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Evaluation and comparison of dietary habits of rural and urban areas school children; A case study

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ABSTRACT

Habits formed in early life are the major determinant of food choice later in life. Accordingly, this study was designed to assess and compare the dietary habits of children (5-9 years) from rural and urban areas of Punjab, Pakistan. The anthropometric measurements were also taken which included weight, height and then compared with BMI percentiles. Intake of breakfast was assessed by taking 24 hour dietary recall method. Independent t-test was used to compare the dietary habits of rural and urban areas children. The results showed that majority students of rural and urban were healthy but 36.7% students of rural school were underweight whereas 30% students of urban school were at risk of overweight. The trend of skipping breakfast was more prevalent amongst urban respondents. The intake of junk food which include chocolate, soft drink and instant noodle; macaroni was more common amongst urban children than rural students because of the availability of fast food in urban area. In conclusion, dietary habits of children are affected by parent's education, geographical area, availability, affordability, media and taste of food. Furthermore, it is recommended that nutrition education should be provided to children to improve their dietary habits to ensure adequate growth and development.

Keywords: Dietary habits, rural school, urban school, children

INTRODUCTION

Food preferences and eating patterns of children are developed early in life. To maintain a healthy weight as well as to provide protection against premature mortality and chronic diseases, it is important that eating habits should be healthy (Dudley, Cotton, & Peralta, 2015). The active growing phase of childhood is school age and it signifies a dynamic period of mental development as well as physical growth of the child. The dietary habits of children are important to assess not only because it affects the health but it also plays an important role in cognitive development of children and their performance at school (Abdelaziz, Youssef, Sedrak, &Labib, 2015; Belot, James, & Nolen, 2013).Childhood is the most important phase of life as the eating patterns are established in this age. Healthy and regular diet is a major factor in the promotion and maintenance of good health throughout life (Erjavec *et al.*, 2012). According to Simeon *et al.* (2015), it is required to assess the nutritional status of children in urban and rural areasbecause it is an important indicatorin the progression of child's growth and development. Anwer and Awan (2003) investigated that in Pakistan, urban and rural areas children have significant difference amongthem as well as the difference among male and female children in terms of lifestyle and economic status.

Many researchers have showed that due to presence of health services, availability of food and possibility of employment opportunities, the children of urban area have good nutritional status than rural area children (Smith *et al.*, 2005).There are many factors that affect the dietary habits and eating patterns of children. Caregiver is one of those factors that affect the children's dietary habits. If dietary habits of caregiver are poor then they need to improve their dietary habits for their children (Black and Hurley, 2007). According to San Juan (2006) the eating patterns are established early in life. The younger children's diet is almost similar to their parents than other children's diet. Similarly, Abideen, & Saleem

(2011) investigated that individual's attitude, lifestyle and behaviour is not only influenced by television but also it affects the culture of country because television is one of the strongest medium of advertisement. The study done by Doko Jelinić et al. (2009) demonstrated that geographical factor is considered as a strong determinant which affects the dietary habits of children. Many other factors include individual preferences, income, prices, individual belief, local traditions, culture as well as social, environmental and economic factors that are responsible to shape the dietary consumption pattern. Mother's education is one of the factors that affect the dietary habits of children. The quality and type of diet that children consume predicts the mother's as well as fathers educational level (San Juan, 2006). The current study assesses and compares the dietary habits of rural and urban areas children. The study also highlights some factors that affect the dietary habits of children.

MATERIALS AND METHODS

Research Design

This research was cross-sectional study about "the dietary habit of rural and urban school children" in Punjab, Pakistan. The government schools from both rural and urban area were selected. The systematic random sampling technique was applied in this research to select the subjects. In this technique, each member of the population has an equal chance of being selected as subject. The students of both genders from each school were chosen.

Inclusion and exclusion criteria

There were 60 students of childhood group ranging from the age of 5-9 years chosen from each school. The children under age 5 and above 9 years were excluded.

Data collection methods

The instruments administered were the self-constructed questionnaire and each child was interviewed alone. It covered the information about demographic data which includes parent's education, age and anthropometric measurements like weight, height and BMI. It collected the information about their dietary habits in the context of area difference specifically. It also contained 24-hour dietary recall of the students to determine the frequency of intake of breakfast as well as questions about eating at fast food restaurants, consumption of soft drinks and eating snacks were also included in this section of the questionnaire. Weight was measured on a weighing machine and height was measured with a wall mounted ruler. Body mass index (BMI) was computed as weight (in kg) divided by the square of height (in meter) and further categorized as underweight, normal, overweight according to BMI percentile chart.

Ethical Consideration

Informed consent was taken from principal of both schools prior to conducting survey. All the students fully contributed their attention towards the interview, the response of children was great and thus data was collected quite comfortably. The staff of both schools was also very cooperative. Confidentiality of information was ensured.

Data Analysis

Data was analyzed using SPSS version 20. The frequency distribution, percentages and graphs were used for the demographic information and factors that effects the dietary habits of children. Independent t-test was used to compare difference between the means of different groups (rural and urban area school children). Allthe tests were considered significant at $p< 0.05$ level.

RESULTS AND DISCUSSION

Demographic information

The current study showed that majority of children (58.3%) of rural area are 6-7 years old while most of the respondents (60.0%) of urban area are 8-9 years old. The majority of rural children (55%) are boys whereas majority of urban children (65%) are girls. The results of current study showed that there is significant difference between parent's education levels of rural and urban area students (p=.000). The results of current study showed that 83.3% mothers and 86.7% fathers of urban area respondents are educated. Whereas 70% mothers and 83.3% fathers of rural area students are uneducated. The mean 0f urban student's mother and father education level are $1.17\pm.376$ & $1.13\pm.343$ respectively. The mean values of rural respondents' mother and father educational level are $1.70\pm.462$ & $1.83\pm.376$, respectively. Numerous respondents of both urban area (91.7%) and rural area (85%) said that their meals are prepared by their mother. The current study showed that parents of urban area school children are more educated than rural area respondents. Likewise, Abuzaid (2012) found that urban student's parent's educational level was higher as compared to parents of rural students. Similarly, Nabag (2011) found that the level of illiteracy among parents of rural school

Table 1. Demographic information of urban-rural children

Parameters		Urban area school-children		Rural area school-children	
		N	%	N	%
Age	5 years	3	5.0%	2	3.3%
	6-7 years	21	35%	35	58.3%
	8-9 years	36	60.0%	23	38.3%
Gender	Boys	21	35.0%	33	55.0%
	Girls	39	65.0%	27	45.0%
Person preparing food	Mother	55	91.7%	51	85.0%
	any other	5	8.3%	9	15.0%
Mother educational level	Educated	50	83.3%	18	30%
Father educational level	Uneducated	10	16.7%	42	70%
	Educated	52	86.7%	10	16.7%
	Uneducated	8	13.3%	50	83.3%

Children were higher as compared to urban school children.

Nutritional status of children

The current study showed that there is a significant difference between urban and rural children's nutritional status (p=.012). The majority of urban (56.7%) and rural students (53.3%) were healthy. On the other hand, percentage of underweight was higher in rural children (36.7%) and the prevalence of overweight higher amongst urban respondents (30.0%). The mean values for urban and rural students are 2.17±.64 and 1.73±.63, respectively. According to BMI percentile chart if children have 10^{th} to 85^{th} percentile then they have normal weight, if $<10^{th}$ percentile then they areunderweight and if $>85^{th}$ percentile then overweight.

Dietary habits of rural and urban area school-children

The current study showed that there is a significant difference between rural and urban students' consumption of vegetables and fruits (p=.000). The researcher of current study determined that 86.7% of rural children consume vegetables daily and majority of urban respondents (56.7%) eat vegetables weekly. Whereas majority of urban children (66.7%) consume fruits daily while most of rural respondents (53.3%) consume fruits rarely. The mean values of vegetable intake for rural and urban children are 1.13±.34 & 1.90±.65 respectively. Whereas the mean values of fruits intake for rural and urban children are 2.20±.91

& 1.53±.81 respectively. There is a significant difference between rural and urban student's meat intake (p=.000).The researcher found that majority of respondents (66.7%) of rural area eat meat rarely whereas the most of the students (73.3%) of urban area responds that they consume meat weekly. The mean values for urban and rural area students are 1.87±.50 & 2.87±.56 respectively. There is no significant difference between rural and urban children's milk consumption (p=.097). The results of current study showed that majority of rural students (80%) and urban children (66.7%) consume milk daily. The mean values for rural and urban children are 1.27±.57 & 1.47±.72 respectively. The current study showed that there is a significant difference between junk food intake of rural and urban children (p=.000). In current study the researcher demonstrated that the majority of urban children (50%) consume chocolate daily while children (66.7%) of rural area eat chocolate rarely. About 36.7% urban students and 20% rural respondents consume soft drink daily. Numerous students (43.3%) of urban area eat noodles daily while majority of rural students (56.7%) don't eat noodles. The mean values for rural & urban children chocolate consumption are 2.70±.86 & 1.87±.99 respectively. The mean values for intake of soft drink by rural and urban students are 2.60±.88 & 1.97±.92 respectively. The values of mean and standard deviation of urban and rural children's intake of noodles are 2.23±.85 & 3.23±1.03 respectively. The current study showed that there is a significant difference between rural and urban children eat dine outdoor (p=.000). The majority of respondents (53.3%) of urban school rarely dine outdoor whereas most of the students (66.7%) of rural school never dine outdoor. The mean values of rural and urban school are 3.13±.676 & 3.67±.475 respectively. The result showed that there is significant difference between rural and urban student's intake of breakfast (p=.001). According to 3 days 24-hour dietary recall numerous students (90%) of rural school eat breakfast daily whereas majority of urban children (65%) consume breakfast daily. The mean values of urban and rural children are 1.35±.481 & 1.10±.303 respectively.

The present results showed that rural children are more prevalent to under-nutrition while urban children are at risk of overweight that are similar with the findings of Simeon *et al.* (2015).They found that the higher proportion of rural area children was underweight as compared to urban area. Likewise, Islam *et al.* (2014) concluded that the higher percentage of children in rural and urban area was normal weight while the percentage of underweight children in rural area was greater than urban.

The data of current study showed that vegetables are more consumed by rural children than urban while fruit intake is more common in urban children. The findings of current research are supported by the study of Aziz and Devi (2012) who concluded that vegetables are consumed more by rural children than urban. Likewise, Nabag (2011) demonstrated that intake of fruits is more common amongst urban children than rural respondents. Similarly according to Davis et al. (2008) analyses indicated that urban children were more likely to consume fruits rather than vegetables, and rural children were just the opposite, more likely to consume vegetables rather than fruits. The researcher found that the meat is consumed more by urban children as compare to rural and it is supported by Nabag (2011) that urban children consume more meat and meat products than rural children. The result of current study showed that the children of both rural and urban areas drink milk daily. Likewise, a group of researchers, McNaughton *et al.* (2006) found that there was no significant difference between urban and rural children regarding milk consumption. The current findings regarding junk food consumption amongst rural and urban children are supported by the study of Aziz & Devi (2012) that instant noodles and chocolate are more consumed by urban children than rural children. Likewise, McNaughton et al. (2006) also investigated that children living in urban areas consumed greater amounts of soft drink and fast food as compared to children living in rural areas. The results of dine outdoor by rural and urban school children are in concordance with the finding of Abuzaid (2012) who demonstrated that eating out at fast food restaurants was higher among urban than rural.

Factors affecting the dietary habits of children

The majority of students (43.3% & 40%) of urban school respond that the major factor that affects the intake of meat & soft drink is affordability. While most of the urban area respondent's 46.7%, 60% & 36.7% intake of fruits, vegetables and milk are affected by mother's demand. The children (50%) of urban school are influenced by TV and consume chocolate. Whereas numerous students 53.3%, 60% & 38.3% of rural school consume fewer amounts of meat, fruit and soft drink due to less affordability. Majority (60%) of rural children consume food due to their mother's demand. About 66.7% students don't eat noodles; macaroni etc because of non-availability in their area. While 56.7% & 36.7% children drink

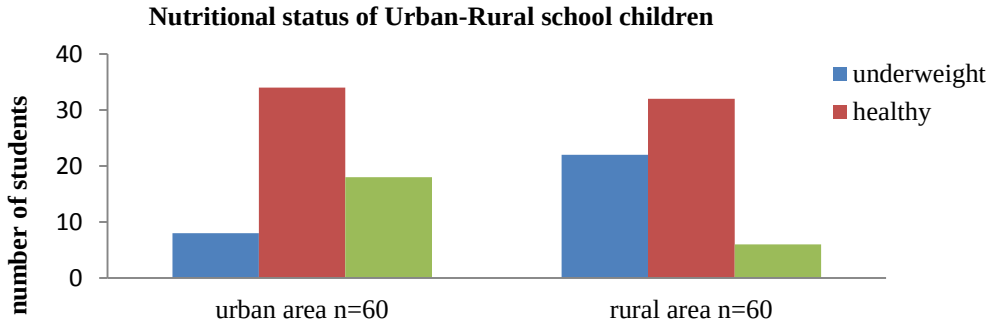

Fig 1. Nutritional status of Urban-Rural school children

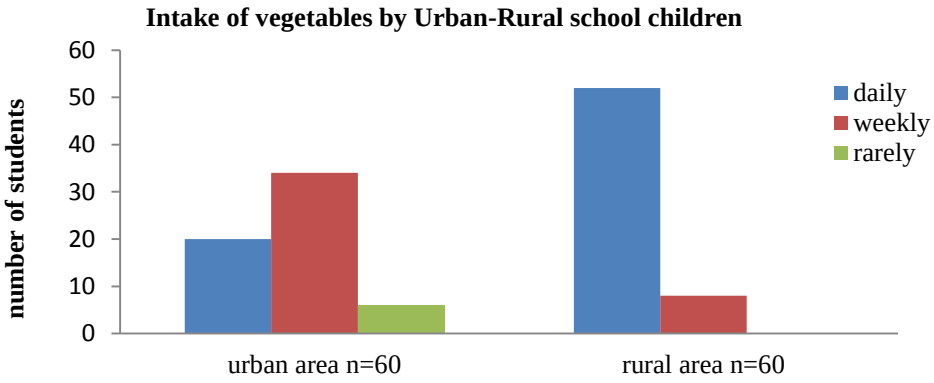

Fig 2. Intake of vegetables by Urban-Rural school children

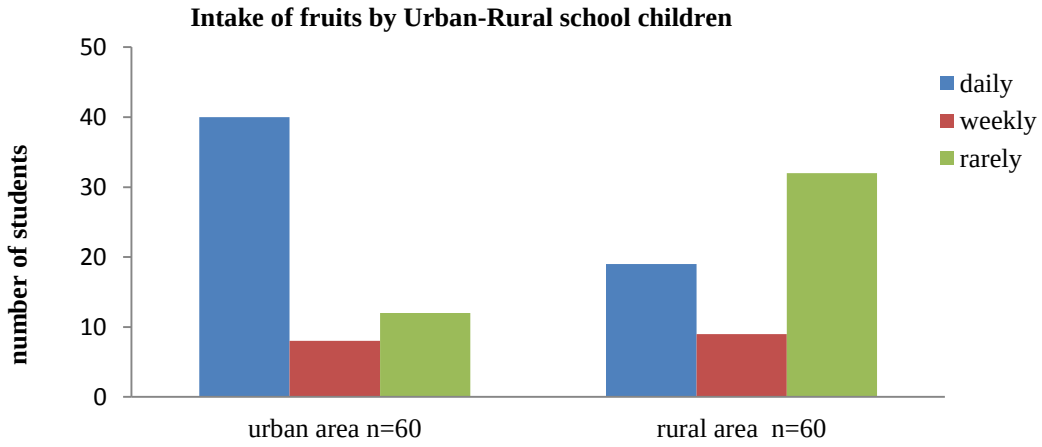

Fig 3. Intake of fruits by Urban-Rural school children

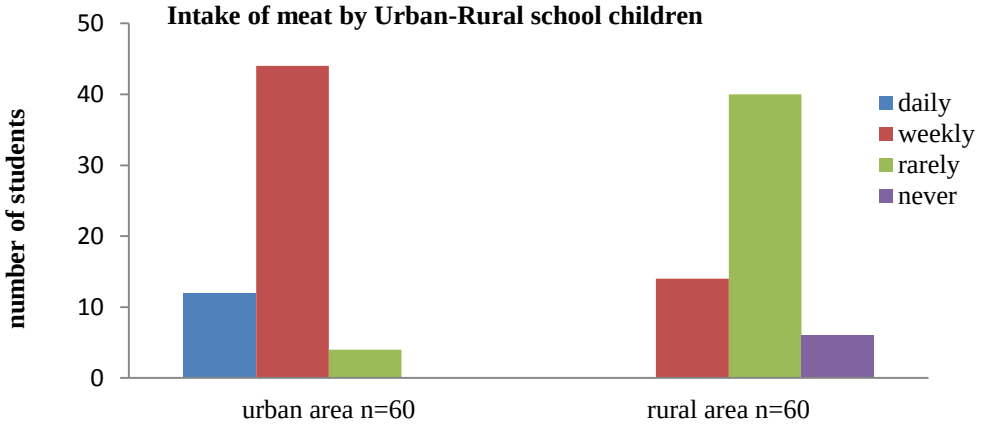

Fig 4. Intake of meat by Urban-Rural school children

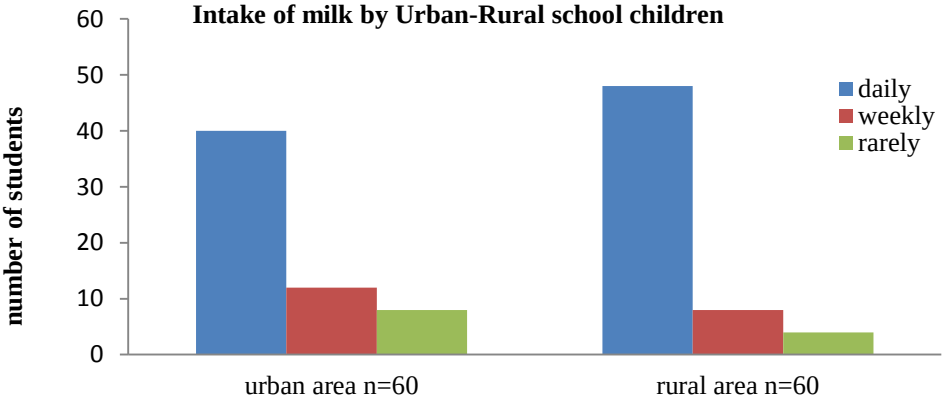

Fig 5. Intake of milk by Urban-Rural school children

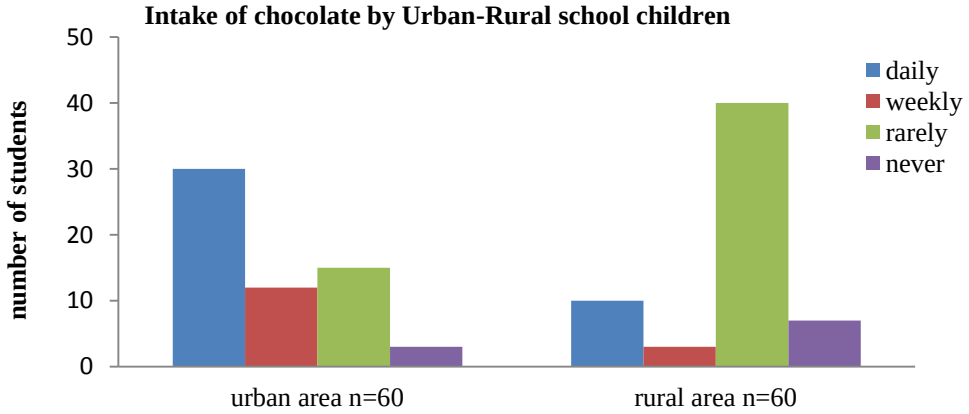

Fig 6. Intake of chocolate by Urban-Rural school children

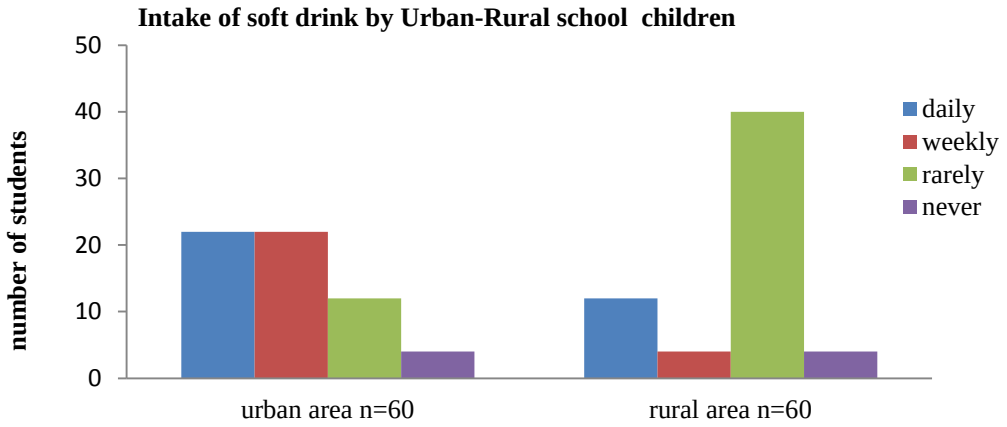

Fig 7. Intake of soft drink by Urban-Rural school children

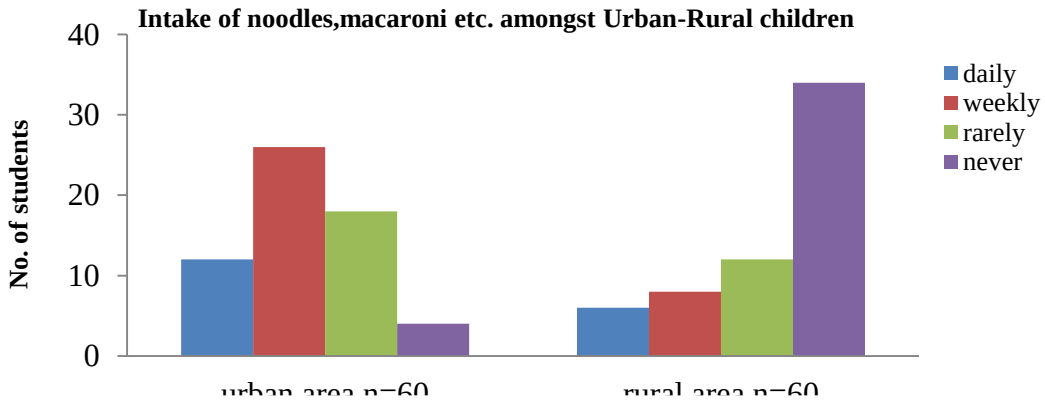

Fig 8. Intake of noodles, macaroni etc. by Urban-Rural school children

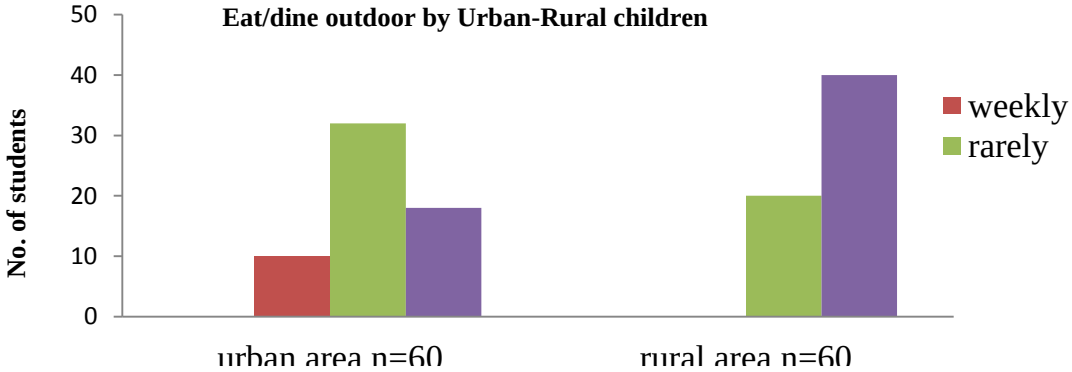

Figure 9. Eat/dine outdoor by Urban-Rural school children

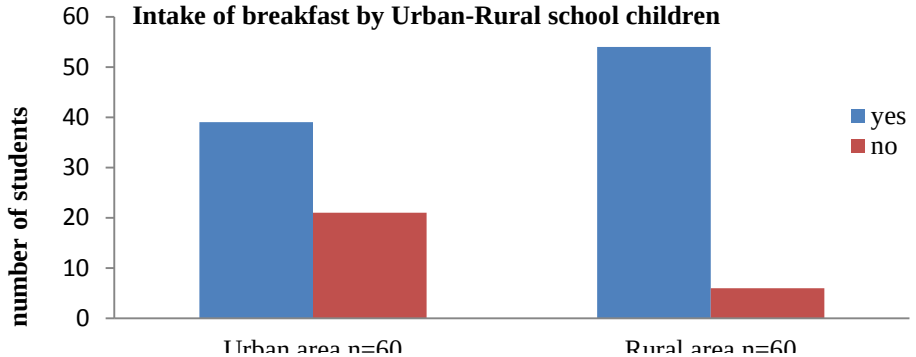

Fig. 10. Intake of breakfast daily by Urban-Rural school children

milk and eat chocolate due to their likeness. Current study indicated that the trend of skipping breakfast is more common amongst urban school children than rural students. Likewise, Davis *et al.* (2008) demonstrated that urban children were more likely to skip breakfast than rural children. In the previous research study, Abuzaid (2012) found that the fast food chains and restaurants are easily available and accessible in urban area so the junk food intake is more prevalent in urban children than rural. The study done by Black & Hurley (2007) determined that children accept or reject food based on qualities of the food such as taste, texture, smell, temperature or appearance, as well as environmental factors such as the setting and the presence of others.

CONCLUSION

The health and nutrition of children is important as their eating behavior and nutrition will affect their future health. The current study showed that dietary habits of rural area's children are quite different from urban area's children in many aspects. The consumption of fruits, meat and junk food was more common amongst urban children whereas the intake of vegetables and trend of daily breakfast consumption was more prevalent amongst rural children. The factors that affect the dietary habits of children were parent's education, TV influence, availability, affordability, likeness and dislike. As childhood group is that period of life in which eating habits and patterns are developed and follow up into the adulthood. The intake of five food groups was present in both groups because in Pakistan there is not concept of pure vegetarian and non-vegetarian as in other countries but their dietary habits differ in the rates of food intake. So improving the dietary habits of children either belonging fromrural area or urban is very important. The on-going primary health care programs should be implemented in urban & rural area.

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Evaluation of the antioxidant and antimicrobial effects of ziziphus leaves extract in sausage during cold storage

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ABSTRACT

Effects of ziziphus leaves extracts as natural alternative preservatives on TBA value, antimicrobial activity, microbial count, color and sensory characteristics of beef sausage were investigated during cold storage at 2°C for 14 days. Five treatments of sausages were prepared; Control, tert-Butylhydroquinone (TBHQ) added, 0.5 and 1.0mg/100g ziziphus leaves ethanolic extract/sausage mixture for T1 and T2 respectively, and 1.0g/100g ziziphus leaves aqueous extract/sausage mixture for T3. In ziziphus leaves; e-vanillic and ellagic were the predominant phenolic compounds represents 39.09 and 36.38 mg/100g of ziziphus leaves powder, while quercetin (8.38 mg/100g) was the major flavonoid in it. Ziziphus leaves extracts inhibited the growth of all tested foodborne bacteria strains, and decreased the total microbial count in sausage samples compared to control and TBHQ containing sausages. During cold storage, sausage samples with the addition of 1% ziziphus leaves extract (ethanolic or aqueous) showed; better color stability, lower TBA values compared to control and TBHQ added samples. Sensory scores of sausage samples with ethanolic or aqueous extracts were as good as control and TBHQ added sausages. T3 (1% added aqueous extract of ziziphus leaves) scored better aroma, taste, tenderness and over-all acceptability compared to control and the TBHQ added sausage samples.

Keywords: Ziziphus extract, nitrosamine alternatives, antioxidant, antimicrobial, phenolic and flavonoids compounds, sausage.

INTRODUCTION

Microbial growth in meat and processed meat products probably hurry the lipid oxidation and other oxidative processes which cause a deterioration in sensory characteristics such as flavor, and it is responsible for the nutritional value reduction (Saggiorato *et al*. 2012). Microbial contamination comprise a major public health hazards and economic loss in terms of food poisoning and meat spoilage (Sallam *et al*. 2004).

Sodium nitrite has been used for a long time as a meat products preservative, because it could effectively inhibit *Clostridium botulinum* growth and other food Spoilage microorganisms (Skibsted, 2011). Drabik-Markiewicz *et al*. (2011) reported a positive correlation between the amounts of sodium nitrite added tp processed meat products and the amount of Nitorsamine formed in it. The use of artificial antioxidants such as TBHQ, BHA and BHT as food preservatives is now banned, or under strict regulation in many countries because of their toxic and carcinogenic side effects (Arshad et al 2013; Jo *et al*. 2006). The increased demand of natural foods and natural preservatives as well has obligated the food industry to include natural antioxidants in foods. Natural antioxidants are recently used instead of synthetic ones to limit lipid oxidation in foods to improve their quality and their nutritional value. Lipid oxidation in meat products can directly affect their color, flavor, texture, nutritive value, and safety (Ruiz *et al*. 1999; Camo *et al*. 2008; Velasco and Williams, 2011).

Ziziphus Spp. Lam., a member of family Rhamnaceae, is a medicinal plant largely distributed to the Mediterranean region, native to the Indo-Malaysian region, southern Africa, China, Australasia and the Pacific Islands (Yoon *et al*. 2010; Ahmad *et al*. 2011). It is called as Red date, Chinese date, and Bera (Pushto) (Naik *et al*. 2013). Ziziphus fruits, barks, roots and leaves are edible, rich in carbohydrate, minerals, potassium, iron, vitamin C, phenolic compounds, flavonoids, tannins, saponins, triterpenic acids, polysaccharides, sterols, pectin, glycosides and alkaloids (Zhao *et al*. 2008; Yusufoglu, 2011; Goyal *et al*., 2012; Naik *et al*., 2013). Ziziphus leaves extract has an antimicrobial activity against *Staphylooccus aureus, Escherichia*

coli, Salmonella typhi, Pseudomonas aeruginosa, Klebsiellapneumoniae, Proteus vulgaris and Bacillus subtilis (Najafi, 2013; Priyanka *et al*. 2015)**.** Thus, it could be used as an alternative source of antibiotics, as its leaves were used in the folkoloric medicine to get rid of internal parasites (Dangoggo *et al*. 2012). The antimicrobial activity of ziziphus extracts might be duo to the presence of phytochemical compounds such as flavonoids, tannins, saponins, *etc*, (Hassan and Abdel-Gawad, 2010). Polyphenolic compounds present in ziziphus leaves and juice are quite safe for human health, as the administration of ziziphus leaves did not cause any changes neither in liver nor in kidney functions (Al-Marzooq, 2014).

This study aimed to investigate the effects of ziziphus leaves (aqueous and ethanolic) extracts addition of different levels, on lipid oxidation, microbial growth and sensory acceptability of beef sausage during cold storage at 2°C for 14 days.

MATERIAL AND METHODS

Five treatments of sausages were prepared to accomplish aim of this study; Control treatment, TBHQ added sausage samples, 0.5 and 1.0mg/100g ziziphus leaves ethanolic extract/sausage mixture for T1 and T2 respectively, and 1.0g/100g ziziphus leaves aqueous extract/sausage mixture for T3.

Phenolic and flavonoid compounds determination

The phenolic and flavonoid compounds of ziziphus leaves were determined according to the method described by Goupy *et al*. (1999) and Mattila *et al*. (2000) by using HPLC instrument (Hewlett Packard, Series 1050, USA) composed of column C18 hypersil BDS with particle size 5 µm. The separation was carried out using methanol and acetonitril as mobile phase, at flow rate of 1 ml/min. Quantification was carried out based on calibration with standards of phenolic and flavonoid compounds.

Determination of free radical scavenging activity (DPPH assay)

The free radical scavenging of different extracts according to the method ofTepe et al. (2005). Briefly, 3ml of 0.1mM ethanolic solution of DPPH was added to 1 ml of ethanolic extracts at concentration 100 µg/ml. The absorbance was measured against a blank at 517 nm at 0, 30, 60 and 120 min. Inhibition of free radical (DPPH) in percent was calculated using the following equation:

DPPH scavenging activity% = (Ac- As/ Ac) ×100

Where Ac is the absorbance of control reaction (containing all reagents except the test extract) and as is the absorbance in the presence of the tested extracts.

Antimicrobial activity of extracts using disc diffusion method

The antimicrobial activity of the ziziphus leaves ethanolic and aqueous extracts (100µg/ml DMSO) was determined compared to commercial antimicrobial agent (ampicillin) by using the Agar diffusion method reported by Singh et al. (2003). Culture plates were prepared with 10 ml of plate count agar (Difco Laboratories, Detroit, MI, USA), plates were overlaid with 5ml of tryptic soy agar (soft agar) (TSA with 8.0 g/kg agar) at 40±1°C containing 1ml of bacterial culture (1x109 cfu/ml). Six sterile paper discs (diameter 6.0 mm) (Becton Dickinson MicrobiologySy stem, MD, USA) were placed at different locations on the surface of culture plates. Five microliters of different ziziphus leaves extract was dropped on each paper disc, and a disc soaked with absolute ethanol was used as the positive control to detect any possible antimicrobial effect due to ethanol content of the dilutions. The treated plates were incubated at 37±1°C for 48 h and were visually inspected for any zone of inhibition around the paper discs. The pathogenic bacteria strains (Bacillus subtilis, Escherichia coli, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Staphylococcus aureus and Staphylococcus faecalis) were obtained from microbial culture collection (MIRCN), Faculty of Agriculture, Ain Shams University, Egypt. The diameter of inhibition zone was measured by calipers at four different places.

Preparation of sausage

Sausage was prepared by mixing minced meat with the other ingredients which are listed in Table (1) according to the method of (Azizkhani and Tooryan, 2014) with minor changes in the formula especially spices amounts to suit the local consumer favorability. Five batches of sausage were prepared according to the following addition system; Control (as described in Table. 1. (With no additives), TBHQ (with the addition of TBHQ at 0.05 g/100g), T1 (with the addition of 0.5 mg/100g of ziziphus leaves ethanolic extract) T2 (with the addition of 1.0 mg/100g of ziziphus leaves ethanolic extract), and T3 (with the addition of 1 mg/100g of ziziphus leaves aqueous extracts). TBHQ or ziziphus extracts were thoroughly mixed with ice water before adding to the sausage formula. Formulated sausage mixtures were filled into sheep casing using filler (moulinex, France). Sausage samples of every treatment (triplicates) were stored at fridge at 2°C for further

use and analysis. A part of each sample was separated for the analysis at zero time, and a part of each batch was cooked for sensory evaluation.

Table 1. Control sauage base formula composition

Ingredients	Percentage	Amount (g)
Beef meat	70.9	650
Fat	10.9	100
Ice water	10.9	100
Starch	3.3	30
Skimmed milk	2.2	20
Garlic	0.3	3
White pepper	0.2	1.5
Ginger powder	0.1	1
Coriander	0.2	1.5
Nutmeg	0.1	1
Salt	1.0	9
Total	100	917

Total microbial count in the prepared sausage samples

The microbial count was performed on the sausage during the storage period according to the method described by Kim and Choi (2014). A 10 gram of sausage sample was homogenized with 90 ml of sterile peptone water using lab-dancer, then serial dilutions was prepared. 100µl of samples of different dilutions were spread on plate count agar (Difco Laboratories, Detroit, MI, USA). Plates were then incubated at 35°C for 48 hours. Number of colonies were count and expressed as $\log^{10}$ CFU per gram sausage sample.

Measurement of Thiobarbituric acid reactive substance (TBARS) of sausages

TBARS number was assessed in triplicates by the TBA method of (Liaros *et al*. 2009) with some modifications. Briefly, ten grams of sausage sample was well homogenized with 25ml of distilled water for 2 minutes, mixed with 25ml of 10% trichloroacetic acid (TCA). Sample was filtrated (through whatman filter paper No. 1), one ml of thiobarbituric acid (0.06 M) in 90% acetic acid (TBA reagent) was added to 4 ml of the filtrate in vial and mixed well. Vials were capped and heated in a boiling water bath for 10 min to develop the chromogen, cooled to room temperature. Absorbance at 538 nm was recorded, against a blank prepared with 4ml distilled water and 1ml TBA-reagent, using a JENWAY 6705 UV/VIS Spectrophotometer. The TBA numbers were calculated as mg malondialdehyde/kg sample according to the following equation:

TBARS number (kg) = Absorbance × 7.8

Color values of sausage

A color hunter lab (Hunter Lab Color Flex EZ, USA) was used for the fulfillment of the procedure of Jayawardana *et al*. (2012) for color determination. CIE (L*, a* and b*) of cross-section of each piece were determined. Color values were recorded in triplicates and mean was recorded.

Sensory evaluation of sausages

Sausage samples were organoleptically evaluated immediately after production according to the method of Mansour and Khalil (2000). Sausage samples were served to twenty panelists (Staff of Food Science Department, Faculty of Agriculture, Zagazig University, Egypt) without care of age or sex. Panelists were asked to evaluate sausage samples for colour, taste, flavour and overall acceptability on a nine points headonic scale system on basis of: 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, 9= like extremely.

REAULTS AND DISCUSSION

Phenolic and flavonoids compounds

Phenolic and flavonoid compounds concentrations in ziziphus leaves (mg/100g leaves powder) are shown in Table (2). Data showed that, twenty four phenolic compounds were identified and quantified in ziziphus leaves. The e-vanillic was the most abundant phenolic compound in ziziphus leaves (39.09 mg/100g) followed by ellagic (36.38 mg/100g), *p*-cumaric (14.32 mg/100g), Pyrogallol (12.86 mg/100g) and benzoic (12.02 mg/100g). Ramanathan and Das (1993) reported that, ellagic acid was one of the most active compound behind the antioxidant activity of ziziphus. Quercetin, which comprise 8.48 mg/100g, was the major flavonoid compound in ziziphus leaves powder followed by quercetrin (3.95 mg/100g), hesperidin (3.40 mg/100g) and rutin (1.52 mg/100g). That was inline with the results of Shahat *et al*. (2001), who reported that, *Ziziphus spina*-Christi leaves contained flavonoids compounds such as quercetin and quercetrin. Flavonoid compounds have also related to the antioxidant capacity, especially quercetin and its glycoside conjugates that it has the ability to chelate the metal ions, free radicals and thus inhibit the oxidation process (Lu *et al*. 2011). Several studies reported that phenolic and

flavonoids compounds from plants origin have showed a high antioxidant activity (Sohaib *et al*., 2015; Mohammed *et al*. 2013; Pontis *et al*. 2014).

Antioxidant activity (DPPH scavenging activity) of ziziphus leaves ethanolic extract

Antioxidant activity of ziziphus ethanolic extract at 250, 500 and 1000µg/ml was presented in Table 3. As could be noted, antioxidant activity of the 250, 500µg/ml ethanolic extract of ziziphus leaves was high, at a maximum scavenging activity of 57.74 and 69.62% after 120 minutes of incubation, but these antioxidant activities were not comparable to the antioxidant activity of the 1000µg/ml TBHQ which scored 91.2% at the same incubation time (120 min). The 1000µg/ml of ziziphus ethanolic extract had lower scores of DPPH scavenging activity at zero time and after 30 minutes (58.88 and 77.85%) comparing to the 1000µg/ml TBHQ (84.83 and 85.15%). But after 60 and 120 minutes of incubation, DPPH scavenging activity of ziziphus extract at 1000 µg/ml was comparable to the TBHQ (85.91 comparing to 86.9% at 60min for ziziphus extract and TBHQ respectively and 92.42 comparing to 91.2 at 120 min for ziziphus extract and TBHQ respectively). Those results were in agreement with the findings of Benammar *et al*. (2014), who stated that, ziziphus fruit, leaves and seeds exhibited *in vitro* antioxidant properties.

Antimicrobial activity of ziziphus extracts

The ability of the plant extracts to inhibit bacterial growth on agar or any suitable media was always considered as an antimicrobial activity. Table (4) shows the inhibition zones of ethanolic and aqueous extracts of ziziphus leaves against different foodborne bacteria strains. Both extracts at a dose of 100µg/ml showed considerable antibacterial activity against all tested pathogenic strains including *B. subtilis, E. coli, Neisseria gonorrhoeae, P. aeruginosa, S. aureus and S. faecalis*, within inhibition zones in the range of 17.6-21.7mm. Ziziphus leaves extracts exhibited stronger antimicrobial activity compared to that of ampicillin (commercial antimicrobial substance) except for *B. subtilis and E. coli*. The antibacterial activity of ziziphus may be related to its content of phenolic compounds or because of its content of glycoside, phenol, tannin and saponin compounds which have a powerful antibacterial effect. Gök and Bor (2012) stated that the antimicrobial effect of ziziphus may be related to the oxygenated mono and sesquiterpene hydrocarbons such as eucalyptol, caryophyllene, caryophyllene oxide. Ahmad *et al*. (2013) found that the leaf extracts of *Ziziphus oxyphylla* and *Cedrela serrata* showed considerable antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Proteus mirabilis, Salmonella typhi, Escherichia coli* and *Citrobacter* spp.

Antibacterial effect of ziziphus ethanolic extract was also reported by (Al-Reza *et al*. 2009; Naili *et al*. 2010; Najafi, 2013) who found that ziziphus inhibited the growth of *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi* with an inhibition zones ranged between 7-18mm. The ethanolic extract had higher antimicrobial activity (18.4 – 21.7mm inhibition zones) than the aqueous extract (17.6 - 19.3mm inhibition zones), that might be because ethanol could extract more active compounds which have antibacterial activity including essential oils and phenolic compounds compared to water which was not as able as ethanol in extraction of the active compounds and essential oil of ziziphus leaves. These results are in line with the findings of (Abdel-Salam *et al*. 2014) who reported that aqueous extract of red onion, garlic and leek were lower antibacterial activity when compared to ethanolic extract and essential oil of these plants.

Total microbial count of sausage during cold storage

The effects of ziziphus extracts on total microbial count of sausage compared to the TBHQ added and control sausage samples, during cold storage (2°C) are shown in Figure (1). Total microbial count of sausage samples was determined at the day of production (0 day) and after 3, 7, 10 and 14 days of cold storage. As expected, the increase of storage time resulted in gradual increase in total microbial count. However, ziziphus leaves extract significantly delayed the total microbial count of sausage samples compared to the control. The total microbial count in the initial time (day 0) ranged from 2.20 and 2.25 $\log_{10}$ CFU/g for sample treated with 0.01% TBHQ and control, respectively. However by the day 14 of cold storage, the total microbial count was 3.69 and 5.83 $\log_{10}$ CFU/g for sample treated with 1% ethanolic extract and control, respectively. The addition of ziziphus leaves showed lower change in total microbial count of beef sausage samples after 14 days of cold storage at 2°C compared to the control. Sausage samples treated with 1% ziziphus ethanolic extract had the lowest total microbial count (3.69 $\log_{10}$ CFU/g) after 14 days of cold storage compared with those treated with 0.01% TBHQ (3.86 $\log_{10}$ CFU/g) and control (5.83 $\log_{10}$ CFU/g). This is probably due to the inhibitory effect of ziziphus phenolic and flavonoid compounds on spoilage bacteria. Wenjiao *et al*. (2014) reported that the tea polyphenols inhibited the spoilage bacteria growth

Table 2. Phenolic and flavonoid compounds of Ziziphus leaves

Sr. No	Phenolic compound	Concentration (mg/100 g)	Falvonoid compound	Concentration (mg/100 g)
1	e-vanillic	39.09	Quercetin	8.48
2	Ellagic	36.38	Quercetrin	3.95
3	p-Coumaric	14.32	Hisperidin	3.4
4	Pyrogallol	12.86	Rutin	1.52
5	Benzoic	12.02	Hisperetin	0.63
6	Salicylic	7.2	Rosmrinic acid	0.55
7	Ferulic	5.38	Naringin	0.39
8	3-OH-Tyrosol	5.3	7-OH flavone	0.37
9	3,4,5-methoxy-cinnamic	5.13	Narengenin	0.34
10	p.hydroxy-Benzoic	2.91	Apegenin	0.3
11	Vanillic	2.29	Kampferol	0.22
12	Chlorogenic	2.13	-	-
13	Alpha-coumaric	2.11	-	-
14	Protocatechuic	2.04	-	-
15	Catachein	1.91	-	-
16	4-Amino-benzoic	1.55	-	-
17	Epicatachein	1.45	-	-
18	Catechol	1.4	-	-
19	Cinnamic	1.26	-	-
20	Iso-ferulic	1.04	-	-
21	Caffeic	0.96	-	-
22	Reversetrol	0.57	-	-
23	Caffeine	0.45	-	-
24	Gallic	0.16	-	-

Table 3. Antioxidant activity at different incubation times and concentrations of Ziziphus leaf extract

Incubation time	Ziziphus leaves ethanolic extract			TBHQ
	250µg/mL	500µg/mL	1000µg/mL	1000µg/mL
0 min	33.99	43.49	58.88	84.83
30 min	42.70	52.74	77.85	85.15
60 min	49.33	61.65	85.91	86.90
120 min	57.74	69.62	92.42	91.20

Table 4. Effect of ziziphus leaves extract on some pathogenic bacteria compared with commercial antibacterial substance

Extract (100µg/ml)	Inhibition zone diameter (mm)					
	Bacillus subtilis (*G^+)	***Escherichia coli*** (G^-)	***Neisseria gonorrhoeae*** (G^-)	***Pseudomonas aeruginosa*** (G^-)	***Staphylococcus aureus*** (G^+)	***Streptococcus faecalis*** (G^+)
Ethanolic	18.4 ± 0.22	20.8 ± 0.19	21.7 ± 0.30	19.2 ± 0.15	19.5 ± 0.22	19.4 ± 0.18
Aqueous	17.9 ± 0.27	18.4 ± 20	19.3 ± 0.19	18.7 ± 0.23	17.6 ± 0.31	19.1 ± 0.16
Ampicillin	20.5 ± 0.21	22.3 ± 0.11	20.6 ± 0.28	17.4 ± 0.17	18.4 ± 0.26	18.9 ± 0.23

Data are mean values ± S.D. (n=3); *G = Gram reaction

Table 5. Color values of sausage during cold storage

Samples	Storage time (day)								
	L*			a*			b*		
	0	7	14	0	7	14	0	7	14
Control	41.11a	40.36a	40.49a	7.56e	3.81e	3.62d	11.37d	12.57a	11.84a
TBHQ	35.12e	34.96e	35.48e	11.27b	4.32b	3.91b	11.24e	11.54d	11.43e
T1	36.14d	36.49c	36.41d	9.93c	3.97c	3.68c	12.05a	11.07e	11.65c
T2	36.37c	35.66d	38.61c	11.84a	5.37a	5.34a	11.46c	11.66c	11.45d
T3	38.19b	37.18b	39.82b	9.69d	3.87d	3.43e	11.86b	11.72b	11.75b

TBHQ = 0.01% TBHQ; T1= 0.5% ethanolicextract; T2=1.0% ethanolicextract; T3=1.0% water extract

Table 6. Effect of ziziphus leaves extract on sensory evaluation of sausage

Samples	Color	Aroma	Taste	Tenderness	Overall acceptability
Control	7.77b	7.37a	7.01ab	6.93b	7.65ab
TBHQ	8.09b	7.44a	7.33ab	7.21b	7.65ab
T1	7.44b	7.63a	7.00ab	7.33b	7.73ab
T2	8.44a	7.67a	7.65ab	7.37b	7.95ab
T3	7.49b	8.03a	8.17a	8.83a	8.48a

TBHQ = 0.01% TBHQ; T1= 0.5% ethanolic extract; T2=1.0% ethanolic extract; T3=1.0% water extract

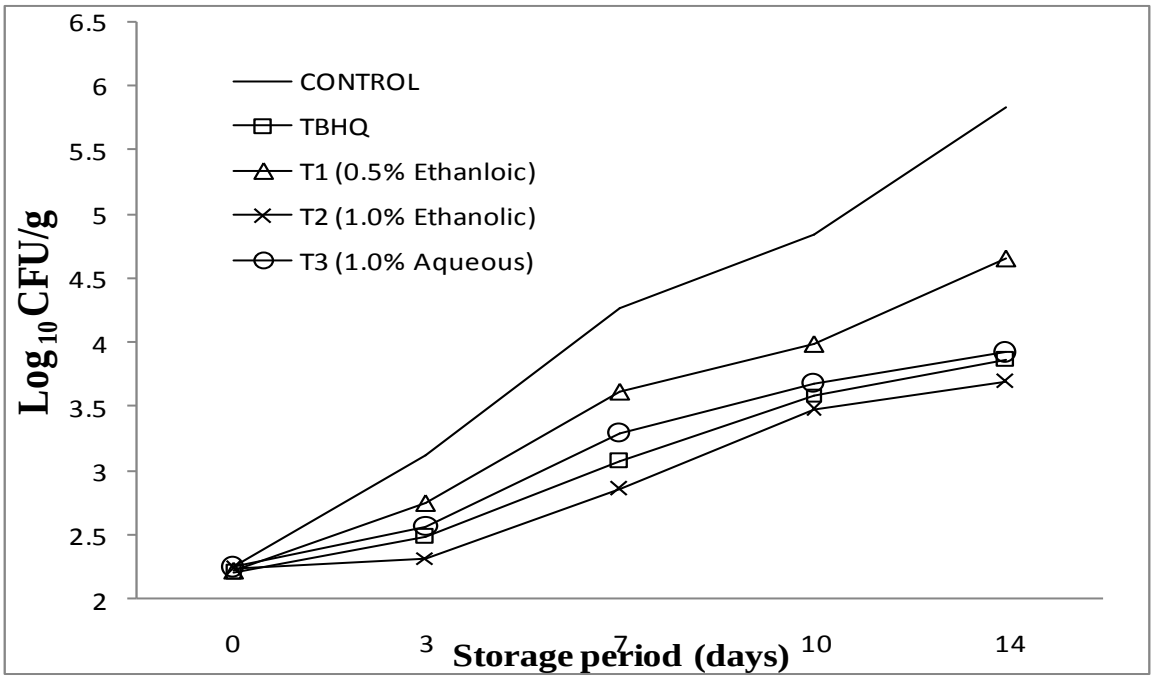

Figure1. Total microbial count of sausage containing ziziphus extracts during cold storage

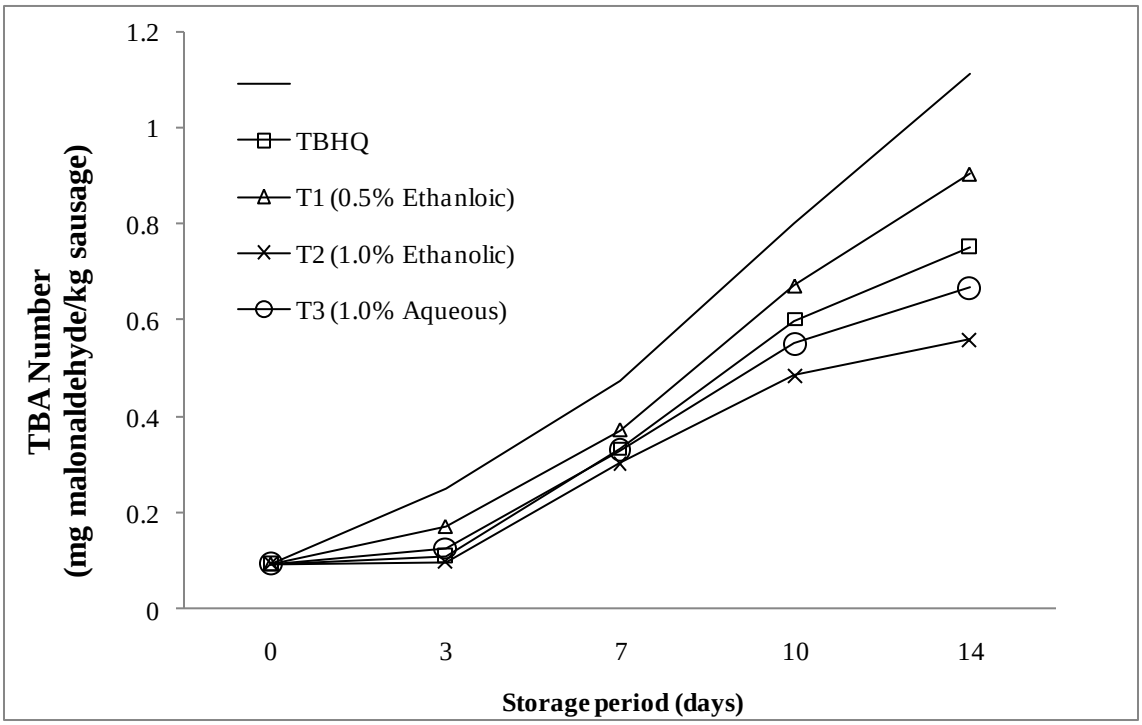

Figure 2. Changes in TBA values during cold storage

and extended the storage life of sausage samples stored at 20° C. These results are in harmony with those found by Valencia *et al*. (2008), who stated that catechins of green tea decreased the total bacterial count of the pork sausages stored at 4 °C.

Changes in TBARS value during cold storage

Off-flavor and off-odor occur in meat and meat products are commonly attributed to the lipid peroxidation. TBARS test is considered as an easy way to monitor lipid oxidation in meat products and it is strongly related to the Sensory attributes of meat products. Maximal acceptable limit of TBARS value in meat products and fatty foods is 1mg.kg^{-1} (1.0 mg malonaldehyde.kg^{-1}) (Ockerman and Kuo, 1982; Wu *et al*. 1991). Changes in TBARS value in beef sausage samples during cold storage are shown in Figure (2). Generally, the lipid oxidation rate, measured as malonaldehyde concentration, increased progressively in all samples during the storage period. However, a slower rate of malonaldehyde production was observed with samples contained ziziphus extract and TBHQ compared to that of control wich led to lower TBA values in the ziziphus added sausage samples comparing to that of control. In the end of storage period, control sample had the highest TBA value (1.12) while sausage sample with 1% ziziphus ethanolic extract had the lowest (0.56). Abalaka, *et al*. (2011) reported that antioxidant from ziziphus leaves was able to inhibit lipid oxidation in sausage during storage period. Pateiro *et al*. (2015) studied the effect of the addition of natural antioxidants (tea, chestnut, grape seed and beer extracts) on shelf-life and oxidative stability of dry-cured sausage. They found that the antioxidant activity of it was equal or even better to those found with BHT. These results is in agreement with that previously found by Amany *et al*. (2012) and Pateiro *et al*. (2014) who reported that lipid oxidation inhibition by natural antioxidant from date pits, green tea, chestnut and grape extract were better or equal to that obtained by synthetic antioxidants.

Color values of sausage during cold storage

Effect of ziziphus leaves extract on color values are shown in Table (5). During the storage period, lightness values in all samples ranged between 34.96 and 41.11 in sausage samples with 0.01% TBHQ and control, respectively. Addition of ziziphus extract significantly decreased lightness compared with control, which might be due to the dark color of the ziziphus extract. Redness values of sausage samples were affected by storage time, and by the added extract. Generally, *a** values were decreased gradually during the storage. Pateiro *et al*., (2015) stated that the color loss of dried sausage could be due to the denaturation of nitrosoglobin by lactic acid produced during ripening time. Also, Wenjiao *et al*. (2014) reported that the decrease in sausage redness may be attributed to the oxidation of nitrosylmyoglobin. In the begaining (day 0), the highest *a** value was recorded with sausage sample with 1% ethanolic extract (11.84) and the lowest was observes with control (7.56). Addition of ziziphus leaves extract maintained the red color of sausage samples, with higher *a** values, during the cold storage compared with control. The addition of ziziphus extract did not effect on *b** values of sausage samples during the storage. Our findings came in accordance with those found by Sebranek *et al*. (2005) and Cao *et al*. (2013).

Sensory evaluation

The effects of ziziphus leaves extract on sensory quality of sausage samples are presented in Table 6. As for color, ziziphus leaves extract enhanced the color of sausage samples compared to control. Samples contained 1% ethanolic extract had the highest color score (8.44), while sausage samples contained 0.5% ethanolic extract had the lowest (7.44). However, ziziphus extract had no significant effect on sausage aroma and taste compared to the control. Jin *et al*. (2015a) stated that the antioxidant extracts from plant origin should be used in a range that does not change the taste of food products. Moarefian *et al*. (2013) stated that addition of *Cinnamomum zeylanicum* essential oil (20 and 40 ppm) did not affect the sensory characteristics of cooked sausages. Also, Muthukumar *et al*. (2014) found that the use of *Moringa oleifera* leaves extract in the range of 600 ppm did not affect the sensory attributes in raw and cooked pork patties. Sausage samples with 1% water extract had the highest tenderness and overall acceptability scores (8.83 and 8.48, significantly) compared with the control. Lipid oxidation in meat products could affect the sensory quality such as flavor, color, taste and texture (Hoffman *et al*. 2014; Jin *et al*. 2015b). Thus, the use of natural antioxidant extracts including ziziphus could prevent these changes and keep the sensory quality of meat products even better than control.

CONCLSUION

Results of this study demonstrate the effect of ziziphus leaves extracts on lipid oxidation, total microbial count and sensory quality of raw beef sausage during refrigerated storage for 14 days at 2°C. Ziziphus leaves contained a considerable amount of phenolic and flavonoid compounds that

provide an antioxidant and antimicrobial benefits to beef sausage during the period of cold storage. Addition of 1% ziziphus leaves ethanolic extract led to retardation of lipid oxidation and lowering the total microbial count in sausage samples in the end of the storage period compared to the control and samples treated with 0.01% TBHQ without significant effect on the sensory quality of the product. Natural antioxidant and antimicrobial extract from ziziphus leaves provides equal or even higher activity than the synthetic preservative (TBHQ) thus, it can be used safely in the preservation of meat products such as sausage.

Conflict of interset

The Authors declare no conflict of interest.

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Evaluation and comparison of Mycotoxin (AFM1) in imported cow milk with Kurdistan milk available in Sulaimani markets by using High performance liquid chromatography

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ABSTRACT

This work determined the quality of milk regarding AFM_1 residue in four types of milk. An highlighted and sensitive method of toxin extraction applied called solid phase extraction (SPE) with using liquid chromatography which was HPLC- fluo-rescence detector (FLD) to determine of aflatoxin M_1 (AFM_1) in pasteurized liquid cow milk and produced fresh raw milk(cow milk). The detection limits of this method were 0.5µg/kg in liquid fresh and pasteurized packaged milk. The rehabilitation achieved in all drink form was between 85.4 and 96.9%, and the RSDs were between 0.8 and 3.6%. In addition, the level of AFM_1 contamination settled in 27 samples from all the evaluated samples; however, AFM_1 residue in contaminated samples were not more than FDA international legal limit (0.5µg/kg) while, AFM_1 in 11 samples were exceed than CR international limits(0.05µg/kg), the rest 33 samples were totally free from AFM_1contamination. OASIS HLB and IAC also used with HPLC to detect AFM1 residue and the optimization of OASIS HLB cartridge were examined to minimize interferences and pre-concentrate analyse. The work totally evaluated three types of milk regarding AFM1 which are available in Sulaimani markets, and a couple of cleaning up technique applied. Finally, all the results statistically analyzed by (Simple *t* test and ANOVA) SPSS-version 18 program.

Keywords: Aflatoxin M1; packaged milk; raw milk; SPE extraction; HPLC

INTRODUCTION

Mycotoxins generally are toxic metabolises produced by fungi growing on a variety of food stuffs including that of animal feeds and affected animals through ingestion contaminated feeds and build-up in the animals circulatory system, liver and other tissues such as adipose and muscle; hence, passes up the food chain(Turner *et al*, 2009). Aflatoxins and Ochratoxins are two common toxins found in milk and the four major AFT are B1, B2, G1, and G2 expected to be in animal products which determined based on their fluorescence and mobility during thin-layer chromatography (Herzallah, 2009). They are heat stable and high in resistance; therefore, threat to human health through ingestion animal products such as milk, cheese, meat and eggs. Ingestion of infected milk or other animal products causes liver damage, cancer, mental impairment, abdominal pain, vomiting, convulsions, pulmonary edema, coma and death. (Sani *et al* 2013) Group of hepatocarcino-genic bisdihydrofurano metabolites are closely related to the aflatoxin and its formed by specific Aspergillus species particularly by specific strains Aspergillus flavus and Aspergillus parasiticus (Richard, 2007). AflatoxinM1 (AFM_1) is a metabolite of aflatoxin hydroxilation which present in the milk products and its reached when cattle consumed contaminated grass and/or ration. Another source is AFB_1, which can provide 0.3- 6.2% of total AFM_1 in milk (Creppy, 2002).

During sterilization, milk-based products, milk storage and pasteurization, the AFM_1 is stable (Dutton *et al*, 2012) whereas consumption of AFM1, even on a small absorption, there is a major threat to community health, particularly for children who are great consumers of milk and its by-products.

Therefore, AFM_1 globally audit and its manage the level of AFM_1 present in milk products. According to the U.S Food and Drug Administration, AFM1 residue milk have no more than 0.5 microgram / kg (Food and Drug Administration, 1996). Indeed, European Union (EU) has settled AFM_1 as 0.05 µg/kg in milk used for adult intake (Commission Regulation, 2001). For the child's milk and its by-product, this amount must be less than 0.025 microgram / kg (Commission Regulation, 2004). In Iraq, there is no specific Iraqi regulation for AFM1 in milk; hence, the EU regulation has been established and applied for authorisation the toxin. For all these reasons, it is crucial to explore the most accurate as well as simple and inexpensive method to determine AFM1 in raw as well as packaged milk. There is many techniques to determine AFM1 in milk such as Enzyme-linked immunosorbent assay (ELISA), which is the existence of a rapid method of screening AFM1, 0.01 microgram/L sensitivity (Kim *et al*., 2000; Rodriguez-Velasco, *et al*, 2003).

Basically the purpose of quantitative analysis including the most popular technique is liquid chromatography (LC), that is typical or reverse phase separation, mainly followed the fluorescence detection. LC mixed with mass spectrometry (MS) also attracted much consideration for its benefits in curing out to identify and determine of analytes at traceelevation (Lee *et al*, 2009). Determining AFM1, a clean-up or enhancement method is generally essential which is Carbograph-4, C18, Multi-functional clean-up column and immune-affinity column (IAC), have desirable purification consequence for AFM1 clean-up of product of milk (Cavaliere *et al*., 2006; Chen, *et al*., 2005 and Manetta *et al*., 2005). Certainly, immune-affinity column is the most classical clean up technique which can select and isolate of analyte from a complex materials; whereas, OASIS HLB sorbent has been established as an adequate method, for polar and non-polar admixture with perfect retention (Fontanals *et al*., 2005). In 20015, Kok-konen's groups extracted AFM1 from cheese, and determined named mycotoxinn in mould cheeses with liquid chromatography tandem mass spectrometry, in which the permit of quantification for AFM_1 was 0.6 µg/kg (Kokkonen, *et al*, 2005). OASIS HLB cartridges have also been used to clean up of mycotoxins in cow milk by HPLC mingled with mass spectrometry. The restoration for AFM1 achieved in this study was more than 80% in the concentration range 0.02-1 µg/L (Sørensen and Elbæk, 2005). In 2011, Beltrán's groups also tried to use OASIS HLB cartridges clean up AFM_1 in cereals in which heavy signal suppression was attended, which make it unattainable to evaluate AFM_1 assuredly; subsequently the matrix effect formed by analogize admixture compounds (Beltrán *etal*., 2011). Although, the usage of OASISHLB cartridge connected with HPLC and FLD, reprivatisation for determination of AFM1 cannot be performed in all types of liquid, and powder milk and still no perfect and highly sensitive method has been improved.

The objective of this work is to determine and evaluate the level of residual AFM_1 in pasteurised imported cow milk with comparing to fresh raw milk are available in Sulaimani markets to confirm that how far they are acceptable in terms of quality and suitability for human consumption regarding the level of AFM1. Moreover, to evaluate the optimization and validation of the OASIS HLB cartridges and IAC clean up technique.

MATERIAL AND METHODS

Collection of Samples

Total of 60 sample of liquid milk were collected in 22 January to 03 February 2015, which involved 15 sample of fresh raw milk and 45 samples of three different types of pasteurised imported packaged milk (Bgah, Ichim, and Nada). All the samples were stored at room temperature before analysis.

Chemicals and Reagents

All chemicals that used in this project were in high performance liquid chromatography grade; chromatographic grade methanol and acetonitrile were purchased from Fisher Scientific (New Jersey, USA). Some other reagents were purchased from Sigma (Sigma–Aldrich, Steinhem, Germany). Immunoaffinity columns were supplied by Vicam (Watertown, MA, USA). OASIS HLB (500 mg, 6 cc) cartridges were achieved from Merck (Darmstadt, Germany).Standard solutions of AFM1 (10 µg/mL in acetonitrile) was purchased from Sigma Chemical (St Louis, MO, USA). AFM1standard were protected from light by putting into amber bottles as AFM1 is relatively light degradation and 0.2-4.0 µg/L working solution was prepared for each injection daily.

High Performance Liquid Chromatography

To follow the (Sørensen, *et al*, 2005) method, the sample extracts are kept at 5–10 ^{o}C until analysis. A volume sample is injected into the chromatographic system and the column flow rate regulated in 600 µl /min and the column temperature kept. The probe voltage also regulated in 4200V in negative ion mode and 5000V in positive ion mode. The SHIMADZU

Prominence HPLC with LC-20AT Solvent Delivery System was equipped with a SIL-20A auto-sampler and RF-10AXL fluorescence detector was applied to separate and determine AFM_1. Isolation was performed on a C18 reverse phase column (250×4.6 mm, ID = 5 µm, Diamonsil®). The mobile phases A and B were acetonitrile and ultrapure water, respectively. Flow rate was held at 1 µL/min and the gradient conditions were as follows: 0-25 min, 25% A; 15-20 min, 30-00% A; 20-30 min, 80% A; 26-28 min, 80-30% A; 28-38 min, 27% A. The temperature of column was controlled at 35 ^{o}C. Injection volume was 50 µL. AFM_1 were detected by FLD and the wavelengths for excitation and emission were 365 and 435 nm, respectively.

Sample pretreatment

By following (Wang *et al*, 2012) method, the procedure was performed as follow. AL All the liquid milk samples were accurately weighed (50 ± 0.1 g) into 100 mL centrifuge glass tube. After centrifugation for 15 min (4000 rpm), fat layer was separated and the supernatant was collected in to 20 mL tube. The supernatant solution (20 mL) was applied to an OASIS HLB cartridge which had previously been conditioned with 5 mL acetonitrile and 5 mL water, successively. The column was washed with 10 mL 20% acetonitrile aqueous solution. The AFM_1 were eluted with 5 mL acetonitrile and the eluate was separated and evaporated to dryness using stream nitrogen. According to (Lee *et al*., 2009) method, the residue was reconstituted with 1.0 mL of 20% aqueous acetonitrile and the achieved solution was forced through a PTFE syringe filter (pore size 0.45 µm). Finally, the cleanup of AFM_1 in milk carried out by using IAC.

RESULTS AND DISCUSSION

Method optimization

On the mode of 250 mm C18 reverse phase column HPLC analysis of AFM_1. Solid phase extraction method was used which includes elution, adsorption, and washing can increase the recovery and the interface, this work compared the optimization of clean up by OASIS HLB and IAC to purify the toxin which are non-expensive method. If these cleanup methods are compared with immunosorbent cartridge which is another common method to clean up AFM1, the cleanup methods are much less in price and so much easier to be applied. In this work, The OASIS HLB cartridge adsorption efficiency initially evaluated from the retrieval values of 10 micro-grams/Liter, and at small flow rate the standard solutions inserted into the cartridge. The outcome presented that the recovery of the AFM_1 was 93.7 ± 1.8%, which indicated that there is no highlighted losses happened during the extraction technique. The admixture of acetonitrile/water at essential wash solvent in different proportions was evaluated to select. A little bit of recovery, when the amount proposed is higher than 30% of acetonitrile. Acetonitrile to keep the maximum amount of analyte retention of about 10% according to the (Beltrán *et al*., 2011). The work presented that the ideal outcome was achieved when concentration of acetonitrile was 30%. Acetonitrile and methanol were tested at various volumes of elution (1, 2, 5, 10 and 15 mL), correspondingly, this is for evaluation of tenor of elution solvent to achieve greater efficiency in the investigation. The primal result was achieved with 5 mL of acetonitrile (91.7 ± 1.8%) and 5 mL of methanol (82.3±2.5%) and the latter was selected as a elution solvent. The acceptable state of extraction based on OASIS HLB cartridges which were showed in (Table1). Corresponding spiked samples which were spiked with 0.050 µg/L or 0.250 µg/kg of AFM1, were determined to check potential interferences in all the blank samples which were include in fresh raw milk (cow milk) and the 3 types of imported milk. All the chromatograms presented in Fig. 1and 2 presented well separated AFM1 peaks and provided desirable optimization condition in both cleanup techniques. The retention time of AFM1 was approximately 8.1 ± 0.2 min. In addition, there is no peak (see arrows in Fig1) in blank samples at the same time; this could be due to the fact that, no components in samples have relatively same retention time as AFM1 to give false positive in four type milk samples. Regarding the cleanup efficacy of OASIS HLB cartridge and IAC columns, there is relatively similar purity peaks were observed in both cartridge, in spite of the fact that tiny differentiation in on the quantitative of AFM_1 been observed.

Method performance

The determination limit of this method is more sensitive than derivative technique of mass spectrometry revealed in previous experiments (Cavaliere *et al*., 2006; Manetta *et al*., 2005). While, Technology presented requires simple laboratory equipment, and detection limit is well below the legal limit Iraq's recent international standards. Hence, this advanced method is applicable for the determination of AFM_1 in imported packaged liquid milk and raw milk. In this experiment, the standard value by following the of 0.10 µg/L to3.00 µg/L was designed and used to detect AFM1 in samples.

Table 1. Optimized parameters for OASIS HLB and IAC

	OASIS HLB	IAC
Adsorption efficiency	93.7%	92.3%
Elution solvent	5 mL acetonitrile	1.5 m acetonitrile methanol (2.2 ± 3, v/v)
Washing solvent	10 mL 30% acetonitrile	10 mL water
Extraction time	21 min	35.0 min

Table 2. Comparison of recoveries in different matrixes cleaned up by IAC or OASIS HLB column (n = 2)

Milk Sample	Spiked (µg/kg)	**Cleaned up by OASIS HLB**		**Cleaned up by IAC**	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Raw milk (Cow milk)	0.010	88.9	4.2	92.6	3.4
	0.150	92.7	3.2	94.8	4.8
Bgah milk	0.100	81.3	4.4	86.4	2.8
	0.025	94.6	2.2	92.5	4.7
Ichim milk	0.250	91.2	3.6	94.6	2.4
	0.050	85.9	2.8	98.4	3.6
Nada milk	0.050	92.5	2.4	90.6	2.6
	0.100	81.6	3.2	88.2	4.1

Table 3. Chromatograms raw data presenting the real AFM_1 in four types of milk samples

Sample type	Raw milk (Cow milk)		*Bgah milk		*Ichim milk		*Nada milk	
	AFM1 µg/kg		**AFM1 µg/kg**		**AFM1 µg/kg**		**AFM1 µg/kg**	
Sample No.	Cleaned up by OASIS HLB	Cleaned up by IAC	Cleaned up by OASIS HLB	Cleaned up by IAC	Cleaned up by OASIS HLB	Cleaned up by IAC	Cleaned up by OASIS HLB	Cleaned up by IAC
1	0.013	0.019	nq	nq	nq	nq	0.008	0.065
2	nq	nq	nq	nq	nq	nq	nq	nq
3	nq	nq	0.093	0.08	nq	nq	nq	nq
4	nq	nq	nq	nq	0.0012	0.0015	nq	nq
5	0.055	0.042	0.011	0.014	nq	nq	nq	nq
6	nq	nq	nq	nq	0.078	0.09	0.098	0.069
7	0.015	0.09	0.03	0.12	nq	nq	0.009	0.003
8	0.013	0.039	0.06	0.044	0.076	0.043	nq	nq
9	0.15	0.01	0.02	0.06	0.0071	0.012	0.0012	0.064
10	0.011	0.014	nq	nq	nq	nq	nq	nq
11	0.014	0.033	0.019	0.034	nq	nq	nq	nq
12	nq	nq	nq	nq	nq	nq	0.016	0.011
13	nq	nq	nq	nq	nq	nq	0.006	0.0017
14	0.012	0.018	0.018	0.0094	nq	nq	nq	nq
15	nq	nq	nq	nq	0.008	0.002	0.0089	0.013

**Bgah, *Ichim,*Nada, are the trade names of 3 types of (Iranian, Turkish and Saudi Arabia) milk respectively*

**nq=non quantified*

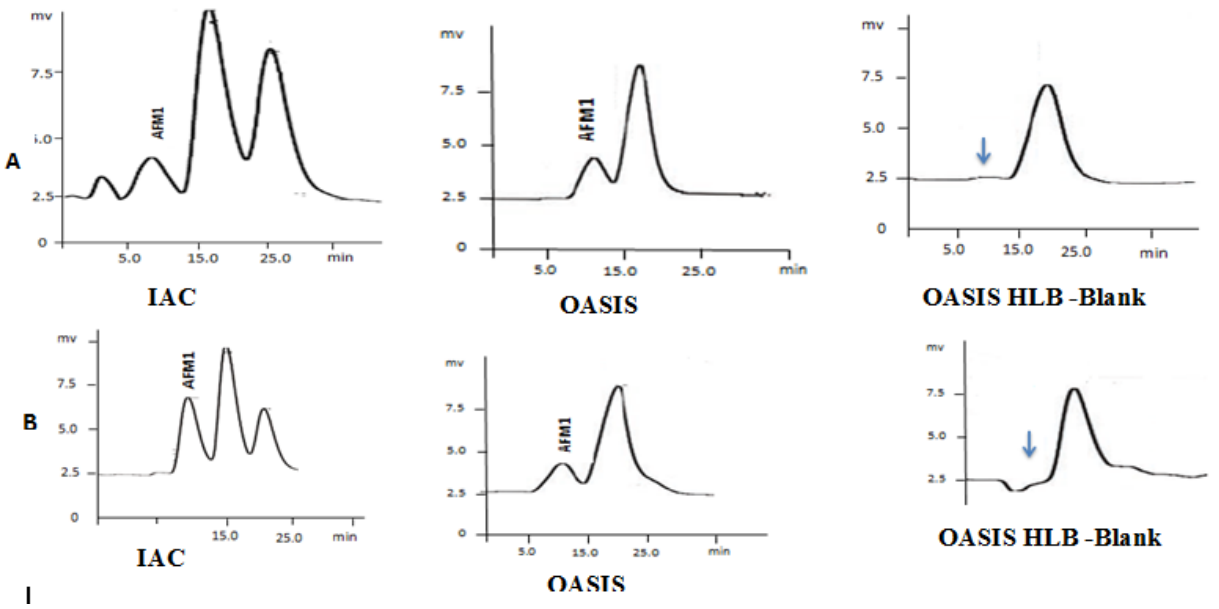

Fig. 1. HPLC-FLD chromatograms for AFM1 in different milk samples cleaned up by IAC and OASIS HLB column: (A) raw milk spiked at 0.050 µg/kg; (B) pasteurized packaged milk (Bgah), spiked at 0.250 µg/kg

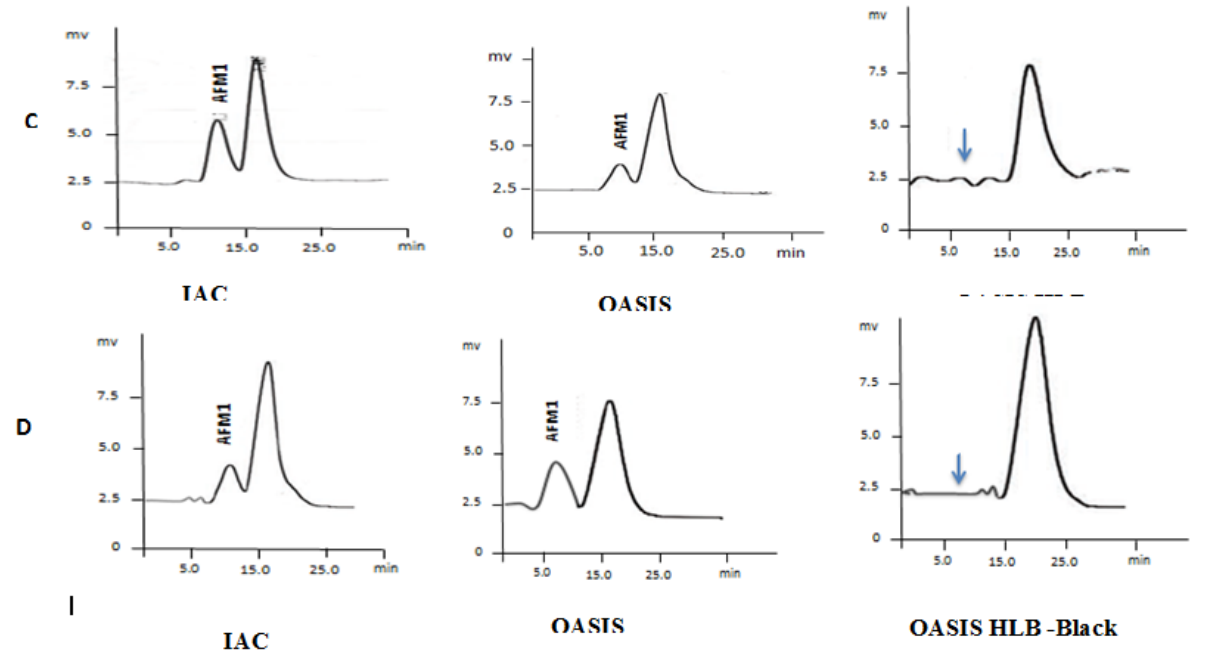

Fig. 2. HPLC-FLD chromatograms for AFM1 in different milk samples cleaned up by IAC and OASIS HLB column: (C) pasteurized packaged milk (Ichim) spiked at 0.250 µg/kg; (D) pasteurized packaged milk (Nada) spiked at 0.250 µg/kg

The linear equation was ("y" = 16,93x - 1332, and" y" = area, "x" = concentration), and the correlation coefficient (R^2) was 0.974. The limits of detection (LOD) of AFM_1 for raw milk and pasteurized milk were 0.050µg/kg and 0.037µg/kg for the limits of quantitation (LOQ). The retrievals and repeatability of Aflatoxin M_1 from packaged pasteurized liquid milk and raw milk and at different levels were attained by (OASIS HLB) column and the detailed data of recoveries conducted which is considered as practical and reliable for detection of AFM_1 from milk. Furthermore, the IAC clean-up column was used as a differentiation to assess the optimization of the method and the results presented that statistically there is no significant difference(p value=0.26). between the two methods.(see table 2).

Application to samples

As it has been shown in table 3, the level of AFM_1 residue fresh raw milk and imported milk was various regarding milk types (fresh and pasteurised packaged milk), also different regarding the milk trade names. The number of positive samples for AFM_1 residue in 15 samples was 8 (53.4%). Furthermore, out of 15 sample of imported milk (pasteurised with ultra-high-temperature (UHT), in Bgah type, 7 (46.6%) were positive, and Ichim was 5 (33.3%) and Nada was also 7(46.6%), respectively. Hence, out of 60 samples of raw and pasteurised milk, there was only 27(45%) of samples contained AFM_1. One more result was that, 53.4%, of raw milk contained AFM_1 residue; whereas, out of 45 pasteurised imported milk samples, only 42% of milk samples contained AFM_1 residue. Moreover, out of all 27 positives, the the values of concentration do not exceed the international law and regulation (0.5micro-g per kilogram) regarding FDA regulation. While, out of all 27 positives, 11 samples contained exceed AFM_1 regarding RC limitation (0.05µg/kg).Fortunately, in Iraq, the FDA regulation is used by ministry of health; hence, the level of contaminationAFM_1 does not seem to be in threatened for public health (see table 3). Finally, both clean-up methods by (IAC) and (OASIS HLB) attained comparatively similar consistent outcomes. Statistically the (t test) results presented that, there is no significant difference between residual AFM1 value in all the milk samples versus to international limit(0.5µg/kg) (p value=0.085).One more interesting (ANOVA) result was that, no significant difference between the level of AFM1 residue in all milk samples cleaned by OASIS HLB versus IAC(p value=0.26).Statistically there was only one significant difference resulted which was between the level of the AFM1 residue between raw milk (Cow milk) and Ichim milk(p value 0.043).

CONCLUSION

High Performance Liquid Chromatography considered a sensitive method to detect components in food and beverages especially if the samples extracted by using one of liquid and solid phase extraction method. In this work, OASIS HLB and IAC used with HPLC to detect AFM1residue in liquid milk and the (OASIS HLB) cartridge were examined optimization to minimize interference and pre-concentration analysis. This performed to reach extreme purification. The technique presented advisable results regarding accuracy, and detected tiny particle of toxin in liquid milk.regarding the results, pasteurised packed milk which is imported in Iran(Pgah),Turkya(Ichim) and Saudi Arabia (Nada) presented safe condition in terms of AFM1 residue. Although, fresh raw milk raw milk in Sulaimani presented AFM_1, the level still would be in safe to public health.

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A treatise on occurrence, regulation and management of aflatoxin M1 in milk

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ABSTRACT

Milk is essential for human health but it is also considered as a source of aflatoxin M1 contamination if lactating animal(s) is fed on the fungus contaminated feed. Milk contamination with AFM1 is more critical as it constitutes an important part of human diet, particularly for the young ones. Aflatoxin M1 belongs to naturally occurring group of mycotoxins and synthesized by toxicogenic filament fungi of specific genera. The causative fungi favor to grow in a wide range of suitable environmental factors and climatic conditions of humidity and temperature. Biosynthesis of AFM1 is a complex multi enzyme involving process which is governed by clusters of genes. AFM1 contamination poses an incredible burden to health care system around the globe being hepatotoxic, carcinogenic and immunosuppressive in its nature and negatively affects both animals and human that outcome in the form of ailments and economic costs. To minimize the risk of exposure numerous regulatory measures have been established worldwide as an official monitoring approach through standardized analytical methods. Although full scale mitigation of AFM1 contamination in milk is difficult to achieve yet there are several strategies that could be implemented as promising lines of future research. However, still no sole strategy has proven a complete solution to this problem.

Keywords: Aflatoxin M1, Milk toxicity, Health, Regulation, Mitigation

INTRODUCTION

Since the recorded history milk has been an important part of human diet. It is a balance diet, being produced by nature, which provides essential nutrients required by the human. Milk is defined as whole fresh, clean, lacteal secretion (produced in mammary glands of mammals) obtained from complete milking of healthy milch animals and is practically free of colostrum. It is a complex colloidal mixture of fat, proteins, minerals, lactose and many other vital components which are present either in suspension or in dissolved form. Adults and children regularly include it in their daily diet (Baskaya *et al.*, 2006). Besides its countless benefits it has also bear the paramount potential for leading aflatoxin (AFM1) to the human's diet. The dairy sector faced lack of checks on quality and contamination of milk with natural toxic compounds i.e. mycotoxins and particularly with AFM1 is the most ignored part of entire system (Jalil *et al.*, 2009). At small scale level, farmers mainly contribute towards total milk production in the country. They do not have adequate

conditions for the storage of feed/forage on their dairy farms due to economic backwardness. The conditions at small scale farms often do not meet the standards and this dilemma leads to the incidence of presence of mycotoxins in the feed which ultimately pass onto the milk as (Garcia *et al.,* 2003). Many previous studies have proved the evidences of likely perilous human acquaintance of AFM1 through milk (Sassahara *et al.,* 2005; Unusan, 2006). As milk consumption is reasonably high by human race, principally among the children, therefore risks of milk contamination with AFM1 are also increasing and constantly posing threats for consumer's health. In developing countries like Pakistan, the milk production contributes a significant share and occupies a distinct position for economic development and to fulfill the domestic needs. Pakistan is blessed with high yielding milch animals; buffalo, cow, sheep, goat and camel are five major species accounts for the abundant milk production in the country (Tipu *et al.,* 2007). Pakistan enjoys the fourth position among milk producing countries of the world behind India, China and USA (Iqbal *et al.,* 2011a). Milk and milk products should not be limited to the local market. They are trade oriented and should compete in the international market. To achieve this goal milk should meet the international standards regarding the presence of all contaminants, especially the AFM1 contaminant. This will certainly enhance the quality of milk and its products as well as ensure the safety of consumer's health. Human are the most likely exposed to this deadly metabolite. Indeed, even low level of AFM1 in milk is of great concern for a large numbers of consumers. Keeping in view the health perils of AFM1 and importance of milk, it is of utmost need of hour to make sure its safety in prospective of AFM1 contamination. In this current review we have debated on the occurrence of AFM1, it toxicity and possible management approaches.

Mycotoxins and aflatoxins

Mycotoxins, a group of naturally occurring toxins, are synthesized by toxicogenic filamentous fungus belonging to specific genera. The causative fungi are capable of growing on wide range of prone crops under suitable climatic conditions of humidity and temperature (Razavilar, 2003). Literally mycotoxin refers to "poison from fungi" (myco = fungus, toxin = poison). Even though more than 300 different kinds of mycotoxins have been documented but as far as human are concerned there are only a few mycotoxins which are of an importance. There are five major genera of fungi responsible for the synthesis of majority of the mycotoxins. They include Aspergillus, Penicillium, Fusarium, Alternaria, and Claviceps (Geisen, 1998; D'Mello et al., 1998). Aspergillus produced mycotoxins are: aflatoxins (B, G and M), sterigmatocystine, ochratoxin A (OTA) and cyclopizonic acid. Penicillium produced mycotoxins are: patulin, citrinin, cyclopiazonic acid, ochratoxin A (OTA) and penitrem A (PA). Fusarium synthesizes: moniliformin, nivalenol, deoxynivalenol (DON), diacetoxyscipenol, T-2 toxin, fumonisins and zearalenone. Alternariol, alternariol methyl ether and tenuazonic acid are synthesized by Alternaria. Ergot alkaloids are produced by Claviceps (Steyn, 1995). Aflatoxins are toxic metabolites of particular group of mycotoxins. They are produced by Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius. If fungi are allowed to grow in an opportunistic way they produce aflatoxins by growing on crops which are poorly managed. Moreover, they also get a chance to produce aflatoxins by appearing on crops during drying and processing and while the commodity is either in storage or in transportation. In 1961, Aspergillus flavus was recognized as the toxin-producing fungus and the toxin was termed as aflatoxin. The word is composed of three words i.e. Aflatoxin (A = Aspergillus flavus, toxin = poison) (Blount, 1961). B aflatoxins are only produced by A. flavus fungus while the other two species (A. parasiticus and A. nomius) can produce both G and B aflatoxins (Ardic et al., 2008; Sidhu et al., 2009). The most toxic aflatoxin is B1which serves as the biosynthetic precursor of AFM1 (hydroxylated metabolite of B1). AFM1 is bio-synthesized as a result of metabolism in animal's rumen and can be assessed in milk of lactating animal which is being fed on AFB1 contaminated feed. Therefore, the M designation is used for the aflatoxins which are present in milk. The B designation of aflatoxin B1 and B2 denotes the capacity to exhibit blue fluorescence under UV-light and the G designation denotes the yellow-green fluorescence of the related aflatoxins under UV-light (Pei *et al.,* 2009; Fallah *et al.,* 2011).

Biosynthesis of AFM1

AFM1 biosynthesis is a complex process. Multi-enzymatic reactions are involved in it. Several specific enzymes with conversions in the aflatoxins pathway have been purified (Yabe et al., 1991a; Bhatnagar et al., 1992). Genes maintained in clusters governed it. With the discovery of the structures of aflatoxins many studied have been made till now to interpret bio-synthetic pathway of aflatoxins synthesis. Studies have evidenced that two stages are involved in the synthesis of aflatoxins from malonyl CoA. The first stage is hexanoyl CoA formation and the second stage is decaketide anthraquinone

formation. Highly well-organized oxidation-reduction reactions governed the sequence of synthesis of aflatoxins (Bhatnagar *et al.,* 1992; Townsend, 1997). The most acceptable scheme of biosynthesis of AFM1 is: hexanoyl Coa precursor → norsolorinic acid → averantin → hydroxyaverantin → versiconal hemiacetal acetate → versiconal → versicolorin B → versicolorin A → demethyl-sterigmatocystin → sterigmatocystin → O-methylsterigmatocystin → aflatoxin B1 and aflatoxins G1. Aflatoxin B2 and aflatoxin G2 are formed from dihydro O-methylsterigmatocystin (Yu *et al.,* 2002). Aflatoxin B1 is broken down in the dairy animal rumen. Minor but important part is re-absorbed and metabolized into AFM1 by a process that causes the conversion of aflatoxin B1 to AFM1 by hydroxylation at the tertiary carbon of the difuran ring system and bio-activated by cytochrome P450 enzyme system in the liver. AFM1 is comparatively stable and excreted in milk, bile or urine after circulation in the blood (Cupid *et al.,* 2004).

Factors affecting AFM1 production

The AFM1 production by toxigenic Aspergilli is effected by a number of environmental factors. In the areas where temperature and humidity is high, the production is accelerated and poses more serious threat to feed and food contamination. 80-85% equilibrium relative humidity, 17% equilibrium moisture contents and temperature ranging between 24-35 ^{o}C are the suitable conditions for the growth of Aspergillus and for consequent production of toxins. For the growth of A. flavus 28-30 °C temperature and 8-10% minimum moisture contents are considered as optimum conditions. In case of some kind of stress in plants the fungus attack and thus in turn the aflatoxins contamination become more severe. This stress may be in the form of extended periods of high temperature, high crop density or competition from weeds, drought (below-normal soil moisture) that weakens the plant system and enhance the damage from insects or birds. As a result of these conditions, the host become weak and offer means of entrance to the spores of fungus to develop a foothold in or on the host (Bennett and Papa, 1988; Höhler, 2000).

AFM1 carry-over in milk

Milk is essential for human health but it is also considered as a source of AFM1 contamination if lactating animal(s) is fed on the fungus contaminated feed. After consumption of aflatoxin B1 via feed, a fraction of it is degraded in dairy animal's rumen. Until its secretion in milk, bile or urine the AFM1 circulates in the blood as it is a stable compound. So, the carry-over of AFM1 in milk is of great importance for investigation. A linear connection has been perceived relating the level of AFM1 in milk and animal's consumption on aflatoxin B1 contaminated feed (Fallah et al., 2011). Nearly 0.3 to 6.2% of AFB1 from the feed is passed on to the milk as AFM1 (Pei et al., 2009). After approximately 12-24 hours of aflatoxin B1 ingestion the AFM1 starts appearing in milk (Rahimi *et al.,* 2010). The concentration of AFM1 in milk reduces to an untraceable level within seventy two hours if the contamination source is withdrawn (Sassahara *et al.,* 2005). Numerous previous studies have been carried out to explore the carry-over of AFB1 from feedstuff to milk as AFM1. The transfer percentage of the consumed AFB1 to AFM1 in milk is wide ranging. Very initially, the transfer percentage was reported to vary from 0.18 to 3.94% (Applebaum et al., 1982; Van Egmond, 1989). Pettersson et al. (1989) and Veldman et al. (1992) reported the highest carry-over of 2.6% and 6.2 % in Sweden and Netherlands, respectively. A large number of surveys and studies have been conducted in various countries to find out incidence of AFM1 contamination in milk due to its detrimental effects on human health. The increasing evidences show incidence of AFM1 contamination in milk. Studies conducted in the last decade to explore the level of AFM1 in milk have been summarized in Table.1.

AFM1 Toxicity and Health Concerns

Incidence of AFM1 in milk is a global apprehension since milk consumption is very high around the globe. Demonstration of possible perilous human acquaintance to AFM1 via consuming contaminated milk had been revealed previously by numerous studies (Sassahara *et al.,* 2005; Unusan, 2006). AFM1 is hepatotoxic, carcinogenic and immunosuppressive fungal metabolites and responsible for harmful effects on animals and human that outcome in the form of ailments and economic costs. It is linked with many ailments. Even in minute concentration it poses a serious threat to human and animals. It is related to the prevalence of many kinds of cancer. This has pulled the international apprehension over the food contamination with AFM1 (Gong *et al.,* 2004). International Agency for Research on Cancer (IARC), in 1987, declared aflatoxins as cancer causing for human (human carcinogens). After re-evaluation in 1992, the classification was confirmed. Later, on the basis of proved toxic and carcinogenic effects of AFM1 the toxin initially classified by IARC as a Group 2B human carcinogen has now moved to Group 1 (IARC, 2002). The carcinogenicity of AFM1 seems to be alike or somewhat less than that of AFB1. It is nearly 2-10% that aflatoxin B1 (Asi *et al.,* 2012).

Table 1: Occurrence of AFM1 in milk and its Contamination rate

Aflatoxin	Samples size	Milk type	Technique	Contamination rate	References
AFM1	40	Pasteurized, UHT half-skimmed	ELIZA	27.5%	Duarte *et al.* (2013)
AFM1	153	UHT milk	ELIZA	54.9%	Zheng *et al.* (2013)
AFM1	107	Raw whole milk	HPLC	71%	Iqbal and Asi (2013)
AFM1	48	Dairy farm milk	LC-FD	27%	Marnissi *et al.* (2012)
AFM1	232	Dairy farm milk	ELIZA	76.3%	Sadia *et al.* (2012)
AFM1	45, 52	UHT, Raw whole milk	HPLC	38%, 62.5%	Siddappa *et al.* (2012)
AFM1	356	Raw whole milk	HPLC	55%	Asi *et al.* (2012)
AFM1	77	Raw whole milk, Pasteurized, Powder milk	ELIZA	73.6%, 68.0%, 35.7%	Assem *et al.* (2011)
AFM1	89	Dairy farm milk	ELIZA	46%	Moghima and Shirzad (2011)
AFM1	311	Raw whole milk	ELIZA	42.1%	Rahimi *et al.* (2010)
AFM1	210	UHT milk	ELIZA	55.2%	Heshmati and Milani (2010)
AFM1	225	Pasteurized, UHT	ELIZA	67.1%, 71.5%	Fallah (2010)
AFM1	169	Dairy farm milk	HPLC	34.5%	Hussain *et al.* (2010b)
AFM1	23,67	Raw whole milk, Processed milk	ELIZA	30.4%, 29.8%	Polovinski-Horvatović *et al.* (2009)
AFM1	50	Pasteurized milk	ELIZA	62%	Ghazani (2009)
AFM1	126	Raw whole milk	ELIZA	80%	Ghanem and Orfi (2009)
AFM1	113	Raw whole milk	ELIZA	42.5%	Nuryono *et al.* (2009)
AFM1	125	Powder, Pasteurized, UHT	HPLC	95.2%	Shundo *et al.* (2009)
AFM1	100	Raw whole milk	HLPC	96.3%	Lee *et al.* (2009)
AFM1	160	Breast milk	ELIZA	98.1%	Sadeghi *et al.* (2009)
AFM1	480	Dairy farm milk	HPLC	52.5%	Hussain *et al.* (2008)
AFM1	319	Raw whole milk	HPLC	54%	Tajkarimi *et al.* (2008)
AFM1	316	Raw whole milk	HPLC	1.6%	Nachtmann *et al.* (2007)
AFM1	186	Raw whole milk	HPLC	63.9%	Ghiasian *et al.* (2007)
AFM1	48	Raw whole milk	HPLC	24%	Ayar *et al.* (2007)
AFM1	128	Pasteurized	ELIZA	78%	Oveisi *et al.* (2007)
AFM1	45	Pasteurized milk	HPLC	88.8%	Zinedine *et al.* (2007)
AFM1	129	UHT whole milk	ELIZA	53%	Unusan (2006)
AFM1	241	Retail store whole milk	HPLC	79.4%	Diaz and Espitia (2006)
AFM1	107	Raw whole milk, Pasteurized, UHT milk	TLC	73.8%	Shundo and Sabino (2006)
AFM1	240	Sheep whole milk	HPLC	81%	Bognanno *et al.* (2006)
AFM1	27	Raw whole milk	HPLC	117.9%	Gürbay *et al.* (2006)
AFM1	75	Raw whole milk	ELIZA	88.23%	Çelik *et al.* (2005)
AFM1	111	Raw whole milk	ELIZA	73.1%	Kamkar (2005)
AFM1	21	Skim milk powder	HPLC	9.5%	Deveci and Sezgin (2005)
AFM1	115	Raw cow milk	ELIZA	48%	Oruc *et al.* (2005)

Table 2. Maximum residual limits (MRLs) of AFM1 in milk in various countries

Aflaoxin	Country	MRL (ng/kg)	Food(s)	References
AFM1	USA	0.050	Milk	Creppy (2002); Dashti *et al.* (2009)
	France	0.30, 0.5	Children milk, adult milk	
	Belgium	0.050	Milk	
	Switzerland	0.050, 0.025	Milk, milk products	
	Sweden	0.050	Milk	
	Austria	0.050	Milk	
	Germany	0.050	Milk	
	Netherlands	0.020, 0.200	Butter, cheese	
	Czech Republic	0.1, 0.5	Children milk, adult milk	
	Turkey	0.05	Milk and milk products, cheese	
	Syria	200	Milk	
	Italy	0.250	Milk	
	Honduras	.0.250	Milk	

Human are the most likely exposed to this (AFM1) deadly metabolite. Indeed, a very low level of AFM1 in milk is a great concern for a great number of consumers (González-Osnaya *et al.*, 2008). Hence, for its reduction and elimination in milk, it is necessary to develop monitoring and regulating strategies.

AFM1 Economic Impact and Regulations

The worldwide contamination of foods and feeds with aflatoxins is a noteworthy problem. There are diverse criteria to assess the economic impacts of aflatoxins on both human and animal health. The key considerations include: loss of human and animal life, intensive burden on health care system, loss of livestock production and veterinary care costs, loss of forage crops and feeds, regulatory cost and above all is the cost of research focusing on finding the solution(s) to lessen the severity of aflatoxins associated malicious problems. It is difficult to develop the formulas that can measure up the worldwide economic impacts of aflatoxins. Hence, most of the reports on economic impact are based of single criteria of aflatoxin exposure or contamination (Hussein and Brasel, 2001). Although it is difficult to remove AFM1 from human and animal diets, it is possible to decrease the risk of exposure through the establishment of regulatory limits and official monitoring plans to control the compliance of commodities with regulations through standardized analytical methods. Considering the health risks associated with AFM1, many countries have established legal limits for maximum residue limits (MRLs) of AFM1 in milk and milk products (Table 2.) In order to decrease the menaces, firm monitoring limits are currently implicated. 0.050 µg L-1 is the maximum residual limit (MRL) of AFM1 in milk which has been established by European Commission (EC, 2010).The rationale for the establishment of specific regulations in each country varies widely, depending on factors such as results of risk analysis (toxicological data and information on susceptible commodities), analytical capabilities (sampling and detection limits), and socio-political issues (adequacy of food supply, economic condition of a country, trade requirements) (Giovati *et al.*, 2015). Efforts should be made in attempting to gain further and extensive knowledge on human health hazards related to long term exposure to low level AFM1, providing scientific basis to standardize the already existing regulatory limits and to implement policies to reduce contamination in low resource countries. Maximum residual limits (MRLs) of AFM1 in milk in various countries have been summarized in (Table 2).

AFM1 Mitigation in Milk

To minimize risks associated with exposure to AFM1, regulation and monitoring measures must be supported by in field (pre-harvest) and storage (post-harvest) interventions which may be applied to minimize the contamination. AFM1 is excreted in milk of dairy animals following metabolism of AFB1 ingested with feed. Thus, contamination of milk maybe reduced either directly, decreasing AFM1 content of contaminated milk, or indirectly, decreasing AFB1 contamination in feed of dairy animals. Many methodologies have been developed to reduce AFM1 contamination with both direct and indirect approaches have been extensively reviewed (Jard *et al.*, 2011). Beyond ongoing research to improve efficiency, safety, and reliability of these interventions, there is a growing interest in developing environmental friendly, cost-effective, and specific alternatives for AFM1 mitigation. Highly promising technologies proposed for this scope exploit microorganisms, purified microbial

enzymes, dietary clay minerals and specific antibodies induced by vaccination.

CONCLUSION

The reduction of AFB1 in the cattle feed is the best way to control the level of AFM1 in milk. Good manufacturing practices and good storage conditions proved to be helpful in reduction of AFB1 concentration in feed and ultimately the lower level of AFM1 in milk. Apparently fungus attacked feedstuffs and long standing storage commodities should be discouraged and fresh feedstuffs for milking animals should be used because during storage fungal growth and mycotoxins contamination is suspected. The farmers and the farm owners should be made aware of the hazardous effects of AFM1 contamination. This could be possible by following the integrated management programme. Organizing seminars, symposia and launching a campaign on print and electronic media can educate the people about the menace of AFM1 contamination of milk and its consequent health hazards.

Conflict of interest

The authors declare no conflict of interest.

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Bread fermentation using synergistic activity between lactic acid bacteria (*lactobacillus bulgaricus*) and baker's yeast (*sacchromyces cerevisae*)

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ABSTRACT

Lactic acid bacteria (*Lactobacillus bulgaricus*) and Baker's yeast (*Saccharomyces cerevisiae*), independently and in combination were used in the fermentation of bread. Their effects on the shelf life and on the sensory characteristics were determined. Three treatments were used for the research, they include; dough fermented with yeast starter (T1), dough fermented with only lactic acid starter (T2) and dough fermented with both lactic acid and yeast starters (T3). The results showed that T2 had longer shelf life due to the presence of the lactic acid produced by *Lactobacillus bulgaricus* during its fermentation process. It was also recorded as the highest when tested for the sensory characteristics like aroma and taste. T3 which was fermented with both lactic acid bacteria and yeast had extended shelf life and its sensory properties were also enhanced due to the presence of organic acids, amino acids and a group of group B vitamins being produced by the lactic acid starter. It also had T1 which was fermented with just yeast (*Saccharomyces cerevisiae*) was observed to have the shortest shelf life. T1 also scored least when tested for its sensory properties. After all three samples (T1, T2 and T3) were generally examined, it was observed that T3 which was fermented with both yeast and lactic acid bacteria starter cultures was regarded as the most generally acceptable amongst all three treatments. Spoilage organisms which include *Aspergillus, Rhizopus, Penicillium, Mucor* and *Bacillus species* which causes ropiness in bread. The results showed that synergistic activity between lactic acid bacteria (*Lactobacillus bulgaricus*) and baker's yeast (*Saccharomyces cerevisiae*) improved the sensory properties of bread and also extended its shelf life.

Keywords: Fermentation, lactic acid bacteria, baker's yeast, sensory characteristics, shelf life.

INTRODUCTION

Bread has been considered as a staple food for humans for thousands of years. Its exceptional nutritional value is suggested by its major constituents: carbohydrates, proteins, vitamins, minerals, fats and water all of which are substrates for microorganisms (Mohammed *et al*., 2003). As a solid, bread is "soft", and like many other foodstuffs, at macroscopic level is comprised of 2 stages: a solid (cell wall material) and a fluid (air) (Campbell & Mpugeot, 1999). The solid structural matrix consists of polymers, which are completing amorphous (gluten) or partially crystalline (starch) (Peleg, 1986). The constituents that are used to make basic formula dough are: flour, water, leavening agent, and salt. To change these mix constituents into finishing aerated structure, a number of processing operations are performed (Davidson, 1999). Many types of bread exist and they include brown bread, white bread, wheat germ, whole meal bread, roti, bread, rye, unleavened bread, sourdough bread, flat bread, crisp bread, hemp bread, quick bread and gluten-free bread (Davidson, 1999).

Dough is mainly leavened by using yeast that can ferment sugar and produces carbon dioxide and alcohol that can affect the taste and quality of baked products. Nevertheless, other gas generating microorganisms e.g., coliform bacteria, wild yeasts, *Clostridium species*, Saccharolytic, hetero-fermentative lactic acid bacteria and its naturally present combinations used for leavening of dough rather than bread yeast only (Vollmer and Meuser, 1992; Bratovanova, 1996). In the fermentation of bread, yeasts have proven to be very useful because they contribute to its aroma, taste and leavening. The yeast that is basically employed in the bread fermentation is known as *Saccharomyces cerevisiae* which is also stated to as baker's yeast. Fermentable sugars which is present in the dough changes into carbon dioxide and ethanol as the chief products. Enzymes which are made by the yeast cells containing maltase, invertase and zymase complex which played a role as natural catalysts in bread fermentation process (Madigan *et al*., 2003).

Lactic Acid Bacteria produces many organic acids such as propionic, lactic and acetic acids as end

product of fermentation which give acidic environment unfavorable condition for the development of many spoilage and pathogenic microorganisms. These acids apply their antimicrobial effect by maintenance the cell membrane potential, preventing active transport, decreasing intracellular pH and preventing a variety of metabolic roles (Ross *et al.*, 2002). They prevent both gram-negative and gram-positive bacteria along with molds and yeast. Fungal development is the often cause of spoilage in bakery products mainly caused by *Penicillium*, *Aspergillus* and *Fusarium* genera.

The final characteristic of bread, influenced by the synergestic activity of microorganism which produce acidification and souring particularly the texture (Corsetti *et al*., 2000) and generate typical flavor compounds yielding typical sourdough sensoryattributes (Katina *et al*., 2006). Sour dough bread flavor compounds can be changed, because the results of the lactic acid bacteria fermented components and ingredients choice (Schieberle and Grosch, 1992; Schieberle and Grosch, 1994). Furthermore, lactic acid bacteria by preventing the growth of pathogenic microorganisms or by removing pollutants or toxic chemicals that can help to produce safer food.

MATERIALS AND METHODS

Collection of starter cultures

Isolates of *Lactobacillus bulgaricus* were gotten from the University of Calabar Culture collection center, Calabar, Nigeria. While commercial industrial yeast (*Saccharomyces cerevisiae*) was gotten from Watt market in Calabar, Nigeria.

Sample preparation

Bread dough was prepared using lactic acid bacteria and yeast as starter cultures. In the preparation of the first sample, using just yeast as starter culture, 3 table spoons of industrial yeast and 3 table spoons of sugar were dissolved in warm water and allowed to froth. This solution was then kneaded with 2 cups of flour and then 5 tea spoons of oil was added and kneaded along. The kneaded dough was then allowed to ferment for about 3 hours at room temperature ($28^oC\pm2$). The fermented dough was then placed in a greased aluminum pan and baking was carried out at a temperature of 220^oC for about 15 minutes using a gas heated oven. The bread was then cooled at a room temperature for a period of 90 minutes before it was analyzed.

In the preparation of the second treatment sample, using just lactic acid bacteria (*Lactobacillus bulgaricus*) as starter, all ingredients were mixed to produce dough and then kneaded. During the kneading process, 3mls of *Lactobacillus bulgaricus* inoculum was introduced and then placed in aluminum pan and allowed to ferment for 3 hours at room temperature ($28^oC\pm2$). The third sample was prepared using lactic acid bacteria and yeast as starter cultures. The yeast and sugar were dissolved in warm water and allowed to froth for about 20 minutes and then all ingredients were added and kneaded together with the flour. 2ml lab inoculum was introduced in the kneading process and then allowed to ferment for 3 hours at room temperature ($28^oC\pm2$). The fermented dough was then placed in a greased aluminum pan and baking was carried out in a kitchen gas heated oven at a temperature of 220^oC for 15 minutes. Then the samples were cooled at a room temperature for a period of 90 minutes before analysis.

Sensory analysis

Sensory evaluations of bread samples were carried out and the parameters used include taste, color, texture and overall acceptability. And this was determined by a panel of judges.

Proximate analysis

This was carried out on the loaves on dry matter basis according to conventional method of A.O.A.C. (1980). Moisture, ash, protein, fat, fiber and carbohydrate contents were determined.

Shelf life determination

The bread produced was packed in the conventional transparent polyethylene bags used in bakeries and stored at room temperature (28±2°C). The bread was observed daily through the transparent polythene bags to determine when signs of spoilage would occur. At the end of the incubation period the colonies were enumerated and expressed as colony forming units per gram (Vanderzannt and Splittstoesser, 1992).

Microbial identification

Total bacterial and mold colony counts and identification of molds were carried out at 0, 24, 48, 72, 96 hours storage intervals (Cappuccino and

Sherman, 1996).1 gram of bread sample was properly mixed in 9ml sterile peptone water. Tenfold serial dilution was subsequently done using sterile peptone water as diluents. 0.1 ml aliquots were pour plated on Nutrient Agar and incubated at 37^{o}C for 24 hours while 1ml from each sample were aseptically plated using the spread plate technique on Malt Extract Agar (MEA) containing0.05g/ml chloramphenicol and the MEA plates were incubated at 30^{o}C for about 48-72hrs. Colonies were counted and expressed as cfu/g of samples. Same procedure was used at 0, 24, 48, 72 and 96 hours storage intervals for the determination of microbial load on each storage day. Also, observed colonies were sub cultured repeatedly on media used for primary isolation to obtain pure cultures.

Characterization and identification of isolates

The bacterial isolates were characterized using gram reactions and biochemical tests and were identified by comparing their characteristics with those of known taxa. Fungal isolates were characterized based on macroscopic and microscopic examination.

RESULTS AND DISCUSSION

Table 1 shows the different treatments and their compositions. Treatment T1 was composed of yeast starter culture, treatment T2 was composed of Lactic acid bacteria starter culture while treatment T3 was composed of yeast and starter cultures of lactic acid bacteria. Table 2 shows the effects of yeast and lactic acid bacteria starter cultures in the sensory properties of bread.

Proximate composition of various processing methods of bread

The proximate composition of the three (3) samples was carried out 90 minutes after bread production so as to avoid alterations in results. The result showing the proximate composition is shown in table 3.

Bacteria/ mold Count

Bacterial count of samples at different storage intervals was carried out and the results presented in table 4 below. Table 5 further shows the number of total mold counts at different storage intervals. Figure 1 showed the percentage occurrence of fungal isolates in the research work. From the figure 1, *Mucor sp*. had the highest percentage occurrence (40%) while Penicillium had the lowest percentage occurrence (15%). Figure 2 showed the bacterial isolates from the work, Bacillus had the highest percentage occurrence (81%) while Lactobacillus had a low percentage occurrence (19%).

Generally, lactic acid bacteria are widely used in food industries due to their ability to display numerous anti-microbial activities in fermented foods. Lactic acid bacteria are used as a co-culture or as a starter culture in the bread making industry in order to increase their organoleptic properties and also enhance their shelf life. Bread nutritional value is increased due to the production of a variety of group B vitamins, organic acids and amino acids. The anti-fungal compounds formed by lactic acid bacteria which are very important to the food industry for either reducing or replacing the usage of chemical preservatives. In other words, lactic acid bacteria act as a preservative in bread.

Table 2 shows the result of the proximate analysis being carried out among the three samples. T1, which was made with just yeast (*Saccharomyces cerevisiae*) as the starter culture, contained the highest amount of crude fat which was about 17.0±0.1, it also had a high amount of carbohydrate at 53.38±0.02 but was recorded 46 to have the lowest ash content which was at 1.12±0.1. T2 which was fermented with just lactic acid bacteria had the highest amount of moisture content which was about 31.50±0.1, it also had the highest amount of ash and fiber content which were 6.52±0.2 and 1.64±0.1 respectively, but was recorded to be the lowest in carbohydrate content. Then T3 which was fermented with both yeast and lactic acid bacteria was recorded to have the highest protein content which was at about 1.15±0.01. The results obtained from the sensory test are represented in table 3. The samples prepared differently were evaluated both internally and externally for their sensory characteristics which included volume, crust color, symmetry of form, the evenness of bake, the character of crust, grain of bread, color of crumb, aroma, taste, texture and general acceptability. All these were affected by various treatments this is in line with results obtained by Corsetti *et al*., 2001.

The results also showed that treatments which contained lactic acid bacteria had higher scores for sensory evaluation than T1 which did not contain LAB. This result is in line with earlier findings of Salim *et al*., (2007). They found out that the sensory properties of bread were improved when fermented with lactic acid bacteria.

Table 1. Showing the different treatments and their compositions

Treatments	Compositions
T_1	Yeast starter culture
T_2	Lactic acid bacteria starter culture
T_3	Yeast and lactic acid bacteria starter cultures

Table 2. Effects of lactic acid bacteria and yeast starter cultures in the sensory properties of bread

Characteristics (score)	T1 (Yeast)	T2 (LAB)	T3 (Yeast+LAB)
Volume (10)	8.0	6.0	7.5
Crust colour (5)	3.5	3.0	3.8
Symmetry of form (5)	3.5	3.0	4.0
Eveness of bake (5)	2.8	2.4	2.6
Character of crust (5)	4.2	4.0	4.5
Grain of bread (10)	8.5	8.0	8.5
Colour of crumb(10)	8.0	9.0	8.8
Aroma (10)	8.0	9.5	8.5
Taste (15)	11.8	13.5	12.5
Texture (15)	11.8	10.0	13.0
General acceptability(10)	8.7	8.0	9.0

Table 3. Proximate composition of various processing methods of bread (g/100g dry matter)

Treatments	Moisture	Ash	Protein	Fat	Fibre	Carbohydrate
T_1	18.52±0.02	1.12±0.1	8.74±0.01	16.70±0.1	1.54±0.02	53.38±0.02
T_2	31.50s±0.2	6.52±0.02	9.62±0.02	13.20±0.1	1.64±0.01	37.52±0.02
T_3	22.67±0.2	3.40±0.1	10.40±0.1	12.20±0.1	1.15±0.01	50.18±0.02

Each value represents the mean of 3 determinants ±SD

Table 4. Bacterial count of samples at different storage intervals

Treatments	0 hour	24 hours	48 hours	72 hours	96 hours
T_1	$7x10^1$	$9x10^1$	$1.2x10^2$	$1.9x10^2$	$2.7x10^2$
T_2	-	$4.2x10^1$	$5.7x10^1$	$8.9x10^1$	$1.9x10^2$
T_3	$3.2x10^1$	$7x10^1$	$9.1x10^1$	$1.5x10^2$	$2.1x10^2$

Table 5. Total mold counts at different storage intervals

Treatments	0 hour	24 hours	48 hours	72 hours	96 hours
T_1	$1.3x10^2$	$1.5x10^2$	$1.9x10^2$	$2.4x10^2$	$2.8x10^2$
T_2	$7.5x10^1$	$8.3x10^1$	$1.1x10^2$	$2.2x10^2$	$2.3x10^2$
T_3	$7.8x10^1$	$9.7x10^1$	$1.6x10^2$	$1.9x10^2$	$2.1x10^2$

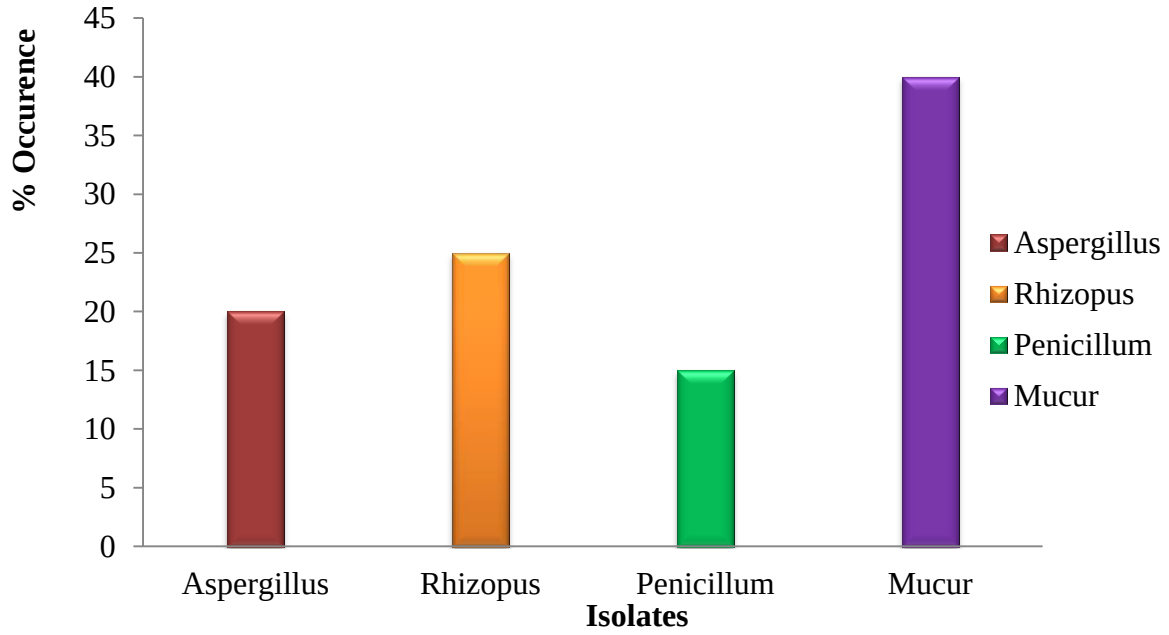

Figure 1. Percentage occurrence of fungi genera

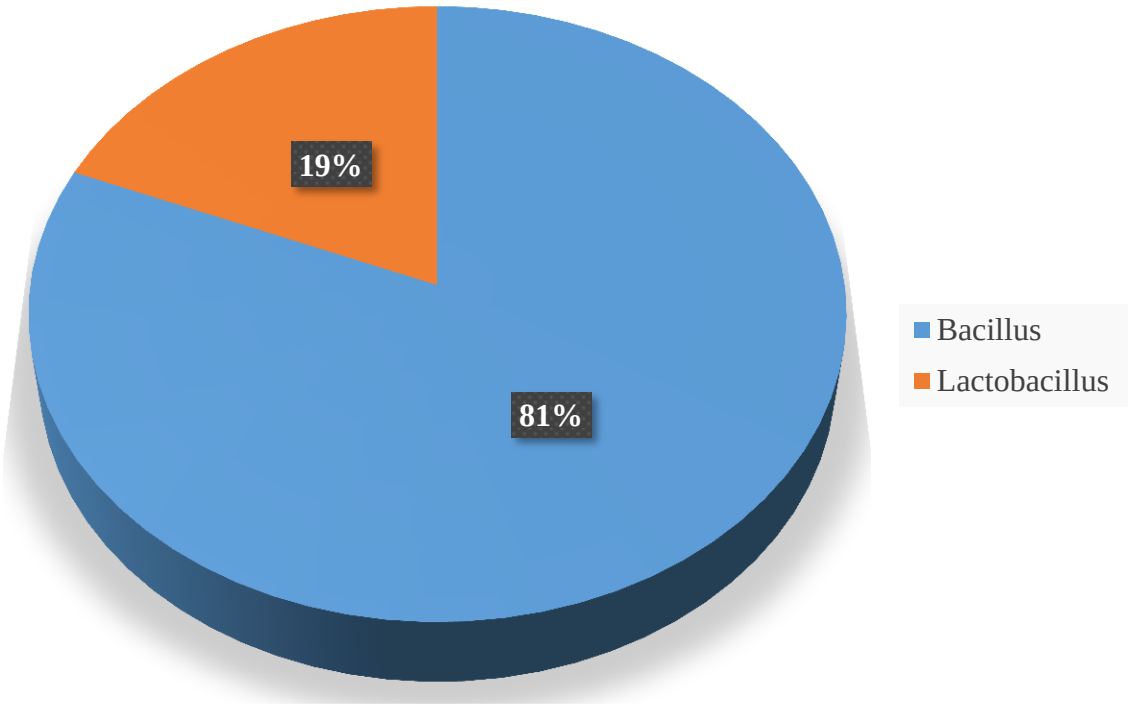

Figure 2. Percentage occurrence of bacteria isolates

The data on the bacterial colony counts in the samples at different storage intervals are provided in table 4. Minimum numbers of bacterial colonies were observed in T1 and minimum in T3. T2 proved to the best in the inhibition of bacterial bread spoilage organisms as no records of colonies were made. After 24 hours, colonies appeared on T2 but it was lowest when compared to other treatments. Bacterial colonies appeared on all samples at 24, 48, 72 and 96 hours storage. This result showed that starter culture of lactic acid (*Lactobacillus bulgaricus*) was much active against the bacterial growth on bread as recorded in the work of Mente *et al*., (2007). Also, on bread mold colony count noticed at different intervals of storage. Maximum numbers of mold colonies were recorded in T1 which was fermented with only yeast starter. T2 which was fermented with only lactic acid starter culture was recorded as the lowest with a total of $7.5x10^{1}$ on the 0 hour. T2 proved to be the most effective in the inhibition of mold growth followed by T3 which was fermented with both yeast and lactic acid starters. This result shows that lactic acid bacteria (*Lactobacillus bulgaricus*) have the ability to inhibit both bacterial and fungal growth when fermented with bread.

The results showed that lactic acid bacteria had the ability to improve the taste, aroma, texture and other sensory characteristics and also, prolong the life of bread by retarding the molds growth. This anti-fungal process is due to their ability to produce organic acids during fermentation (Rocken and Voysey 1995). The results of Martinez *et al*., (1990) also show that the lactic acid bacteria in bread making is useful to improve its organoleptic properties along with avoiding spoilage. Morphological, microscopic and biochemical examination showed that all the bacterial isolated obtained were *Bacillus species* after due comparison of the results obtained with guidelines in Bergey's Manual of Determinative Bacteriology (1994). The mold isolates obtained were subjected to microscopic and macroscopic examination and were found to belong to the following Genus; *Rhizopus*, *Aspergillus*, *Penicillium* and *Mucor* species.

CONCLUSION

The results of this study demonstrate the influence of lactic acid bacteria and yeast starter cultures on bread fermentation. *Lactobacillus bulgaricus* was able to extend the life of bread. The physical properties of bread such as volume, symmetry of form, colour of crumb etc., were assessed objectively were affected by the lactic acid bacteria. However, the key differences between yeast breads and breads fermented with lactic acid bacteria were observed in the sensory characteristics which include the enhancement of the nutritional and sensory qualities and extension of shelf life.

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Estimation of capsaicin in different chilli varieties using different Extraction Techniques and HPLC method: A Review

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ABSTRACT

Capsicum is the major compound found in hot pepper chilies and is responsible for the hotness and pungency of the fruit. It imparts aroma, color and hotness in foods and has wide applications in food and pharmaceutical industries as capsaicin having a number of benefits besides adding flavor to foods. The human body is equipped with capsaicin sensitive receptors TRPV1 present in the neurons which respond accordingly to the body environment when administered chemically. Due to high potential applications of capsaicin, it is extracted using different techniques using organic solvents like methanol, ethanol and acetonitrile prior to its qualitative and quantitative analysis by HPLC (High performance liquid chromatography) which estimates sample based on its retention time. HPLC is considered as the most steadfast and rapid technique for the identification and quantification of capsaicin in various chilly varieties.

Key words: Capsaicin, Extraction techniques, HPLC, Chilli

INTRODUCTION

Capsaicinoids are the compounds that are responsible for the pungency, aroma and flavor of the hot chili peppers. Capsaicin is the most abundant capsaicinoid found in chili peppers. There are many varieties of capsaicinoids like nordihydrocapsaicin, hydrocapsaicin, homocapsaicin, nornorcapsaicin, nornornorcapsaicin, nonivamide (Barbero *et al.*, 2006). 90% of the total capsaicinoids are capsaicin and dihydrocapsaicin and capsaicin makes about 71% in total (Ravishankar *et al*., 2003; Losuge and Furuta, 1970). The commercial quality of hot peppers is solely determined by amount of capsaicin (hotness) present in them (Jarret *et al*., 2007). There concentrations in different capsaicin fruits is regulated by factors such as light intensity, age of fruit and plant's growing temperature.

Capsaicin is the compound found in fruits of plants belonging from the genus Capsicumand family Solanacae. These fruits are generally called peppers. Most of the species belonging to Capsicum genera contain capsaicin such as C. anum, C. frutescens, C. chinence. It is a compound of no flavor and is accumulated in veins of capsicum fruits. Capsaicin is located in vesicle like structures present in epidermal cells of placenta in the pod (Cheema and Pant, 2011). The levels of capsaicin in tip and ovaries are high and in seeds its concentration is low (Supalkavo *et al*., 2007). 90% of the capsaicinoids are produced in placenta portion. Seeds are not source of capsaicinoids but absorb them from placenta (Andrews, 1984).

For centuries capsaicin was used unknowingly in the form of chili peppers in foods in order to enhance their taste, aroma, color and hotness (Goodwin and hertwig, 2003). Besides it was used in food industry, capsaicin has found its application in pharmaceutical industry as well providing many health benefits and treatment strategies for medical conditions. Capsaicin is also known to be active against neurogenic inflammation which causes burning and stinging sensation in hands mouth and eyes (Szolcsanyi, 2004). These properties make capsaicin an active ingredient in different pepper sprays (Kempaiah *et al*., 2005; Reilly et al., 2001; Spicer and Almirall, 2005).

Health Benefits and Clinical Uses

As Analgesic/ Anti-inflammatory

Capsaicin has been known to help in reducing pain (analgesic property) as well as in reducing

inflammatory heat (anti-inflammatory property) like bladder inflammation (Fraenkel *et al*., 2004). It also provides pain relief from arthritis (Kaale *et al*., 2002) and fibromyalgia. Capsaicin is also used in some topical ointments sold over the counter as pain reliever cream (Knotkova *et al*., 2008; Kaale *et al*., 2004) for chronic pain syndromesneurolgia, diabetic neuropathy, musculoskeletal pain and osteoarthritis (Backonja *et al*., 2010; Tesfaye, 2009; Fusci and Giacovazzo, 1997).

Anticancer Activity

Capsaicin has been recognized as anti-cancer agent for many years (Surh, 2002). In an experiment capsaicin was seen to hinder the movement of breast cancer cells and to retard growth of prostate cancer cells. Also, in this experiment, dihydrocapsaicin was reported to kill HCT116 human cancer cells (Oh *et al*., 2008; Thoennissen et al., 2010; Yang et al., 2010). Another study showed that leukemic cell's growth can be retarded by natural capsaicin (Ito *et al*., 2004). Capsaicin attacks the abnormally dividing cells and hinders the proliferation of these malignant cells by cycle arrest, apoptosis, autophagy or through deactivating the cellular metabolism (Choi *et al*., 2010; Ghosh and Basu, 2010 and Thoennissen *et al*., 2010). Isoform of enzyme cytochrome P450 is inhibited by capsaicin. This enzyme detoxifies many low molecular weight carcinogens (Singh *et al*., 2001). Capsaicin identifies malignant cell lines and specifically attaches the immortal dividing cancerous cells and retards their growth (Kim and Moon, 2004). Studies show that cancer mutation in the DNA can be caused by the activity of metabolites of capsaicin (Baez *et al*., 2010).

Weight Management

Since the weight of the body is dependent on the energy metabolism and the heat produced by it, chili peppers are considered as one of the major weight reducing food ingredients (Cui and Himms-Hagen, 1992; Joo *et al*., 2010; Leung, 2008). Clinical studies and animal experimentation reveal that capsaicin cuts down the weight by increasing temperature of the body which causes consumption of calories (Reinbach *et al*., 2009; Shin and Moritani, 2010). Capsaicin notably alters the proteins which are involved in thermogenesis and lipid metabolism, which shows that the target of capsaicin is energy metabolism. Capsaicin can also have side effects which lessens its medical use to reduce weight (Joo *et al*., 2010). CH19 sweet pepper is a non-pungent type which can reduce weight and oxidize fat upon thematic inkate (Reinbach *et al*., 2009).

Benefits to Gastric Pathway

Gastrointestinal system is equipped with numerous sensory nerves which are capsaicin sensitive. They serve to maintain the mucosal integrity of the gastrointestinal tract (Arora *et al*., 2011). Research in this regarded has shown that capsaicin can exert both beneficial and harmful effects which depend upon the usage during treatment gastrointestinal mucous. Studies show that high dosage of Capsaicin can capsaicin sensitive nerves (Arora *et al*., 2011). Low dosage of capsaicinoids in rat's stomach has shown to protect against ethanol ulceration (Holzer and Lippie, 1988). On the other hand, administration of high dosage in the same case had worsened the condition (Szolcsanyi, 1990).

TRPVI or the transient receptor potential vanilloid 1 are the receptors which are expressed on sensory neurons (Sterner and Szallasi, 1999; Szallasi and Blumberg, 1996; Nunn and Qian, 2010). These are the receptors specific for capsaicin binding and are located in the small fibres of nociseptic neurons (Arora *et al*., 2011) known as the C-fibre. These C-fibre contain substance P which stimulates different physiological responses such as inflammation, pain, thermoregulation, burning sensation, irritation, lacrimation (Bevan and Szolcsanyi, 1990). These receptors are present throughout the body in locations like brain, kidney, intestine, liver and keratinocytes. These responses are triggered release of neuro-peptide such as substance P and the calcium gene related peptide.

Extraction techniques

When extracted, Capsaicin ($C_{18}H_{27}NO_3$) is an odorless, white crystal having harsh flaring pungency. Its melting point is 65^{o}C and boiling point is 210^{o}C-220^{o}C. It is slightly soluble in water and adequately soluble in ether, benzene, alcohol and chloroform (Yao, 1992). Its molecular structure shows cis-trans isomerism and doesn't allow internal rotation due to presence of double bond. Its molecule is most active in trans form. As mentioned above, capsaicinoids are mostly soluble in organic solvents such as methanol, ethanol, acetonitrile, n-butyl chloride, ethyl acetate, acetone, propanol and hexane (Peusch *et al*., 1997) but the recommended solvent used for capsaicin extraction is acetonitrile because of its high extraction yield with minimum impurities present (Karnka *et al*., 2002).

Using these solvents, capsaicinoids are extracted by a number of methods such as Magnetic Stirring Extraction (Kaale *et al*., 2002), Solid Phase Microextraction (Tapia *et al*., 1993), Liquid Liquid Extraction (Tapia *et al*., 1993), Pressurized Liquid Extraction (Barbero *et al*., 2006), Supercritical Fluid Extraction (Sato *et al*., 1999; Uzunalic *et al*., 2004), Enzymatic Extraction (Santamaria *et al*., 2000), Reflux (Peusch *et al*., 1997), Microwave Assisted Extraction (Barbero et al., 2006), Maceration (Titze *et al*., 2002), Soxhlet Extraction (Korel *et al*., 2002) and Ultrasonic Assisted Extraction (Karnka *et al*., 2002). Gnererally the fruit from which the capsaicinoids are to be extracted are first dried and smashed (Koleva *et al*., 2013) then further dehydrated using desiccator. This material is then treated with organic solvents like methanol, ethanol or acetonitrile and is incubated at temperature of 40^{o}C (Garces *et al*., 2006). Organic extraction of capsaicin is obtained by filtration (Collins *et al*., 1995) or centrifugation (Juangsamoot *et al*., 2012). This extract needs to be ultra-filtered before its estimation by HPLC. Techniques like SPE are further used to purify the extract. Although there are many techniques for extraction, this review focuses on some of the modern techniques used for extraction like Accelerated Solvent Extraction, Solid Phase Extraction and Magnetic Stirring Extraction.

Magnetic Stirring Extraction (MSE)

In MSE, the extraction is brought about by macerating the ground chili samples using magnetic stirrer with three types of polar solvents which are 80% ethanol in water, methanol and acetonitrile. This process is done at four heating temperatures that are 60, 70, 80 and 90^{o}C for three extraction time intervals. The extracts obtained are filtered with whatman filter paper no. 42 and are re-dissolved in a suitable solvent after solvent evaporation (Juangsamoot *et al*., 2012).

Accelerated Solvent Extraction (ASE)

ASE can be considered as a modified form of MSE as ASE is a rapid technique which utilizes very small amount of organic solvents at high temperature and pressure (more than that of MSE) which facilitates the accelerated extraction (Chanthai *et al*., 2012). ASE can be used in absence of light which facilitates the extraction of light or air sensitive compounds which can undergo oxidative degradation (Barbero et al., 2006). The sample mixture along with silica gel was placed into extraction cell. Two pieces of filter paper no. 42 were stacked at the outlet and on a cellulose disk of the extraction cell. Another filter paper on the top of the sample was placed in the extraction cell. The extract obtained was made solvent free by evaporation and then again diluted in a suitable solvent. This extraction process was run in 3-5 extra cycles with the extraction temperature range of 60-200^{o}C and pressure 10MPa. The solvent used was 80% ethanol in water, methanol and acetonitrile (Chanthai *et al*., 2012). These extraction techniques require additional cleanup techniques like SPE, Solvent Solvent Partition and Liquid Chromatography (Kim et al., 2002; Korel *et al*., 2002; Monnerville, 1999; Attuquayefio and Buckle, 1987) to further purify the extract prior to analysis by HPLC.

Solid Phase Extraction (SPE)

SPE is a technique used to prepare the extract sample for HPLC analysis by concentrating and purifying Substances from the solution by the process of sorption in a disposable solid phase cartridge (Karnka et al., 2002; Korel et al., 2002). Through SPE, in some capsicum fruits, extraction yield was found to be six times higher as compared to other organic solvent extractions (Peusch *et al*., 1997). The downside of this technique is that it is cost effective and is not common in routine laboratories (Juangsamoot *et al*., 2012). Generally, an ordinary extraction method like magnetic stirring solvent system is opted (Kaale *et al*., 2002).

In studies conducted by Juangsamoot et al., and Chanthai et al., the solid phase cartridge was first conditioned with 2ml of methanol followed by 2ml of de-ionized water. The extract obtained earlier which was around 400 micro liters was diluted with 600 micro liters of de-ionized water and then subjected to the conditioned cartridge. This process allowed separation of capsaicin and dihydrocaopsaicinfrom other capsaicinoids in the extract as they remain trapped in cartridge which was then eluted with 2ml of methanol twice (Juangsamoot *et al*., 2012 and Chanthai *et al*., 2012). In a separated study conducted by Musfiroh *et al.* (2013), 4mL acetonitrile and 1ml 1% acetic acid solution was used as an eluting buffer instead of methanol.

Analysis of Capsaicin by HPLC

HPLC is considered to be the most reliable, highly efficient, rapid and precise analytical method of capsaicinoid estimation. This technique is primarily associated with fluorescence detection (Cooper et al.,

1991; Uzunalic *et al*., 2004; Peusch *et al*., 1997, UV absorption detection (Lui *et al*., 2007; Bajaj and Kaur, 1997; Betts, 1999 and Korel *et al*., 2002) or photodiode array (Estrada *et al*., 2002; Cooper et al., 1991). A new HPLC method developed recently known as LC-MS (Liquid Chromatography-Mass Spectrometery) also provides an accurate analysis of these compounds (Perucka and Oleszek, 2000; Martin et al., 2004; Kozukue et al., 2005; Lui *et al*., 2007) but it is proven to be non-economical and is rarely used in routine laboratories. Therefore, keeping in mind the practical and reasonable characteristics of this method, RP-HPLC (Reversed phase high performance liquid chromatography) is the most common analytical method of choice for capsaicin estimation (Chanthai *et al*., 2012). HPLC has the ability to separate a component from its mixture and can identify it on the basis of its retention time.

HPLC-PDA (photo diode array detector) is a technique employed routinely in the analysis of capsaicinoids. The PDA is considered to be a vital tool for the identification of capsaicinoid structures. It works by using absorption and peak purity for identification. In a study conducted by Musfiroh et al., qualitative and quantitative analysis of capsaicin was carried out by RP-HPLC method with C-18 column and used acetonitrile –acetic acid 2% (6:4) as mobile phase. The flow rate was maintained at 1ml/min and injection volume was 10 micro liters and a UV detector at 280nm was used. For each sample, area under curve and retention time was recorded (Musfiroh *et al*., 2013).

Analysis of standards and samples showed 2 peaks. The peak shown at 5 min retention time was always higher than the other peak at 6.7 min retention time. As capsaicin is the major capsaicinoid compound, it was concluded that the higher peak was of capsaicin. Another study reveals that using acetonitrile; formic acid (5.5; 4.5) capsaicin appears at over 10 min of retention time. It was found that out of 12 chili varieties selected for this experiment, capsaicin content was highest in Ravit chili (C. frutescence) which was 2.10 %. In another study carried out by Othman et al. 2011, for the estimation of capsaicin, similar C-18 column with column temperature of 60^{o}C was used. Sample temperature was kept at 20^{o}C and injection volume was 5 microliters. UV detection wavelength was maintained at 222nm. A binary mixture of water-acetonitrile at 50:50 was used as mobile phase at a flow rate of 1.5 ml/min. Total of six capsicum fruits were analyzed to determine their capsaicin. It was found that the hot chili contained the highest amount of capsaicin followed by the red chili which had moderate concentration. Green chili was found out to be containing mild concentration of capsaicin with green, red and yellow pepper containing trace amounts of capsaicin.

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