.0	0.014	0.004	3.500
				0.011		**2.750**
Acephate (12.9%)	B15	Spinach, nightshade	0.30	0.001	0.030	0.033
	B33	Kale, nightshade	0.42	0.001	0.030	0.033
	B54	Nightshade	12.4	0.012	0.030	0.400
	B34	Nightshade	2.03	0.002	0.030	0.067
	B41	Nightshade	1.97	0.002	0.030	0.067
	B74	Nightshade	0.33	0.001	0.030	0.033
		Nightshade (average)		0.004		0.140
	B39	Kale	4.60	0.003	0.030	0.100
	B44	Kale	2.04	0.005	0.030	0.167
		Kale (average)		0.004		0.130
	B49	Cabbage	1.97	0.005	0.030	0.167
				0.004		**0.130**
Malathion (2.8%)	B1	Nightshade	6.31	0.000	0.300	0.001
	B4	Nightshade	4.63	0.005	0.300	0.017
		Nightshade (average)		0.003		**0.009**
Profenofos (8.6%)	B15	Spinach, nightshade	16.6	0.069	0.03	2.300
	B51	Nightshade	6.64	0.011	0.03	0.367
	B46	Kale	6.53	0.015	0.03	0.500
	B73	Kale	7.24	0.004	0.03	0.133
		Kale (average)		0.010		0.320
	B61	Cabbage	7.07	0.001	0.03	0.033
	100	Spinach	6.63	0.015	0.03	0.500
				0.019		**0.630**
						11.00*

Note: [1] mg $kgbw^{-1}d^{-1}$ is mg per kg body weight per day; Source (ADI): (FAO & WHO, 2015); The bolded values in the column of EDI are the mean exposure values for the particular pesticide in the vegetables; The bolded values in the HQ/HI column are the HQ values for particular pesticide in the vegetables *The HI for the organophosphates

Table 6b. Risk of dietary pesticides exposures above ADIs for organophosphate (including non-detects assigned with 0.5 LOD); EDIs in (mg kg $bw^{-1}d^{-1}$)

n	Vegetable	Acephate		Dimethoate		Profenofos	
		Mean EDI	HQ	Mean EDI	HQ	Mean EDI	HQ
31	African nightshade	0.001	0.018	0.002	0.813	0.000	0.011
15	Kale	0.001	0.018	0.005	2.570	0.001	0.046
10	Cabbage	0.000	0.012	0.002	0.928	0.000	0.005
3	Spinach	0.000	0.000	0.000	0.002	0.005	0.166
2	Ethiopian mustard	0.000	0.000	0.000	0.003	0.000	0.000
2	Amaranthus spp	0.000	0.000	0.000	0.001	0.000	0.000
1	Chinese	0.000	0.000	0.000	0.005	0.000	0.000
6	Mixed vegetables	0.000	0.011	0.000	0.002	0.011	0.382

Table 6b. Risk of dietary pesticides exposures above ADIs for organophosphate (including non-detects assigned with 0.5LOD); EDIs in (mg kg $bw^{-1}d^{-1}$)[1] cont…

n	Vegetable	Dichlorvos		Malathion	
		Mean EDI	HQ	Mean EDI	HQ
31	African nightshade	0.000	0.112	0.000	0.001
15	Kale	0.001	0.116	0.000	0.000
10	Cabbage	0.000	0.001	0.000	0.000
3	Spinach	0.000	0.001	0.000	0.000
2	Ethiopian mustard	0.000	0.001	0.000	0.000
2	Amaranthus spp	0.000	0.001	0.000	0.000
1	Chinese	0.000	0.002	0.000	0.000
6	Mixed vegetables	0.000	0.001	0.000	0.000

Table 7a. Risk of dietary pyrethroid pesticide exposures below ADIs (positives only)

Pesticide (Prevalence)	Code	Vegetable	Concentration mg kg^{-1}	EDI mg $kgbw^{-1}d^{-1}$	ADI mg $kgbw^{-1}d^{-1}$	HQ/HI
Lambda cyhalothrin (1.4%)	B5	Kale	16.2	**0.012**	0.02	**0.600**
Cypermethrin (1.4%)	B10	Nightshade	2.34	**0.003**	0.02	**0.150**
Permethrin (17.1%)	B15	Spinach, nightshade	2.60	0.011	0.05	0.220
	B4	Nightshade	2.10	0.002	0.05	0.040
	B10	Nightshade	1.70	0.002	0.05	0.040
	B29	Nightshade	2.17	0.002	0.05	0.040
	B28	Nightshade	1.23	0.002	0.05	0.040
	B41	Nightshade	8.18	0.009	0.05	0.180
		Nightshade (average)		0.003		0.068
	B11	Cabbage	3.91	0.013	0.05	0.260
	B62	Cabbage	1.76	0.001	0.05	0.020
	B3	Cabbage	1.45	0.002	0.05	0.040
		Cabbage (average)		0.005		0.110
	B45	Kale	4.45	0.010	0.05	0.200
	B5	Kale	3.27	0.002	0.05	0.040
	B44	Kale	2.62	0.007	0.05	0.140
		Kale (average)		0.006		0.130
				0.005		**0.100**
						0.900*

Note: [1] mg $kgbw^{-1}d^{-1}$ is mg per kg body weight per day; Source (ADI): (FAO/WHO, 2015); The bolded values in the column of EDI are the mean exposure values for the particular pesticide in the vegetables; *The HI for the pyrethroids

Table 7b. Risk of dietary pesticide exposures below ADIs for pyrethroids (including non-detects assigned with 0.5LOD); EDIs in (mg kg $bw^{-1}d^{-1}$)

n	Vegetable	Permethrin		Cypermethrin		Cyhalothrin	
		Mean EDI	HQ	Mean EDI	HQ	Mean EDI	HQ
31	African nightshade	0.001	0.011	0.000	0.005	0.000	0.000
15	Kale	0.001	0.026	0.000	0.000	0.001	0.041
10	Cabbage	0.002	0.031	0.000	0.000	0.000	0.000
3	Spinach	0.000	0.000	0.000	0.000	0.000	0.000
2	Ethiopian mustard	0.000	0.000	0.000	0.000	0.000	0.000
2	Amaranthus spp	0.000	0.000	0.000	0.000	0.000	0.000
1	Chinese	0.000	0.000	0.000	0.000	0.000	0.000
6	Mixed vegetables	0.000	0.036	0.000	0.000	0.000	0.000

Table 8. Average estimated daily intakes and hazard quotients of pesticide residues in vegetables

Pesticide group	Pesticide residue	EDIs	HQ	HI
Organophosphates	Dimethoate	0.002	1.044	1.190
	Acephate	0.000	0.014	
	Profenofos	0.002	0.055	
	Dichlorvos	0.000	0.075	
	Malathion	0.000	0.000	
Pyrethroids	Permethrin	0.001	0.018	0.029
	Cypermethrin	0.000	0.002	
	Cyhalothrin	0.000	0.009	

3.5 Association of Pesticide Exposure and Application Practices

Sources of vegetable for household consumption, knowledge and awareness on pesticide use, vegetable cropping systems and lack of advice from agricultural extension officers, pesticide application rates and adherence to pre-harvest interval were assessed in this study in order to establish their association with exposures to pesticide residues through vegetable consumption.

3.5.1 Source of Vegetables

Among the 70 vegetable farmers interviewed only 12 (17%) reported to obtain their vegetables from market or neighbours as discussed in the previous section of pesticide residues in ready-to-eat vegetables. This finding indicates that most of the vegetable farmers consume what they produce. The farmers who bought vegetables reported that they usually do so while waiting for the pre-harvest interval to elapse after they have sprayed their own vegetables or prefer a different type of vegetable than what they have on their farm. It was revealed in this study that the odds of exposure to pesticide residues were 4.062 higher for farmers who consume own grown vegetable than for those who bought vegetables. However, the association was not statistically significant.

Results of pesticide exposure for vegetable farmers who reported to obtain the vegetable samples from their own farms (n=58) were used in the logistic regression analysis in order to clearly associate the practices and exposure levels. Results showing the association between exposure to pesticide residues and knowledge or application practices for pesticides are presented in Table 7.

3.5.2 Knowledge and Awareness of Pesticide Application

Knowledge and awareness of pesticide application are important for appropriate pesticide application and handling. Among the 58 vegetable farmers interviewed, only 20 (34.5%) had attended some form of training on pesticide application. Among those 38 out of 58 who had no training, 52.6% were exposed to pesticide residues. Linear regression analysis shows that there is a significant association (P=0.043) between lack of training on pesticide application and exposure to pesticide residues. The adjusted odds of exposure to pesticide residues are 3.73 times higher for the vegetable farmers who had no training than for those who had undertaken training on pesticide application.

It was reported that 81% (47) of the farmers had a low level of education (only up to primary level) and the others (19%) had a higher level of education (secondary to university). A similar level of literacy is reported in other developing countries. In Nigeria, 96.2% of farmers had a low level of education (only up to primary level). The odds of exposure to pesticide residues for the farmers with a low level of education were 1.745 higher than for those who had a higher level of education but the results were not statistically significant (p=0.634). These results suggest that continuous training and awareness raising among vegetable farmers on pesticide application regardless of their level of education can significantly reduce dietary exposure to the pesticide. The training should include provision of knowledge on health and environmental effects associated with indiscriminate use of pesticides and provide other options for pest and disease control so that farmers can willingly shift from relying on the indiscriminate use of synthetic pesticides to safer pest management methods such as the integrated pest management (IPM) approach. This approach combines various means of pest and disease control including the use of cultural and mechanical means, biological control such as introduction of beneficial insects and mites and minimum use of IPM compatible pesticides (Dijkxhoorn, Bremmer, & Kerklaan, 2013; Lahr, Buij, Katagira, & Valk, 2016). The approach is currently applied in Europe, and in parts of East Africa, particularly in Kenya for farmers who grow vegetables for export and who apply this practice in order to meet the stringent requirements that vegetables are not allowed to contain pesticide residues above MRLs (Maredia, Dakouo, & Mota-Sanchezs, 2003).

Table 9. Association between dietary exposure to pesticide residues with knowledge and pesticide application practices

+	Farmers exposed (%)	*p*-value	OR[1]	CI[2] (95%)	AOR[3]	*p*-value	CI (95%)
Primary or lower level of education (n=47)	23	0.634	1.745	0.176-17.261			
Source of vegetables (own grown n=58)	44.8	0.087	4.062	0.817-20.201			
Lack of a formal training on pesticide application (n=20)	34.5	**0.133**	**2.317**	**0.822-8.179**	**3.73***	**0.043**	**1.04-13.363**
Vegetables intercropped with cabbage (n=33)	51.5	0.961	1.889	0.652-5.476			
Lack of advice from extension officer (n=13)	15.4	**0.031**	**6.768**	**1.188-38.57**	**6.56****	**0.031**	**1.187-36.291**
Prepare pesticide at over-dosage (n=24)	58.3	**0.032**	**4.12**	**1.127-15.06**	**3.751**	**0.038**	**1.078-13.06**
Non-adherence to PHI[4] (n=31)	32.3	0.038	3.83	1.166-11.659	3.223	0.057	0.964-10.768

[1]odd ratio, [2]confidence interval; [3]adjusted odd ratio; [4]the time that lags between last pesticide spraying and harvest of the vegetables; *odds ratio of exposure to the residues for lack of training after be

ing adjusted from influence of low level of education and lack of advice from extension officer; **adjusted odds ratio of exposure to the residues for lack of advice from extension officers after being adjusted for confounding influence of lack of adherence to PHI and over-dosage; the bolded values are for practices with significant association.

3.5.3 Vegetable Cropping System

During the field survey, it was observed that vegetables were grown in small farms (mostly ≤0.5 acre) located close to the residential area of the respondents. Vegetables were either intercropped or in separate plots. Most of the farmers who grew cabbage and other types of vegetables claimed that they separated cabbage from other crops because the crop is more frequently sprayed with pesticides. Thus, they separated cabbage from the other vegetables in order to control pesticide cross-contamination. Among the 58 respondents who consumed vegetables from their own farm, 55.2% (32) reported growing cabbage, of which 75% planted cabbage in a separate farm. Among those who intercropped cabbage with other vegetables, 62.5% were exposed to pesticide residues. However, there was no significant association between intercropping cabbage with other vegetables and exposure to pesticide residues (p=0.961, odds ratio=3.769).

Results of pesticide residue analysis in the vegetable samples show that pesticide residues were more frequently detected in cabbage samples than other vegetables. Of the cabbage samples, 60% contained pesticide residues higher than 53.3% of kale and 35.5% of nightshade (Table 4). However, the occurrence of multiple pesticide residues was higher in nightshade 18.75% and kale 10.5% than in cabbage (10%), indicating that the farmers' claim is not valid. Literature reports that intercropping of cabbage with appropriate vegetables (referred to in the literature as companion crops) has potential for controlling pests in vegetables and thus minimising pesticide use. For instance, intercropping cabbage with alliums and tomato were found to significantly minimise pests in the field (Baidoo, 2012; Debra & Misheck, 2014; Luchen, 2001).

3.5.4 Lack of Advice from Agricultural Extension Officers

The role of agricultural extension officers is to provide farmers with knowledge, information, experience, and technology which are important for improved productivity. In the current study, most of the vegetable farmers (84.6%; n=58) reported that they did not seek agricultural officer advice on pesticide application issues. Of those who did not rely on the officers' advice, 53.3% were exposed to pesticide residues. The result from the regression analysis indicates that there is a significant association between exposure to pesticide residues and lack of advice from agricultural extension officers (p=0.031). The adjusted odds for exposure to pesticide residues are 6.56 higher in the farmers who did not rely on extension officers' advice than for those who did.

Literature reports that most of vegetable farmers do not rely on extension officers' advice in pesticide application issues (Issa & Atala, 2012; Lekei et al., 2014; Ngowi et al., 2007). Each ward and two villages (Shiboro and Siwandeti) visited in the current study had at least one agricultural extension officer. Therefore, if agriculture extension services were well-equipped by the government and utilized by farmers, the risk of exposure to pesticide residues would be minimized.

3.5.5 Over-dosage of Pesticides

Appropriate pesticide preparation is crucial for controlling pesticide residues in vegetables. By comparing application rates for pesticides as indicated on labels of pesticides to the rates applied to vegetables, it was

realized that 41.1% of the interviewed farmers prepared pesticides at over-dosage. Among those who prepared pesticides at over-dosage, 58.3% were exposed to pesticide residues. The adjusted odds ratio of exposure to the residues was found to be 3.751 higher in the farmers who prepared pesticides at over-dosage than for those who prepared pesticides accurately. The association was statistically significant at p=0.038. Pesticide dosage has been a great challenge in developing countries because most farmers dose inaccurately which results in excessive pesticide residues in vegetables (Adjrah et al., 2013; Banjo et al., 2010; Sheikh, Nizamani, Panhwar, & Mirani, 2013).

During the field survey farmers reported measuring liquid pesticides with calibrated caps, most of them delivered together with the pesticide package. However, in the case of powdery pesticides such as Linkmil 72WP, Ebony 72WP, and Ivory 72WP, tablespoons were used as measurement tools. This is a bad practice because powdery pesticides should be weighed in grams. Unpublished information from the Horticultural Research and Training Institute Tengeru, Arusha informed that the lack of appropriate measures such as weighing scales for powdery pesticides is a common challenge. Extension officers further have attempted to calibrate commonly used equivalent tools at the farm level, such as spoons, but it is still a challenge because new pesticides which are lighter or heavier than those previously used for calibration, are entering the market. A similar challenge is reported in Ethiopia where farmers use non-calibrated measuring tools (Mengistie, Mol and Oosterveer, 2015). Pesticide formulators and extension officers should find means for farmers to be able to measure an accurate quantity of pesticide. This will minimize the risk of exposure to pesticide residues associated with the inappropriate measurement of pesticide quantities.

3.5.6 Adherence to Pre-harvest Interval (PHI)

The time that lags between the last pesticide spraying and harvest of the vegetables is important to ensure reduction levels of the applied pesticides to at least the recommended maximum residual levels. Field surveys revealed that all vegetable farmers interviewed were aware of PHI. Among the 58 respondents, 31 (53.4%) reported waiting for the recommended PHI whereas 27 (46.6%) harvest earlier than the recommended intervals. Adherence to PHI was in concurrence with the effectiveness of pesticides. More than three quarters (87.1%) of the farmers who reported that the pesticides applied were effective, could adhere to PHI. Among the farmers who reported to harvest vegetables before the recommended interval (n=21), 59.3% were exposed to pesticide residues through vegetable consumption. The odds ratio of exposure to the residues was 3.83 times higher for the farmers who did not adhere to PHI and the result was statistically significant at p=0.026. However, after adjusting for confounding influences such as the lack of advice from extension officers, the results showed no significant association (p=0.057) suggesting that the lack of advice from extension officers was the cause for non-adherence to PHI. It is therefore suggested that farmers should be advised on the importance of adherence to PHI so that safe vegetables are produced for their own consumption and for other consumers.

4. Conclusion and Recommendations

The findings of the present study indicate that 18.6% of vegetable farmers in Arusha district are at potential risk of exposure to organophosphate pesticide residues through vegetable consumption. The risk is due to high levels (above MRLs) of organophosphate pesticide residues that were detected in almost one-third of vegetable samples. Dimethoate was the main contributor to the exposure to high levels of organophosphates with a hazard index above one. Other organophosphate pesticides detected were dichlorvos, acephate, profenofos, and malathion whose HQs were below one. Pyrethroids including permethrin, cypermethrin, and lambda-cyhalothrin were also detected having HQ and combined HI below one, indicating a minimum potential health risk. Our findings showed that lack of formal training on pesticide application, non-reliance on agricultural extension officers' advice and over-dosage of pesticides are the main factors for the observed potential risk of exposure to pesticide residues. Since vegetable farms were closer to the residential houses, there are possibilities that individuals, especially pregnant women and children, are at higher risk of exposure through other routes such as inhalation and skin contact. For that reason, we recommend that an exposure assessment for the general population be carried out using a more robust approach that includes other potential routes including consumption, inhalation and skin contact. The risk may be minimized by observing extension service advice, specifically by observing pre-harvest intervals for these pesticides and applying pesticides at an appropriate dosage.

Conflict of interest

The authors declare no conflict of interest

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Improvement in the Extraction of Hass Avocado Virgin Oil by Ultrasound Application

Nadia Segura[1], Miguel Alejandro Amarillo[1], Natalia Ines Martinez[1] & María Antonia Grompone[1]

[1]Área Grasas y Aceites, Depto CYTAL, Facultad de Química, Univerisdad de la República, Montevideo, Uruguay

Correspondence: Miguel Alejandro Amarillo. Área Grasas y Aceites, Depto CYTAL, Facultad de Química, Univerisdad de la República, Montevideo, Uruguay. E-mail: amarillom@fq.edu.uy

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Abstract

The virgin oil extraction from avocado Hass was carried using an Abencor pilot scale plant.

High-power ultrasound (1.73 MHz) was applied after mixing. High-frequency ultrasound device consists of two transductors which each deliver 30 W/L.

Different proportions of water were added before ultrasound treatment (no water added and paste:water relation (1:3, 1:2, 1:1, 2:1, 3:1) and also different ultrasound application times (0, 1, 2, 3, 4, 5, 10, 15, 20 and 25 minutes). The study was carried out by setting one of the two variables considered and changing the values of the other. In the water addition study, ultrasound time was set at 15 min.

It was found that oil recovery increased with the percentage of water added. It was decided to employ a ratio of 1:1 to study the influence of ultrasound application time. Under these conditions, it was found that with 1 minute of ultrasound application, recovery increased by 40 % over the process without ultrasound.

It was observed that the composition in fatty acids and the content of natural antioxidants (tocopherols and polyphenols) are not affected by the ultrasound application.

It is concluded that the application of high-frequency ultrasound with addition of water post malaxing improves recovery of virgin avocado oil without negative effects on the general quality of the oil.

Keywords: extraction, avocado oil, extra virgin, ultrasound.

1. Introduction

1.1 Introduce the Problem

Several authors studied the application of ultrasound in food (Jiménez *et al*., 2006) and its effects on oils (Chemat *et al*., 2004; Chemat *et al*., 2004; Cañizares-Macías *et al*., 2004; Sharma & Gupta, 2006; Benedito *et al*., 2007; Zhang *et al*., 2008; Jerman *et al*., 2010; Izbaima *et al*., 2010; Riera *et al*., 2010; Li *et al*., 2004; Liu *et al*., 2011; Jerman & Mozetic, 2012; Da Porto *et al*., 2013; Goula, 2013; Tian *et al*., 2013; Rodríguez *et al*., 2013; Reboredo-Rodríguez, 2014; Samarama *et al*., 2014).

Ultrasound has been applied in the production of palm oil. Mechanical vibration, acoustic flow and cavitation result in large localized forces that produce physical changes such as surface tension reduction and clustering particle density. Water sonication includes forming hydroxyl radicals. The mechanical effects dominate at low frequencies (20-80 kHz) and at frequencies higher than 1500 kHz; chemical effects take place from 100 to 1000 kHz. Juliano et al. (2013), applied frequency between 20-2000 kHz. The Malaysian company Tai Tak Snd Bhd incorporated this process industrially achieving an annual increase of 1% in the production of crude palm oil with an additional gain of approximately US $ 1,000,000.

Avocado (Persea americana Mill.) is a mexican origin specie that belongs to the family of Lauraceae. This family includes about 150 species, most of which grow in tropical America. Avocado pulp contains more than 20% oil. For example, a fruit Hass contains about 200 grams of pulp and, therefore, about 40 grams of oil.

The oil extracted from the pulp of the avocado has many applications. Yanty *et al*. (2012) studied the composition of avocado oils from different varieties. The food industry uses it to prepare canned foods and salad

dressings. The cosmetics industry uses it in the formulation of lotions, creams and soaps for skin care and hair care. The pharmaceutical industry uses it as a base for ointments, salves and balms. Currently they are studied other ways to use it in medicines and nutraceuticals. It´s international price is very expensive so it is justified to study changes in the extraction procedure in order to increase their performance.

In this paper the application of high frequency ultrasound (1,73 MHz) was optimized in order to increase the extraction yield of virgin avocado oil. The qualities of the oils obtained in the Abencor pilot scale plant with and without the high-frequency ultrasound application were compared

2. Method

2.1 Raw Material

For this study, Chilean origin avocado from "Hass" variety, acquired in the local market in a state of maturity appropriate for their intake were used. This optimum maturity considered is observed when the skin is dark and the fruit is firm but not hard to the touch.

As maturity varies with post-harvest time, avocados cannot be stored for many days. It was intended that all lots had the same maturity degree. Despite these precautions, the lots presented differences in composition (amount of oil and moisture from the pulp). As the oil pulp content from different batches is not constant, it was necessary to define a coefficient of Performance (CP), which considers the relation between the mass of oil obtained for each test on the mass of oil obtained from control, bearing in mind the same average sample of ground pulp (equation 1):

$$C.P = \frac{mass\ of\ oil\ obtained}{mass\ of\ oil\ control} \quad (1)$$

2.2 Oil Extraction of Avocado Pulp in an Abencor Pilot Scale Plant

The extraction of virgin avocado oil was performed in an Abencor pilot scale plant for extracting virgin olive oil. First, the avocados were manually peeled and their seed separated in order to obtain only the pulp. The oil extraction begins with grinding the pulp to obtain a paste by employing a hammer mill, followed by a malaxation step at a defined temperature and finally a vertical centrifuge for separating the liquid phase (oil and water) from the solid phase (rest of the pulp). Instead of the Abencor hammer mill, an electric food processor for grinding the avocado pulp was employed. The next step was the paste malaxation in a Thermo-mixer TB-100 at 40 °C for 6 hours, with addition of water in a ratio 5:1 paste: water at 4,5 hours of mixing, to favor the separation of oil from paste. Finally the paste was centrifuged in a Centrifugal machine CF-100 at 3500 rpm for 1 minute.

2.3 Application of Ultrasound in Oil Extraction of Avocado Pulp in an Abencor Pilot Scale Plant

Ultrasound was applied to the pulp at a frecuency of 1,73 MHz between the malaxing and centrifugation steps.

The ultrasound device consists of two transducers which deliver a power of 30 W/L each. The tests were conducted using a single transducer. The container vessel is a square prism (7.5 x 7.5 x 18 cm) with a 1000 mL capacity. A volume of 500 mL paste was added, so that only one transducer was employed. After the application of ultrasound, pulp was centrifuged in a centrifuge SORVALL SS-4 at 3500 rpm for 15 minutes. The Abencor system centrifuge could not be used because it was not effective due to the high fluidity of the paste, as a consequence of the amount of water added.

2.3.1 Influence of Adding Different Proportions of Water on Yield of Extracted Oil (For the Same Ultrasound Application Time)

To study the possible increase in oil extraction, different proportions of water were added to the paste prior to application of ultrasound. The obtained results were compared to those obtained from the extraction process in the Abencor plant described in section 2.2 (without addition of water and without application of ultrasound, called "control"). The paste:water relations used were 1:3, 1:2, 1:1, 2:1, 3:1 and non water addition (maintaining constant application time ultrasound in 15 minutes).

2.3.2 Influence of Ultrasound Application Time on Yield of Extracted Oil (For the Same Amount of Water Added).

The relation paste:water (1:1) was selected as the most appropriate for the different ultrasound application times. The application times evaluated were: 5, 10, 15, 20 and 25 minutes. The results were compared with the control sample obtained as described in 2.2. In a second step, shorter times of ultrasound application were studied (1, 2, 3, 4 and 5 minutes) as a possibility for improving the industrial application.

2.4 Analytical Techniques for the Characterization of Oils

2.4.1 Oil Content of Avocado Pulp by Soxhlet Method

The oil content was determined on the dry pulp by the Soxhlet method using petroleum ether (62-68 ºC) for analysis during 8 hours. Prior to this solvent extraction the sample was dried in an oven drying with forced convection at 55.0 ± 0.5 ºC for 12 hours.

2.4.2 Biophenols Content

According to International referee method COI/T.20/Doc. nº 29, "Determination of biophenols from olive oils by HPLC".

2.4.3 Tocopherol Content By HPLC

According to Andrikopoulos *et al*. (1991) method.

2.4.4 Acidity Value

According to standard method IUPAC 2.201.

2.4.5 Peroxide Value

According to standard method IUPAC 2.501.

2.4.6 Absorbency in Ultra-violet (K232 and K270)

According to International referee method COI/T.20/Doc. nº 19/Rev. 3, "Spectrophotometric investigation in the ultraviolet".

2.4.7 Fatty Acid Composition

The preparation of the methyl esters was according to official method AOCS Ch 1-91 and its determination by gas chromatography according to official method AOCS Ce 1e-91.

2.4.8 Chlorophyll Pigments Content in Crude Vegetable Oils

According to official method AOCS Cc 13i-96. The content of chlorophyll pigments in vegetable oils is expressed as mg of pheophytin a in 1 Kg of oil.

2.4.9 Statistical Analysis

The data were treated statistically by analysis of variance (ANNOVA) and using the Tukey test of INFOSTAT with a significancy level of 0.05.

3. Results and Discussion

3.1 Influence of Adding Different Proportions of Water on Yield of Extracted Oil (For the Same Ultrasound Application Time)

The results of CP based on paste:water relation are shown in Figure 1. The CP was not increased by applying ultrasound without addition of water nor for the relation 3:1. This could be due to the fact that this type of paste does not allow the propagation of waves within it making ineffective the application of ultrasound. By increasing the amount of water the CP increased dramatically for relations paste: water 2:1, 1:1, 1:2 and 1:3, among which there was no significant difference.

While higher CP was obtained with relation paste:water 1:3, at industrial scale it is better to work with a paste:water ratio 1:1 because of the smaller amount of water required. This involves a reduced capacity of equipment and less effluent volumes to be treated. The CP value of relation paste: water 2:1 showed no significative difference with the corresponding value ratio of 1:1.

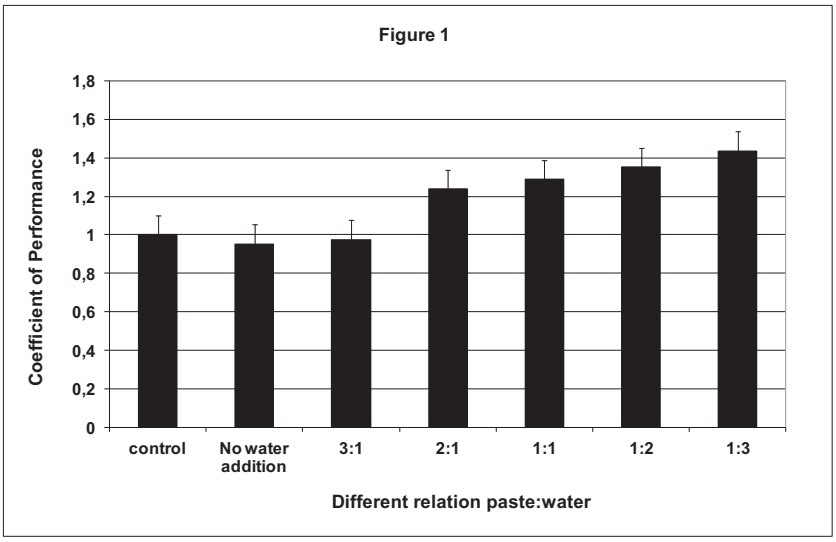

Figure 1. Coefficient of performance as function of different relation paste:water, expressed with it's margin of error

3.2 Influence of Ultrasound Application Time on Yield of Extracted Oil (For a Paste: Water 1:1 Relation)

The CP results according to ultrasound application times 5, 10, 15, 20 and 25 minutes are shown in Figure 2. With 5 minutes of application the CP increased 33 % over the process without application of ultrasound. There was not a significant increase in the amount of extracted oil by prolonging the ultrasound application time (taking into account the margin of error shown in Figure 2).

According to these results smaller application times (1, 2, 3, 4 and 5 min) were evaluated. The results are shown in Figure 3. The CP increased for all application times compared to the control sample. There was no difference among these application times (to take into account the margins of error shown in Figure 3).

It was observed that only one minute of ultrasound application was enough for increasing oil extractability in 40 % (CP = 1.4) under the studied conditions (Figure 1). Martinez-Padilla *et al.* (2018), studied ultrasound application (2 MHz) in avocado puree prior to malaxing step without water addition, they obtained an increase of 15-24 % in oil recovery. Further studies must be carried on in order to get a better understanding of ultrasound and it´s application in food industry, but both researches show how usefull high frequency ultrasound proves to be in oil extraction proccesses such as avocado oil.

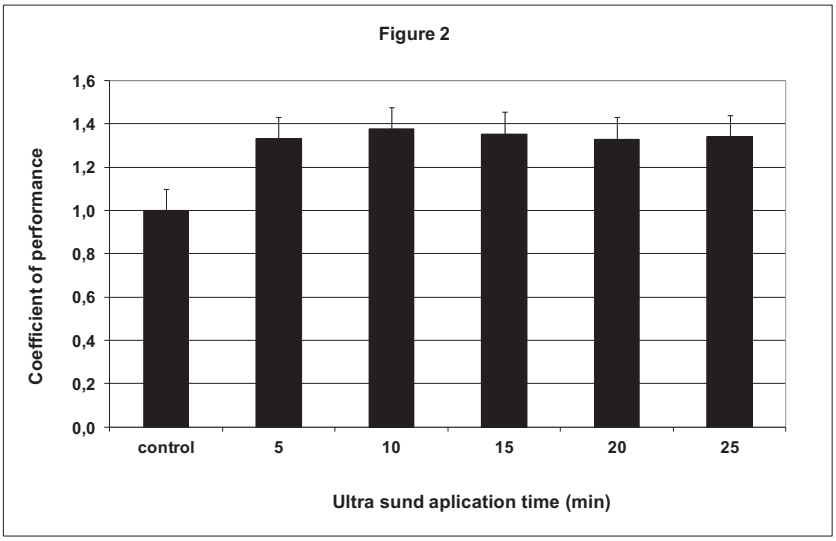

Figure 2. Coefficient of performance as function of different ultrasound application time with relation paste:water 1:1, expressed with it's margin of error

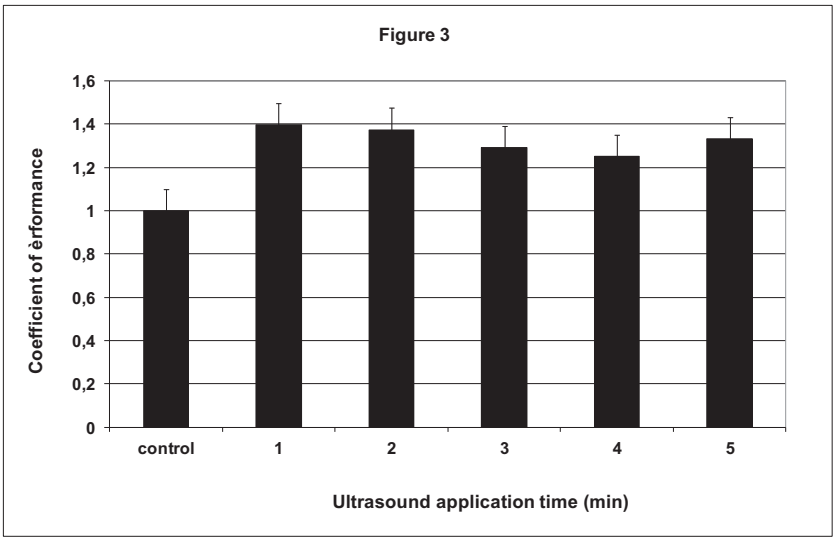

Figure 3. Coefficient of performance as function of different ultrasound application time with relation paste:water 1:1, expressed with it's margin of error

3.3 Influence of the Ultrasound Application on the Purity and Quality of Extracted Avocado Oil

The composition in fatty acids was studied as criterion of nutritional quality and oxidative stability of the oils. The fatty acid profile of the oils extracted by, Soxhlet method and Abencor pilot plant, with and without ultrasound application was evaluated from a single batch of avocado, the results are observed in Table 1.

Table 1. Composition in fatty acids of the oil of avocado extracted by Soxhlet method and in pilot plant with and without the application of ultrasound (US)

		Abencor	
	Soxhlet	with US	without US
16:0	20.7	21.2	21.2
16:1	11.5	11.8	11.9
18:0	0.5	0.4	0.4
18:1	51.3	52.2	51.9
18:2	12.8	13.0	13.1
18:3	0.6	0.6	0.6

It is observed that the extraction method does not affect the fatty acid profile of the oil. It was found that the main fatty acid was oleic acid (52 %) followed by palmitic (21 %). Therefore it can be said that this oil has good oxidative stability considering the fatty acids composition.

Table 2 shows the contents of natural antioxidants (tocopherols and phenols), acidity, peroxide value, absorbancy in ultra-violet and pheophytin a content of extracted oils.

Table 2. Content of tocopherols and phenols, acidity value , peroxide value, absorbency in ultra-violet and pheophytin a content of avocado oil extracted in Abencor plant with and without ultrasound application.

	with US	without US
α -Tocopherols (ppm)	412 ± 2^{a}	357 ± 44^{a}
β + γ -Tocopherols (ppm)	59 ± 1^{a}	58 ± 0^{a}
δ -Tocopherols (ppm)	8 ± 0^{a}	9 ± 0^{a}
Phenols (ppm)	54 ± 5^{a}	59 ± 3^{a}
Acidity (%)	0.33 ± 0.02^{a}	0.52 ± 0.01^{b}
PV (meq O_2/Kg)	8.06 ± 0.27^{a}	9.64 ± 0.31^{b}
K232	2.68 ± 0.02^{a}	3.13 ± 0.16^{a}
K268	0.20 ± 0.01^{a}	0.19 ± 0.02^{a}
Pheophytin a (mg/kg oil)	35.5 ± 0.1^{a}	$33,2 \pm 0,2^{b}$

Note. Different letters in the same column indicate significant differences according to Tukey´s test ($p < 0.05$).

It was observed that the main antioxidants content in virgin avocado oil are tocopherols, in particular α-tocopherol. Although the obtained oils contain a certain amount of the phenolic type, they are the minority. Clearly there are no significant differences between the amount of all antioxidants for oils extracted with and without application of ultrasound. Therefore, ultrasound application after malaxing of avocado paste did not affect the total content of antioxidants.

It is observed that the acidity value is lower in the oil extracted with ultrasound application. The acidity values are lower comparing to those values for the extra virgin olive oil quality. This is why it could be classified as extra virgin according to Trade Standard applying to olive oils and olive pomace oils of International Olive Council.

A decrease in the peroxide value was also found, suggesting that the application of ultrasound does not compromise the oil quality. This can also be observed in the K232 coefficient. The decrease in the K232 coefficient could be due to the decomposition of the peroxides during the process of application of ultrasound, although the products formed would not absorb at 268 nm, since no changes in the value of K268 were found.

As for chlorophyll pigments, a slight increase in the content of pheophytin a was observed with the ultrasound application. This could be reflected in the partial loss of the characteristic green color of chlorophyll, with the appearance of a yellowish hue in the oil. This could slightly affect the visual characteristics of the product.

4. Conclusions

The application of high frequency ultrasound (1.73 MHz) proved to be an effective tool to increase the extraction yield of virgin avocado oil (Hass variety) when water is added to the paste in a 1:1 relation after the thermo-mixing process at 40 °C. It was found that 1 minute of application of ultrasound was enough to increase the extraction of oil by 40 %.

Also, this technology generates partial changes in regards to the quality of avocado oil. It causes a decrease in the acid and peroxide values, while the content of pheophytin a increases due to the decomposition of chlorophyll pigments.

Respect to the antioxidants content, this technology does not affect it, so it could be expected not to modify the oil oxidative stability. It also does not change the fatty acid composition. Consequently, the application of high frequency ultrasound benefits the extraction of avocado oil.

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Effects of *Ilex Paraguariensis Polyphenols* on Magnesium Absortion and Iron Bioavailability: Preliminary Study

Lucila Sánchez Boado[1], Raquel María Fretes[1] & Luis Alberto Brumovsky[1]

[1]National University of Misiones, Faculty of Exact Sciences, Chemistry and Natural Sciences, Argentina

Correspondence: Lucila Sánchez Boado, National University of Misiones, Faculty of Exact Sciences, Chemistry and Natural Sciences, Argentina. E-mail: lsboado@fceqyn.unam.edu.ar

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Abstract

Yerba maté (Ilex paraguariensis) is a native plant of South America widely consumed as hot infusion. It is a vegetable that has many bioactive compounds, such as caffeine, theobromine theophylline and saponins, phenolic compounds, mainly chlorogenic acids and dicaffeoylquinic acids, and minerals as Fe, Ca, K y Mg, which have many beneficial properties for the health. Polyphenols have the property of chelating the transition metals, as iron and magnesium, decreasing their bioavailability. Iron deficiency anaemia and subclinical hypomagnesemia is observed in the American population. Women at childbearing age belong to a specially vulnerable group. The aim of this work was to evaluate the "in vivo" effect of the polyphenols from yerba maté infusions, consumed in their traditional form, on the bioavailability of Fe and Mg in the diet. Eleven healhty young female volunteers participated in the magnesium and iron study. After the first blood sample was taken (time 0), the volunteers ingested 300 mL of yerba maté infusions, ferrous sulfate or yerba maté infusions plus ferrous sulfate at50 °C in no more than 5 minutes. They remained seated at a comfortable temperature throughout the study. Blood samples were taken 20, 40, 50, 60, 80, 100 and 120 minutes after ingestion.The yerba maté infusions prepared in their traditional form of “hot maté”, which containining an average of 4.68 ± 0.54 GAE/L of polyphenols, have an inhibitory effect on the bioavailability of the magnesium and mainly on non-heminic Fe consumed as ferrous sulphate of the 76 ± 10 % .

Keywords: bioavailability, Ilex paraguariensis, in vivo, iron, magnesium, polyphenols

1. Introduction

Yerba mate (YM), *Ilex paraguariensis* is a native plant of the region of South America widely consumed as infusion. It´s a vegetable that has many bioactive compounds, such as caffeine, theobromine and theophylline, phenolic compounds, mainly chlorogenic acids and dicaffeoylquinic acids, saponins and minerals as Fe, Ca, K y Mg mainly, which have many beneficial properties for the health. Consumption of YM infusions would signifcantly contribute to the overall antioxidant intake, providing high amounts of cafeoylquinic acid derivatives (Bravo, Goya & Lecumberri, 2007) (Bastos et al., 2014) (Heck, Schmalko & De Mejia, 2008). It was determined that YM infusions have cytotoxic activity and by inhibition of topoisomerase II may contribute to the general chemopreventive activity (Ramirez-Mares, Chandra &Mejia, 2004) (Bracesco, Sanchez, Contreras, Menini & Gugliucci, 2010). Some authors determined that YM infusion consumption is associated with higher bone mineral density in postmenopausal women (Conforti, Gallo &Saraví, 2012). Other authors report that the presence of polyphenols in the YM infusion has a favorable effect on bone tissue and can mitigate the negative effect of caffeine (Brun et al., 2015). It has also been reported that there has been an inverse association between YM infusion consumption and Parkinson's disease (Gatto, Melcon, Parisi, Bartoloni & Gonzalez, 2015). Antiherpes activity was also detected in the crude extract obtained from Ilex paraguariensis leave (Lückemeyer et al., 2012).

In Argentina, Paraguay, Brazil and Uruguay Yerba mate consumption is very high, either in the traditional form of hot mate, tea mate in tea bags or cold mate (tereré). Hot mate consists of putting between 30 and 50 g of yerba mate in a gourd, and a device similar to a straw (bombilla) with a filter on its end is inserted into the yerba mate. Then fractions of approximately 30 mL of hot water (70 ºC to 85 ºC) are poured repeatedly on the yerba mate, to extract the infusion through the “bombilla”. Yerba mate infusions contain a high amount of polyphenols, mainly caffeoylquinic acids.

Due to the increasing consumption of refined foods and the decrease in vegetable and fruit intake, subclinical hypomagnesemia is observed in the American population. Part of the problem is that soils are becoming deficient in minerals. In the last 60 years the Mg in the content of fruits and vegetables decreased in 20% and 30% (Worthington 2001). In additionto this, the Western diet contains more refined grains and processed foods. Estimates are that 80% to 90% of magnesium is lost during food processing. Thus, it is estimated that 2.5% to 15% of the world population suffers some degree of hypomagnesemia (Belluci et al., 2013). Prevalence of hypomagnesemia varies widely as reported in previous studies, being 14.5% in an unselected German population, 11.8% in Indian subjects and sub-optimal Mg levels were found in 14.6% of the total Iranian urban population (Syedmoradi, Ghasemi, Zahediasl & Azizi, 2011). The Recommended Dietary Allowances (RDA) of magnesium for women aged 29 to 30 years old is 310 mg of Mg and women aged 31 to 50 years old is 320 mg of Mg to according to the Institute of Medicine. Food and Nutrition Board (1997)

Mg is involved in more than 300 enzymatic reactions. The main part of body magnesium is located in the bones. Part of this magnesium—absorbed by the bone surface—is in equilibrium with the extracellular magnesium. At reduced plasma concentrations magnesium can be rapidly released from the bone surface and at increased plasma concentrations magnesium is bound to the surface (Vormann, 2003).

Magnesium that is ingested with food is exclusively absorbed as Mg^{+2} ions within the human small intestine, mainly in jejunum and ileum. Approximately one third of the magnesium consumed is absorbed in the process of renal resorption, since the rest is excreted with the faeces. Reabsorbed magnesium is mainly excreted through the urine and in small amounts through the gallbladder and sweat (Francisco & Rodríguez, 2013) (de Baaij, Hoenderop & Bindels, 2015).

Low serum Mg^{+2} values are associated with a wide range of neurological diseases such as migraine, depression, Parkinson's disease, brain damage, stroke and epilepsy. Magnesium is essential for maintaining the physiological functions of brain, heart and skeletal muscle (de Baaij, Hoenderop & Bindels, 2015). Magnesium deficiency is observed in chronic malnutrition, diarrhea, diabetes, alcoholism, acidosis, renal disease and in patients who consume diuretics (Barbagallo & Dominguez, 2007).

Among the most common nutritional deficiencies is iron deficiency anaemia according to the World Health Organization (2015). One of the causes of this anaemia is insufficient intake of iron in the diet to replace menstrual losses. Especially vulnerable groups are those of children and women at childbearing age. According to studies (Kogan, Abeyá Gilardón & Biglieri, 2008) in northwest Argentina, 22.6% of women between 18 and 49 years old suffer from iron deficiency anaemia, the highest value in the country.

The yerba mate infusions prepared in their traditional form of hot mate have 1.4 ± 0.2 mg of Fe / L, this amount in the case of women at childbearing age can reach 7.1% of the RDA, for a high bioavailability diet (15%). Absorption of Fe vary according to the amount of potentiators and inhibitors present in the diet, thus we have 5%, 10%, 12% or 15% Fe bioavailability diets. For bioavailability diets of 15% the RDA for women at childbearing age is 29.4 mg (Institute of Medicine. Food and Nutrition Board, 1997).

It is known that polyphenols have an antioxidant capacity, which is beneficial to prevent oxidative stress, but one of the mechanisms by which they exhibit antioxidant capacity (AOC) is through the property of chelating the transition metals, decreasing their bioavailability. The magnitude of this interaction is different for each polyphenol.

It is extensively studied and quantified in the case of tea, coffee and wine. The fact that iron absorption can be reduced by tea consumption has been recognized for many years, with the inhibitory effects by the marked iron-binding properties of the phenolic compounds bearing catechol groups in tea. It was demostrated that the simultaneous consumption of black tea and iron-containing foods inhibits iron absorption by about 60 to 70%, independently of the strength of the tea. Between-meal tea consumption inhibits non-heme iron absorption by about 20% (Zijp, Korver & Tijburg, 2000). It was already proven in 1983 that a cup of coffee reduced iron absorption from a hamburger meal by 39% as compared to a 64% decrease with tea (Morck, Lynch & Cook, 1983).

Although traditional views hold that polyphenols affect nonheme iron absorption only, recent experiments on human intestinal monolayer cells have provided evidence that dietary polyphenolic compounds could interfere with absorption of both heme and nonheme iron across these cells and demonstrated a dose-dependent inhibitory effect of polyphenols on heme iron absorption (Kim, Ham, Shigenaga, & Han, 2008).

Up to now, no *in vivo* studies have been conducted on the interaction of yerba mate polyphenols on iron absorption in the diet. The objective of this work was to evaluate the "in vivo" effect of the polyphenols from

yerba mate infusions, consumed in their traditional form, on the bioavailability of iron and magnesium in the diet.

2. Materials and Methods

2.1 Reagents

The following reagents were used: Iron (III) Heptahydrate sulfate (Sigma-Aldrich), commercial Kit Architect Ferritin® (Abbot), commercial Kit Architect Iron® (Abbot), commercial Kit Architect Magnesium® (Abbot), commercial Kit Architect Transferrin® (Abbot), Folin-Ciocalteu Reagent (Fluka). Folin-Ciocalteu (Fluka), anhydrous sodium carbonate (Sigma-Aldrich), monohydrate gallic acid (MP Biomedicals, limited liability company (LLC), anticoagulant W (EDTA 0.342 mol/L, pH 7.2) Wiener lab®.

2.2 Female Volunteers

The Study Protocol was approved by the Ethics Committee of the Dr. Ramón Madariaga Hospital in Posadas city, Povince of Misiones, Argentina. The tests were carried out with 12 apparently healthy female volunteers after giving their informed consent. Before testing, volunteers were evaluated clinically and with hematology tests. All female volunteers were normal weight, non-smokers, between 20 and 40 years old, they followed a diet which consisted in restricting food high in polyphenols 24 hours before the test and they did not consume vitamin supplements or medicines in the week before the test itself. They went to the laboratory to have the tests done having fasted for 10 to 12 hours on three occasions. On the first occasion they ingested 300 mL yerba mate infusion; on the second occasion 40 mg of iron (as ferrous sulphate) were dissolved in 300 mL of water and on the third occasion 40 mg of iron dissolved in 300 mL of weed infusion were ingested. On each opportunity the nutritional status of iron was evaluated. All tests were performed in the luteal phase of the menstrual cycle to avoid confounding factors with variation in Fe absorption during the menstrual cycle (Chandra, Gupta &Patel, 2017). In each test, the iron nutritional status of female volunteers was evaluated in order to guarantee that there was no variation in the avidity of Fe absorption. The tests were erythrocyte sedimentation, hemoglobin, hematocrit, serum iron, transferrin, percentage of tranferrin saturation and serum ferritin, as can be seen in Table 1. The tests were performed in no more than two months.

After the first blood sample (time 0), the volunteers ingested 300 mL of yerba mate infusions, ferrous sulfate or yerba mate infusions plus ferrous sulfate at 50 °C in no more than 5 minutes. They remained seated at a comfortable temperature throughout the study. Blood samples were taken 20, 40, 50, 60, 80, 100 and 120 minutes after ingestion. They were made by antecubital venipuncture using a catheter. All blood test samples were taken by a trained nurse and under medical supervision. In order to obtain plasma, blood was collected on anticoagulant EDTA (0.342 M, pH 7.2) in the relationship 10 µL anticoagulant per blood mL. Plasma separation was performed in a refrigerated centrifuge of 2.500 g for 10 minutes at 4 °C. All samples were refrigerated and protected from light until the processing time.

Table 1. Hematological parameters of the female volunteers during the 3 tests

	Female volunteer	1	2	3	4	5	6	7	8	9	10
Test 1: only YM infusión	GSR 1	3	5	7	6	6	5	3	9	3	4
	Hematocrit 2	37,7	35,5	38,7	38,6	36,5	34	41,1	33,9	38,1	40,4
	Hemoglobin 3	12,8	11,9	12,7	12,3	12	12	13,7	11,3	12,5	13,5
	Serum iron 4	74	54	146	65	80	64	100	90	106	49
	Transferrin saturation percentage 5	22,2	20	50,8	22	26,5	19	36,1	29,9	35,7	20,5
	Transferrin 6	262	212	226	264	238	256	177	237	234	188
	Ferritin 7	37,7	33	28	42,5	35,3	9,6	102	85	15	40,5
Test 2: only Fe	GSR 1	9	3		7	3	8	6	9	8	10
	Hematocrit 2	38,6	35,1		38,8		35	38,1	32,7	37,4	38,8
	Hemoglobin 3	13,2	11,8		12,5		12	12,1	11,5	11,8	12,7
	Serum iron 4	92	92	138	76	63	59	61	76	70	115
	Transferrin saturation percentage 5	26,7	33,7	49,8	20,1	20,2	18	52,9	27,9	21,2	43,7
	Transferrin 6	271	215	218	298	246	259	218	214	260	207
	Ferritin 7	22,1	12,1	21,5	42,1	24,4	7,6	100	44,8	20,6	60
Test 3: YM Infusión YM + Fe	GSR 1	3	8	3	8	10	9	7	6	3	10
	Hematocrit2	37,4	35,3	36,5	43,9	41	35	38,5	33	38,3	40,2
	Hemoglobin 3	12,4	11,9	11,9	13,9	13,4	12	12,6	11	12,2	12,8
	Serum iron 4	117	93	118	126	88	39	90	72	85	142
	Transferrin saturation percentage 5	34,7	32,4	46,1	30,1	29	11	37,5	26,1	27	53,2
	Transferrin 6	265	226	198	314	239	275	189	217	248	210
	Ferritin 7	24,1	26,6	39,2	43,3	28,8	8	78,1	47,6	15,3	83,4

Units and reference values: [1] Globular sedimentation rate (GSR): 0 – 15 mm/ 1 ^{er} h; [2] Hematocrito: 37 -47 %; [3] Hemoglobin: 11,5 16, 5 g/dL;

[4] Serum iron: 50 -170 µg/dL; [5] Ferritin: 21,8- 276,4 ng/mL; [6] Transferrin: 180 – 382 mg/dL; [7] Transferrin saturation percentage: 14 – 31 %.

2.3 Preparation of the Infusion

The infusion was prepared by a method which simulates a traditional "*mateada"* (Scipioni, 2010) (Hartwig, Brumovsky & Fretes, 2012). Briefly, 50 ± 0.10 g of yerba mate were placed in a glass beaker, and a stainless steel straw with holes smaller than 0.8 mm, was connected by a silicon hose to a filter flask with a fallopian vacuum. Then approximately 30 mL of distilled water at 70 °C were poured, 20 seconds after vacuum was applied for another 20 seconds. Then the vacuum was stopped and a new landfill was made, imitating the traditional pouring. This process was repeated until a volume of 500 mL was reached. The infusion was ingested immediately after its production.

2.4 Determination of Total Polyphenols Concentration in the Infusion

The total polyphenols concentration was determined spectrophotometrically according to ISO 14502-1 (2004) and expressed as g of gallic acid equivalents/mL acid infusion (g GAE/mL). Briefly, 1.0 mL of YM infusion was diluted (1:5) and then diluted (1:100) with distilled water. After that, 1.0 mL of the latter solution was taken and placed in a test tube. Then, 5.0 mL of a solution of Folin-Ciocalteu reagent and 4.0 mL of sodium carbonate solution concentration of 7.5 per cent w/v were added. The tubes were stirred, capped and allowed to stand for 60 minutes at room temperature in the dark before performing the absorbance readings at 765 nm. As reagent blank, 1.0 mL of diluted infusion was replaced by distilled water.

2.5 Determination of Serum Magnesium

A colorimetric method was used to measure serum magnesium. Magnesium forms colored complexes with the arsenazo and its absorbance was measured at 572 nm, being proportional to the concentration of Mg. We worked with an ARCHITECT c-Systems 8000 analyzer and with the commercial kit for the determination of the brand Magnesium ABBOTT. The detection limit of the method is 0.152 mgMg / dL and the limit of quantification is 0.66 mg Mg/dL.

2.6 Determination of Magnesium of Yerba Mate Infusion

Determination of the magnesium of yerba mate infusion has been measured with 200.2 EPA 600 / R-94. Briefly, an amount of 100 mL of the infusion is taken and placed in a tared crucible, then in a convection oven at 105 ° C to dryness (about 16 hours), then it is removed from the furnace, cooled in a desiccator, weighed and placed in a muffle at 550 ° C for 6 h to convert the sample to ashes. A dilution is then carried out where the final volume has a concentration of 0.1% lanthanum (La2O 3) to improve the readings a PerKin Elmer AAnalyst 700 in flame mode (Air-Acetylene), with hollow cathode lamp Ca-Mg was used. The detection limit of the method is 0.1 mg Mg / L and the limit of quantification is 0.20 mg Mg / L.

2.7 Determination of Serumc Iron

A direct colorimetric method (Ferene-S* reagent) was used to determine serum iron without deproteinization. We work with an ARCHITECT c-Systems 8000 analyzer. At pH 4.8, iron is released from transferrin to which it is bound and then quantitatively reduced to a ferric state. Iron forms with the Ferene-S reagent, forms a stable color complex whose color intensity is proportional to the amount of iron in the sample. We work with the ABBOTT commercial iron determination kit. The detection limit of the method is 1.3 µ g Fe/dL and the limit of quantification is 5.4 µ g Fe /dL.

2.8 Determination of Iron of Yerba Mate Infusion

The determination of Fe in the infusions was performed using the 200.2 EPA 600 / R-94 method. In a tared crucible, 100 mL of the convection stove infusion is carried to dryness at 105 ° C. Then it is cooled in a desiccator and weighed. After that, it is carried to 550 ° C in a muffle for 6 hours to obtain ashes. They are brought to 100 mL with 10% HCl. The Fe concentration of this solution was determined with a Perkin Elmer Atomic Absorption Unit, Model AAnalyst 700 Spectrometer in flame (air-acetylene) mode with hollow cathode lamp. The detection limit of the method is 0.02 mg Fe / L and the limit of quantification is 0.04 mg Fe / L.

2.9 Calculation of Magnesium and Iron Absortion

To calculate the magnesium and iron percentage absorbed by each female volunteer, we determined the body surface area "S" (Farah, Monteiro, Donangelo & Lafay, 2008) and then we calculated the needed "PV" plasma volume (García Curiel, & Gómez Perales, 2001) as Formulae 1.

$$S = \frac{\left(Weight^{0,425} \times Height^{0,725}\right) \times 71{,}84}{10000} \quad (1)$$

Weight is expressed in kg, height in cm and the surface in m^2. Plasma volume, expressed in mL, was calculated by the following 2 and 3Formulaes 2 and 3. Table 2 shows the anthropometric parameters of the 12 female volunteers:

$$PV_{men} = 1578 \times S \quad (2)$$

$$PV_{women} = 1395 \times S \quad (3)$$

The total amount of polyphenols absorbed by each volunteer was determined by calculating the "area under the curve" with the method of the sum of the areas of the rectangles (base x height) in the absorption versus time charts (data not shown) according to Formulae 4:

$$A = \Delta x \sum H_i \quad (4)$$

Table 2. Anthropometrics parameters of the female volunteers

Volunteer	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Weight (kg)	65,5	67	70	53,4	65	75	60,9	65	56	77	65	74
Height (cm)	169,5	167	164	153	159	157	170	163	158	167	164	176

Where H_i is the average height of each rectangle and Δx_i is the rectangle base it is the time lapse between each test and obtaining the amount absorbed per liter of plasma and multiplying it by the VP.

Calculating the mineral (magnesium or iron) absorption percentage as follows as can be see in Formulae 5:

$$\%\_\bar{Mineral}\_absorbed = \frac{Mineral\_absorbed}{Mineral\_ingested} \times 100 \quad (5)$$

2.10 Statistical Analysis

Data were expressed as the means ± standard deviations (SD). The data were submitted for analysis of variance (ANOVA). Differences between the groups were considered significant when $p \leq .05$. To compare the means, the Student test was used. To analyze the relationship between different absorption profilest linear regression was used. To analyze the variation within each absorption profiles paired samples was used. The statistical program Statgraphics Centurion XVI Plus was used.

3. Results

3.1 Characterization of Ingested Yerba Mate Infusion

It worked with two different lots of the same brand of elaborate yerba mate. For the magnesium bioavailability study it worked with 12 female volunteers, of which only 10 participated of the iron bioavailability study. The first half of female volunteers consumed the infusions of the first batch of yerba mate and the second half consumed the other yerba mate infusions lot. Differences were observed in the composition of the infusions. Thus, the first female volunteers (6 of magnesium bioavailability study and 5 of iron bioavailability study) ingested 300 mL of an infusion of yerba mate equivalent to 10 mates poured contained 50.8 ± 6.2 mg of Mg, 325 ± 18.7 µg of Fe and 1.52 ± 0.04 g GAE of total polyphenols, while the last female volunteers ingested 300 mL of the yerba mate infusion containing 105 ± 9.1 mg of Mg, 426 ± 49 µg of Fe and 1.36 ± 0.09 g GAE of total polyphenols.

3.2 Average Profile of Magnesium Absorption in Serum

The volunteers were divided into two groups, according to the amount of Mg ingested, corresponding Group 1 (G1) to female volunteers 1 to 6 and Group 2 (G2) which corresponds to female volunteers 7 to 12. It was determined that there were no statistically significant differences in the amount of Mg absorbed between the two groups ($p < 0.419718$), being the average absorption in t1 of 3.4 ± 1.6 mg of Mg and in the G2 of 2. 9 ± 0.8 mg

Mg the Mg.

These values indicate that, in spite of the fact that the amount of Mg ingested was doubled in the different intakes, the amount absorbed by the body keeps constant and with an average of 3.1± 1.2 mg of Mg.

When analyzing the 12 individual profiles (data not shown), it was observed that 40% of the female volunteers, started to increase the serum Mg values after 20 minutes of the yerba mate infusion intake as can be seen in Figure 1.

According to the results, in 300 mL of Yerba Mate infusions, we have an average of 77.9 ± 29.5 mg of Mg in solution with an average of 1.290 mg EAG of polyphenols which have the ability of chelating metals, so it can be assumed that the polyphenols could be chelating the Mg, decreasing its average absorption to only 3.1 mg Mg.

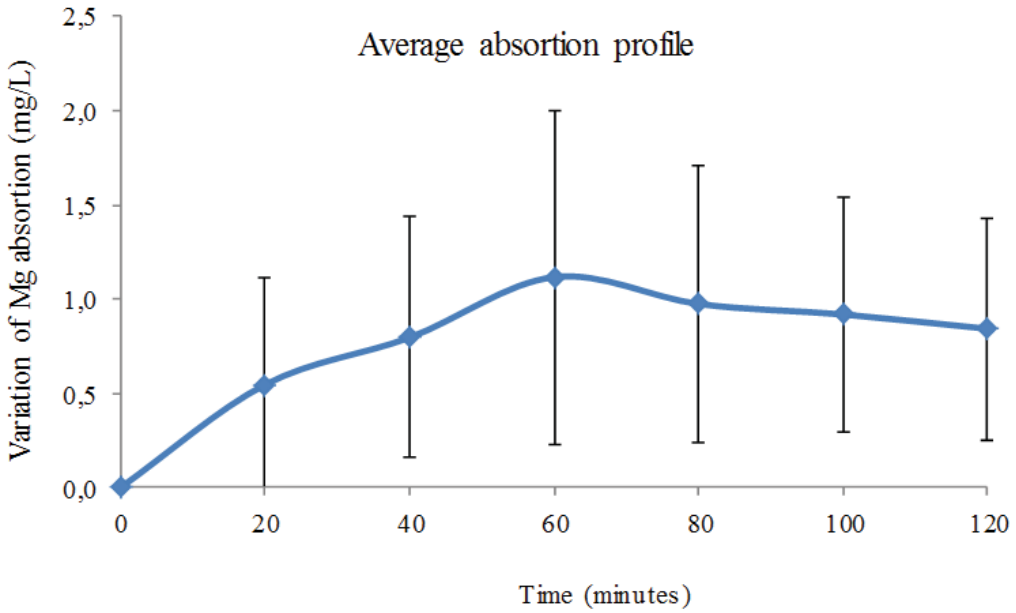

Figure 1. Variation of magnesium absortion (mean ± SD) of the 12 female volunteers.

3.3 Iron Absorption Serum Profiles

It was observed in the iron absorption profiles of the 10 female volunteers (dates not shown) a great interindividual variability in the iron absorption capacity. When comparing the ferrous sulphate absorption profiles to those of iron consumed along with the yerba mate infusions (ferrous sulfate + YM Fe), a decrease of the ferrous sulfate absorption was observed in all the volunteers profiles (data not shown).

A hypothesis test of paired samples ($\alpha \leq 0.05$) was used for the iron absorption profiles. It was observed that there are significant differences between the yerba mate iron absorption profile and the profile of the absorption of iron sulphate plus iron of yerba mate infusion of ($P \leq 10^{-7}$) and also between iron of yerba mate absorption profiles and iron of ferrous sulfate absorption profile ($P \leq 0.012$). It was also determined that there are significant differences between absorption profile of iron absorption of ferrous sulphate and the iron absorption profile of ferrous sulphate plus iron from the yerba mate infusion ($P \leq 10^{-7}$) as can be seen in Figure 2.

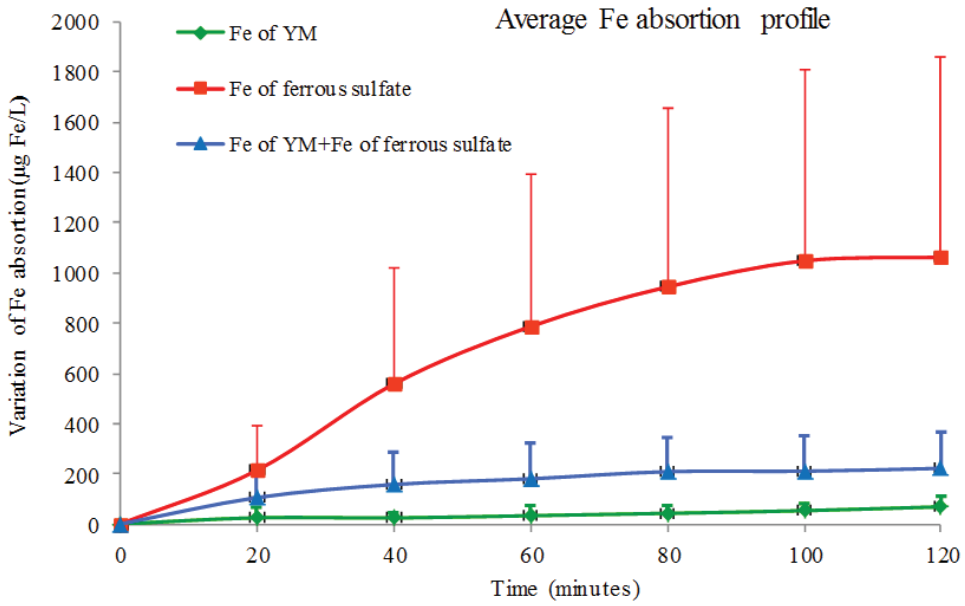

Figure 2. Average Fe absortion profile (mean + SD) of serum iron absorbed by 10 female volunteers in different consumption forms

To identify the mean moment at which the global change in iron absorption was detected, the Fisher's Minimum Difference (LSD) was used with 95.0% confidence level. It was verified that at 40 minutes of the ingestion of yerba mate infusion an increase in iron absorption began to be detected. In the case of the intake of the 40 mg of iron consumed as ferrous sulfate, the absorption began to be detected after 20 minutes of the intake. When yerba mate infusion with the ferrous sulfate was ingested, it was observed that iron absorption is detected at 25 minutes of the acute intake.

3.4 Effect of Polyphenols on Iron Absorption

It was determined that the yerba mate infusions contribute on average 376 ± 21 µg of Fe, taking into account the individual absortions of each of the female volunteers, the average absorption percentage of iron from the 300 mL of yerba mate infusion is 44.6 ± 18.4 % an average of 173 ± 20 µg de Fe.

When the volunteers consumed 40000 µg of Fe in ferrous sulfate form it was observed that only 3310 µg de Fe is absorbed on average 8.3 ± 6.1 % of iron total intake of 40 mg Fe. When ingested these 40 mg of Fe together with 300 mL of the yerba mate infusion, Fe absorption decreased to 803 µg of Fe and 2.0 ± 1.3 % of iron total intake of 40426 mg Fe, as seen in Figure 3.

If we take the absorbed amount when 40 mg of Fe were consumed as the maximum each volunteer can absorb, and we compare it with the amount of ferrum absorbed when digesting the 40 mg of Fe and the 300 mL of the yerba mate infusion together, we will get the percentage of the ferrum absorption. . The average absorption of Fe is 26.6 ± 10%, being so the average decrease in the bioavailability of Fe of 73.2 ± 10.

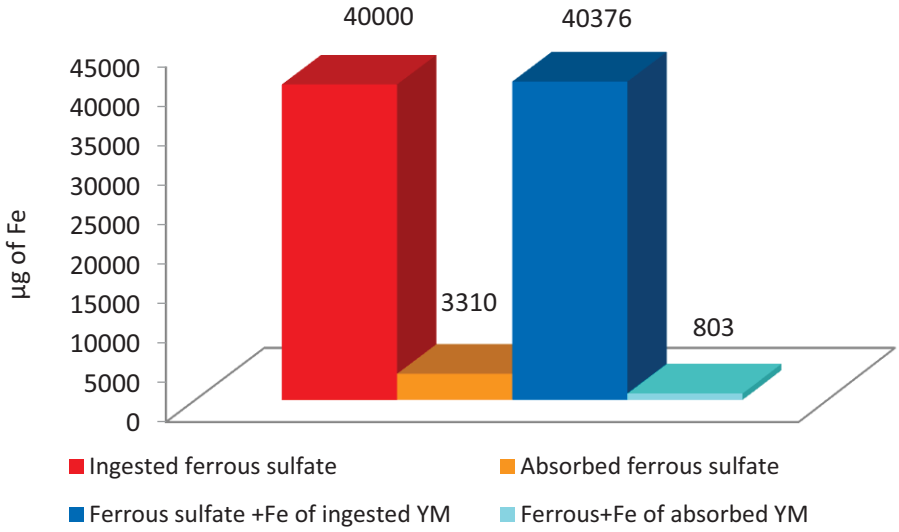

Figure 3. Inhibition of the bioavailability of Fe by action of the polyphenols from yerba mate infusions

4. Discussion

The elaborated yerba mate is a heterogeneous product, constituted by a mixture of leaf and stick. The yerba mate harvest extends for a period of 6 months from April (zafra) to September (zafriña), when sprouting begins. Like any agricultural product, its chemical composition can vary with the type of soil, climate, time of year, age of the plant and the leaves and genetic characteristics. Significant differences are reported in the content of minerals (N, P, Ca and Mg) in yerba leaves from different places. In Brazil there is a higher content of polyphenols and lower mineral content in yerba mate extracts from plants that grow exposed to sunlight (typical yerba mate) compared to those that grow in the shade (native plantations). (Escalada, Brumovsky, Hatwig, 2011)(Oliva, Reissmann, Gaiad, Sturion, De olivera, Wisnewiski &Miaqui, 2006). It was observed that caffeine varied significantly with the harvest time, being lower in the sprouting months and increasing as the leaves mature. (Heck; Schmalko & Gonzalez de Mejia, 2008).

It was found that in the leaves of 9-year-old yerba trees the amount of P, Mg, Ca was greater in the zafriña period than in the zafra, while in the 12-year-old plants those same minerals were found in greater quantity, both in the zafra and in the zafriña the concentration of Fe in young leaves was varied between 62 and 109 mg kg-1, not finding a statistically significant difference with the mature leaves, with values between 85 and 102 mg kg. Some authors also indicate that there is variation in Fe and Mg content of yerba in relation to the progeny to which they belong. (Jacques, Arruda, De Oliveira, De Oliveira, Dariva, De Oliveira & Carama, 2007).

We have found there is a great variability in Mg composition in yerba mate infusions between 50.8 ± 6.2 and 105 ± 9.1 mg of Mg every 300 mL of yerba mate infusions (49% of variation). This agrees with the values (reported by some authors) of 188 mg of Mg/L de infusion with a variation between 168 and 200 mg of Mg/L, corresponding to an average of 56.4 mg of Mg every 300 mL (Heinrichs & Malavolta, 2001) and others informed values of 129 ± 42 mg every 500 mL of infusion, corresponding to 77.4 mg Mg in 300 mL (Maiocchi et al., 2016). These variations could be due to the yerba mate mineral composition varied according to the place of development of plantations (Faria et al., 2008; Bastos et al., 2014) and that the leaves mineral contents drastically change depending on agricultural practices. The fertilizers and soil type have a significant impact on leaf mineral composition (Helena et al., 2007).

While the queantities of Mg of the infusions consumed by the volunteers were diferent, with values of 50.8 ± 6.2 mg of Mg and 50.8 and 105 ± 9.1 mg of Mg, the quantities absorbed were not statistically different with an average of 3,1 ± 1.2 mg of Mg. This value corresponds to an absorption of 7.0 ± 3.7 % and 2.8 ± 0.75 % of the Mg ingested from the first and second infusions respectively. This absorbed amount of Mg constitutes 1% of the RDA. These values of percentage of absorption contrast with those provided by other authors that indicate an absorption efficiency of 50% of the Mg present in solid diets (Coudray, Demigne &Rayssiguier, 2003) and of 80 - 90% of breast milk and about 55-75% in formulated milk contains according to the World Health Organization (2004).

There is currently no data on the in vivo absorption of Mg from yerba mate infusions, it contain on average 77.9 ± 29.5 mg of Mg and 1.41± 0.16 mg of EAG of polyphenols. Due to the fact that polyphenols have the ability to chelate metals, they are the most likely cause of the low absorption Mg values found in yerba mate infusions.

In the case of Fe content, it can be observed that there was also a statistically significant difference between both infusions, but with a much lower variability of 23.7% regarding Mg variability. Many authors have described *in vivo* and *in vitro* polyphenols interaction from various sources on iron absorption, which varies according to the chemical structure of polyphenol. Contrary, some polyphenols have no inhibitory effect on Fe uptake; it is so that, small amounts of grapefruit polyphenols in diets can improve availability of some minerals without significant negative influence on Fe status (Frejnagel, Gomez-villalva, Urbano & Zduñczyk, 2003). Some authors have reported up to 90% Fe absorption inhibition in *Leucaena glauca* leaf in Thailand (Mascitelli & Goldstein, 2013). It was also observed that among elderly participants in Framingham Heart study, a cup of coffee (236 mL) consumed for one week was associated with a 1% decrease in serum ferritin (Petry, Egli, Zeder, Walczyk &Hurrell, 2010). There are data that show that bioactive dietary polyphenols inhibit also heme iron absorption mainly by reducing basolateral iron exit rather than decreasing apical heme iron uptake in intestinal cells (Ma et al., 2010).

On the other hand even some authors have concluded that diets rich in polyphenols may be beneficial for iron overload patients by limiting the rate of intestinal absorption (Lesjak et al., 2014). Polyphenols are linked to iron through the ortho-dihydroxy (catechol) or trihydroxybenzene group (Khokhar & Owusu Apenten, 2003) doing by this mechanism, antibacterial activity due to its strong affinity with iron (Daglia, 2012).

Some authors compared the yerba mate chelation power according to their origin, determining that Brazilian and Uruguayan yerba mates had an 80% iron chelation capacity, while the Argentinian yerba mate's iron chelation capacity was lower but still significant (Colpo et al. 2016). Other authors showed that mate tea and green tea extracts provoke a very significant inhibition of the iron absorption, whereas it is much less significant with red wine extract (Anghileri &Thouvenot, 2000).

There are no *in vivo* studies about the inhibitory power of yerba mate polyphenols on iron absorption. In this study it was found that by ingesting 40 mg of Fe as ferrous sulphate (100%), 8.3 ± 6.1% of Fe was absorbed, while if it is consumed with 300 mL of yerba mate infusion, containing on average 1.41± 0.16 mg GAE of polyphenols, the Fe absorption decreases to 2.0 ± 1.3%.

Thus, the polyphenols could be responsible for the decrease in absorption of iron contained in yerba mate infusions. Therefore, the bioavailability of Fe decreased by 73.2 ± 10 %, reaching its absorption only 26.8 ± 10 % with respect to Fe ingested without polyphenols, as can be seen in Figures 3.

Due to the high prevalence of iron deficiency anaemia in women of childbearing age in addition to the decrease in absorption of Fe produced by polyphenols is that some authors evaluate the way to neutralize this inhibitory effect. The action of ascorbic acid and Ethylenediaminetetraacetic acid was studied. Ascorbic acid was found to be more efficient than Ethylenediaminetetraacetic in a way that lesser quantity is required for completely overcoming negative iron binding effects of polyphenols and similar samples (Tamilmani & Pandey, 2016).

In Argentina there is a habit of consuming yerba mate infusion throughout the day, including before and after meals.

To avoid the inhibitory effect of the polyphenols on the iron absortion from diet, it would be advisable to consume yerba mate infusion at least one hour before and 1.5 hours after the food intake.

5. Conclusions

It was determined that 300 mL of yerba mate infusions contain an average the 3.1 ± 1,2 mg de Mg, 376 ± 21 μg of Fe and 1.41 ± 0.16 mg GAE of polyphenol.

YM infusions contain 25% of the RDI for women of childbearing age, but only Mg amounts representing 1% of the RDI can be absorbed.

It was verified that absorption dynamics present a great interindividual variability, observing that 42% of the population begins to absorb magnesium after 20 minutes of acute intake.

It was determined that the 44.6 ± 18.4% of the iron ingested was absorbed when consuming 300 mL of yerba mate infusion during the two hours the test lasted, constituting approximately 1% of RDA for women at childbearing age and with a diet of high iron bioavailability of 15%.

It was determined that, out of 40 mg of Fe ingested as ferrous sulphate 8.3 ± 6.1% of Fe a was absorbed, while when consuming it with 300 mL of yerba mate infusion, containing on average 1.41 ± 0.16 mg GAE of

polyphenols, Fe absorption decreased to 2.0 ± 1.3% of the initial intake.

It can be concluded that yerba mate infusions prepared in their traditional form of hot mate have a nonheminic iron absorption inhibitory effect of 73.2 ± 10 %.

Due to the prevalence of iron deficiency anaemia in women at childbearing age and Fe absorption inhibitory effect by the polyphenols of hot yerba mate infusions, it is important to advise their consumption away from meals, as well as tea and coffee infusions.

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General

Tel: 1-416-642-2606

Fax: 1-416-642-2608

E-mail: info@ccsenet.org

Website: www.ccsenet.org

Mailing Address

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