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Full Length Research Paper

Microbial quality of artisanal yoghurt and Dèguè products collected in schools of Cotonou and Abomey-Calavi (Benin)

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The main aim of this study is to evaluate the microbiological quality of yoghurt and two types of dèguè (couscous and millet) sold in Cotonou and Abomey-Calavi. Samples of dairy products were collected in 15 schools of Abomey-Calavi and Cotonou from vendors located inside the schools. In this study, 180 samples (60 yoghurt, 60-dèguè millet and 60-dèguè couscous) were analysed. The microbial quality of collected samples was investigated using conventional methods for lactic acid bacteria, staphylococci, faecal and total coliforms and thermo-tolerant Gram-negative bacilli bacteria. The results of the microbiological analyses of the collected samples revealed a contamination of all the samples of yoghurt, dèguè millet and dèguè couscous. The microorganisms load varies according to not only the sampling sites, the sampling period but with the type of microorganisms. The presence of potentially pathogenic bacteria particularly the total coliforms (38%), faecal coliforms (21%), and thermo-tolerant Gram-negative bacilli bacteria (20%) and staphylococci (21%) were observed. In addition, it should be noted that the different samples of yoghurt, dèguè millet and dèguè couscous taken in the morning have a high microbial load compared to that of the evening with regard to faecal coliforms for the two sites. Dairy products sold to school children in Cotonou and Abomey-Calavi are potentially sources of food poisoning.

Key words: Yoghurt, dèguè, microbiological quality, schools, South Benin.

INTRODUCTION

Dairy products have exceptional qualities, which are very important in the diet for both humans and animals. They are rich in a wide variety of essential nutrients, easily

digestible minerals, vitamins and proteins (Haug et al., 2007). The production of milk is not high in Benin (about 112.302 tons) compare to other countries (Kassa et al.,

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2016). However, a significant part of production sold to people is found in the informal sector without any control. Dairy products occupy an important place in the diet of the Beninese population because of its richness in proteins of animal origin and its high concentration of nutrients (Anihouvi et al., 2019). Among those products, yoghurt is consumed by both children and adults because it provides energy and essential elements for growth and maintenance (Dror and Allen, 2014). It also contains probiotics and elements with immune-stimulant properties that help them adapt to environmental variations (Aspri et al., 2017). According to Standard N°. A- 11 of the Codex Alimentarius Commission (1975), yoghurt is a coagulated milk product obtained by lactic fermentation through the action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* from fresh milk and pasteurized milk with or without addition. Milk and its derivatives are mostly sold as ready to eat food dubiously in the local markets and particularly on roadsides and in schools (Tchekessi et al., 2014). In such cases, raw milk is processed and spontaneously fermented at home, before it is transported to the market in calabashes (Tankoano et al., 2016). There are various kinds of dairy products but the most consumed in West African countries are dèguè (millet and couscous), yoghurt, gappal, tchobal (Tankoano et al., 2016) which are foods rich in vitamins and minerals, especially calcium.

Despite their nutritional interest, milk products are excellent environments for the multiplication of all kinds of microorganisms (Bonfoh et al., 2003). They can present a health hazard due to the possible contamination with pathogenic bacteria when consumed unpasteurized or exposed to contaminated environment (Angulo et al., 2009). The microorganisms in the final product must be viable and abundant (FAO, 2008). Thus, more than 200 diseases can be transmitted through food contaminated by pathogenic bacteria, fungi, viruses and parasites (Zagare et al., 2012). The dairy products are mostly packaged in recycled bottles after fermentation of unpasteurized raw milk (Bagré et al., 2014). Several studies reported the presence of *Enterobacteria*, *Salmonella*, yeast and *Staphylococcus aureus* in the raw milks and other milk products sold in Burkina Faso (Bagré et al., 2014; Millogo et al., 2018). The prevalence of food-borne pathogens in milk is influenced by numerous factors such as farm size, hygiene, sampling, and samples analyse methodology, (Zagare et al., 2012). Indeed, to guarantee the safety of food offered for sale, good maintenance and adequate storage of preparation and sales materials and equipment are essential (Becila, 2009). Traditional techniques for improving the production and preservation of yoghurt and dèguè are expensive and less accessible to processors in Africa, including Benin (Akouedegni et al., 2013; Sessou et al., 2013). The quality of the ingredient used in the manufacture and non-compliance with hygiene rules are among factors that can affect the microbiological quality

of the final product that can be source of public health problems (Bagré et al., 2014).

Preventing school health is important, as nutritional deficiencies and infections have serious consequences for school participation and learning (UNESCO, 2000; UNICEF, 2013). Nevertheless, food poisoning has been reported among young schoolchildren in Benin, Ivory Coast and other West African countries (Pinstrup-Andersen and Watson, 2011; Bsadjjo-Tchamba et al., 2015). Foodborne disease is a major public health problem worldwide causing several deaths in developing countries (Baba-Moussa et al., 2006; Havelaar et al., 2015; Van de Venter, 2000). Thus, this study is a contribution to improving of young people's health. It is aimed at evaluating the microbiological quality of yoghurt and its two derivate product (dèguè couscous and millet) sold in secondary schools of Abomey-Calavi and Cotonou in Benin.

MATERIALS AND METHODS

Sampling and samples collection

Samples of dairy products (Yoghurt, Dèguè millet and Dèguè couscous) were randomly collected in 15 schools of Cotonou and Abomey-Calavi from vendors located inside the schools or outside within a radius of 20 m. For each type of dairy products, two samples were taken twice a day (morning and evening) and twice during the week with one day difference. A total of 180 samples were collected from the two sites: 60 yoghurt samples, 60 dèguè millet samples and 60 dèguè couscous samples. Once collected, the samples were transported in ice (4°C) to the laboratory for microbial analysis.

Microbiological analyses

Once in the laboratory, 10 g of each collected samples were homogenized into sterile stomacher bag with 90 ml of sterile tryptone salt water to obtain the stock solution. From this solution, a serial decimal dilution was made with tryptone salt water. The dilutions 10^{-1} to 10^{-3} were used for the detection and enumeration of total and faecal coliforms, *Staphylococcus* sp., and thermo-tolerant Gram-negative Bacilli bacteria whereas dilutions 10^{-1} to 10^{-7} were used for the detection and enumeration of lactic acid bacteria.

Lactic acid bacteria

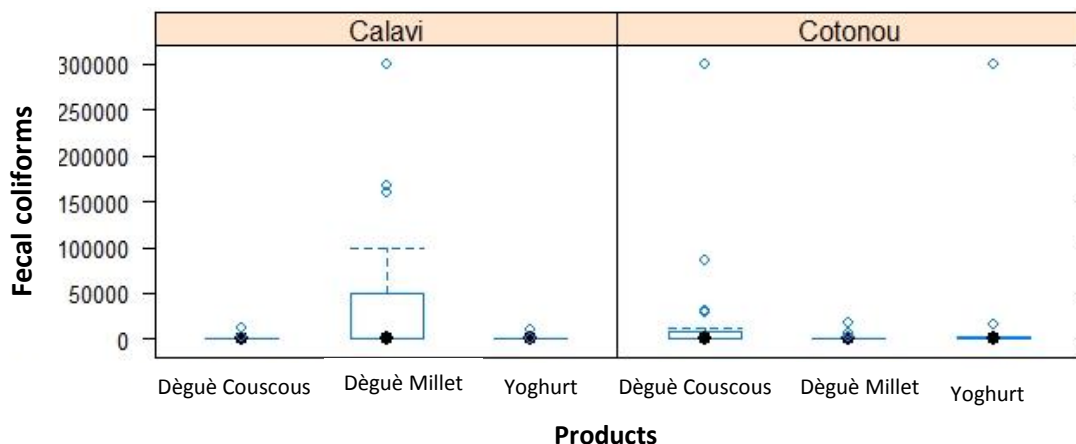
The Man Rogosa and Sharpe Agar (MRSA) medium was used for the enumeration of *Lactobacillus* in dairy products (de Mesquita et al., 2017). One millilitre of each dilution was taken and spread on sterile Petri dishes and 15 ml of the medium was poured into the samples and homogenized. After solidification, 5 ml of the medium was added to the surface of the previous stratum. The dishes were then incubated in anaerobiosis at 30°C for 48 to 72 h (de Mesquita et al., 2017).

Total and faecal coliforms

The enumeration of faecal coliforms was performed on Violet Red Bile Glucose Agar (Oxoid CM0485, England) medium (Milani et al., 2011). One millilitre of 10^{-1} to 10^{-3} dilutions was first spread on

Table 1. Average number of microbial load per germ on dairy products in colony forming units per gram (CFU / g).

	Yogurt (CFU/g)	Dèguè millet (CFU/g)	Dèguè couscous (CFU/g)
Lactic acid bacteria	3.710^{10}	1.15×10^4	2.2×10^{10}
Total Coliforms	1.2×10^5	1.2×10^6	7.7×10^6
Faecal Coliforms	3.9×10^4	1.1×10^6	1.5×10^6
<i>Staphylococcus</i> spp.	7.5×10^5	1.2×10^6	7.7×10^5
Thermo-tolerant Gram-negative bacilli	7.2×10^5	3.1×10^5	1.1×10^6

**Figure 1.** Distribution of the average faecal coliforms.

sterile Petri dishes and 15 ml of the VRBA medium was added and homogenized. A second stratum of about 5 ml was poured on the first before incubation at 30°C for 24 h for total coliforms and at 44°C for 24 h for faecal coliforms.

Thermo-tolerant Gram-negative bacilli

Thermo-tolerant Gram-negative bacilli were enumerated on Tryptone Bile X Glucuronide agar (Oxoid CM0595, England). Thus, 1 ml of the 10^{-1} to 10^{-3} dilutions was poured into petri dishes and 15 ml TBX was added, mixed and homogenized before incubation at 44°C for 24 h. The search for gram-negative bacteria was completed, after isolation on Eosin Methyl Blue Agar (Oxoid CM0069, England), by the indole test and the Api20E (bioMérieux, France) gallery (Riegel et al., 2006).

Staphylococci

Baird-Parker Agar enriched with egg yolk and potassium tellurite was used for the detection and enumeration of *Staphylococcus* sp strains (Baird-Parker, 1990). Briefly, 0.1 ml of the 10^{-1} at 10^{-3} dilutions was spread into petri dishes containing B Baird-Parker Agar (Oxoid CM0275, England) with with egg yolk. After 48 h incubation at 37°C, black, smooth or domed colonies with regular outlines with or without a halo were counted (Yehia et al., 2019).

Data analysis

To assess whether there is a significant difference in the measured

parameters between the products, the period and place of collection, generalized linear models were used. The R 3.6.0 software was used for the realization of graphs (Pinheiro et al., 2019). The threshold of statistical significance was set at $p < 0.05$.

RESULTS

Microbial load of dairy product

The results of the microbiological analysis show that 100% of dairy product (yogurt, Dèguè millet and Dèguè couscous) collected in the schools of Cotonou and Abomey-Calavi has unsatisfactory hygienic quality considering acetic acid bacteria, total coliforms, faecal coliforms, *Staphylococcus*, thermo-tolerant Gram-negative bacilli (Table 1).

Microbial loads in total coliforms and faecal coliforms

The average faecal coliform loadings of the products sampled are represented in Figure 1. It is noted that the average faecal coliform loads are similar for products such as Yoghurt and Dèguè couscous in the two collection cities. However, Dèguè millet samples from Abomey-Calavi mean loads are higher than those collected

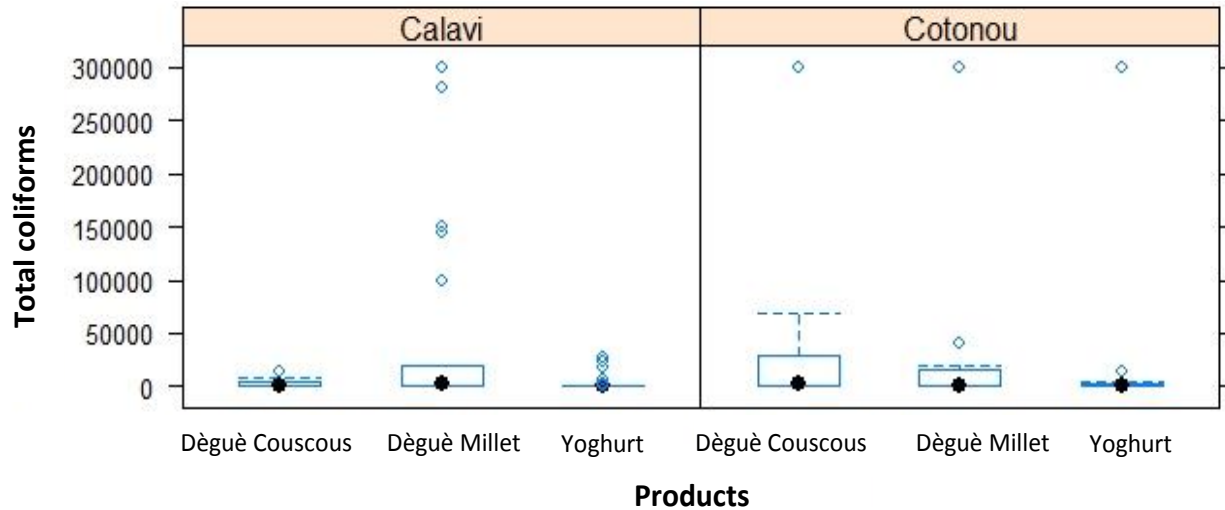


Figure 2. Distribution of the average total coliforms.

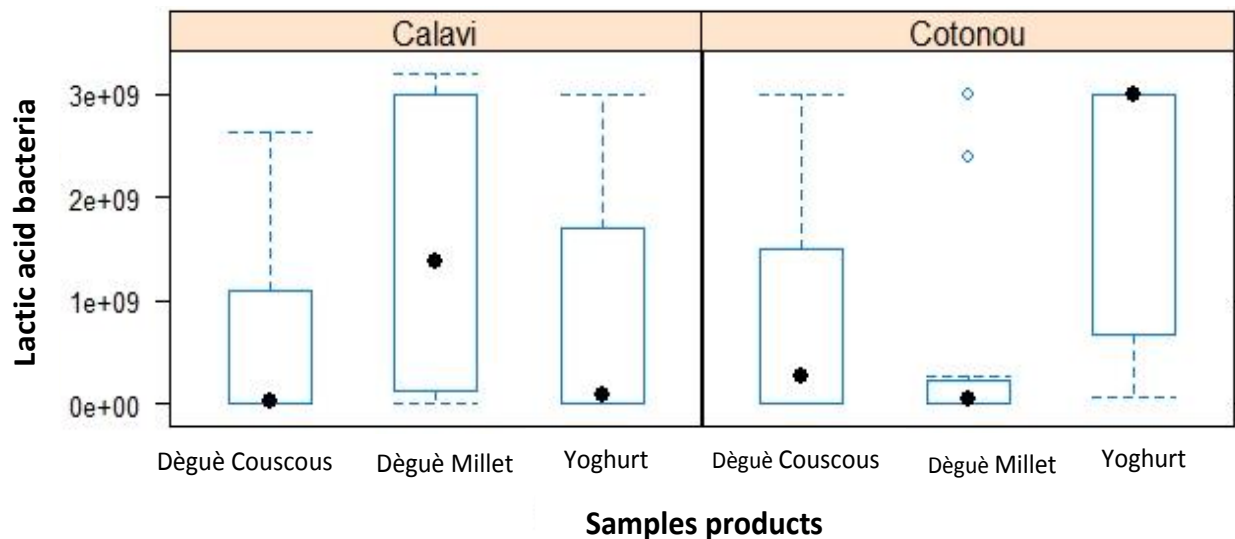


Figure 3. Distribution of the average lactic acid bacteria per product.

in Cotonou.

Concerning total coliforms, these averages are all similar between the two cities for yoghurt and Dèguè Millet and a variation is noted for Dèguè couscous (Figure 2).

Microbial loads of lactic acid bacteria

The Figure 3 shows the variation in lactic acid bacteria loads. It is founded that the average loads are higher in products such as yoghurt and Dèguè couscous in the city of Cotonou while these loads are higher in dèguè millet in the city of Abomey-Calavi.

Staphylococcal microbial loads

The changes in staphylococcal loads are represented in Figure 4. It is founded that the average *Staphylococcus* sp. loads are higher in products such as yoghurt and Dèguè couscous in the city of Cotonou while these loads are higher in dèguè millet in the city of Abomey-Calavi.

Thermo-tolerant Gram-negative bacilli microbial loads

It was found that the average load of thermo-tolerant Gram-negative bacilli (Figure 5) is similar between products

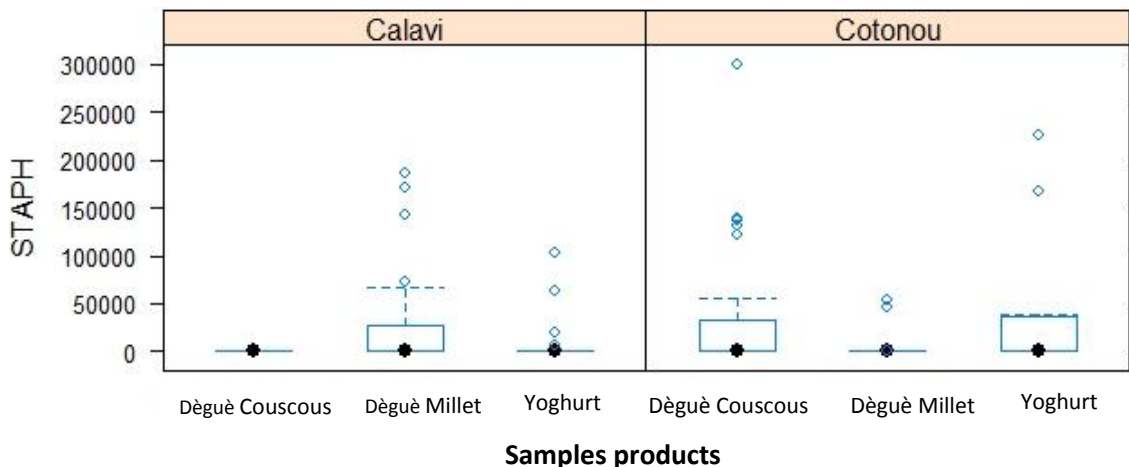


Figure 4. Average of *Staphylococcus* by product.

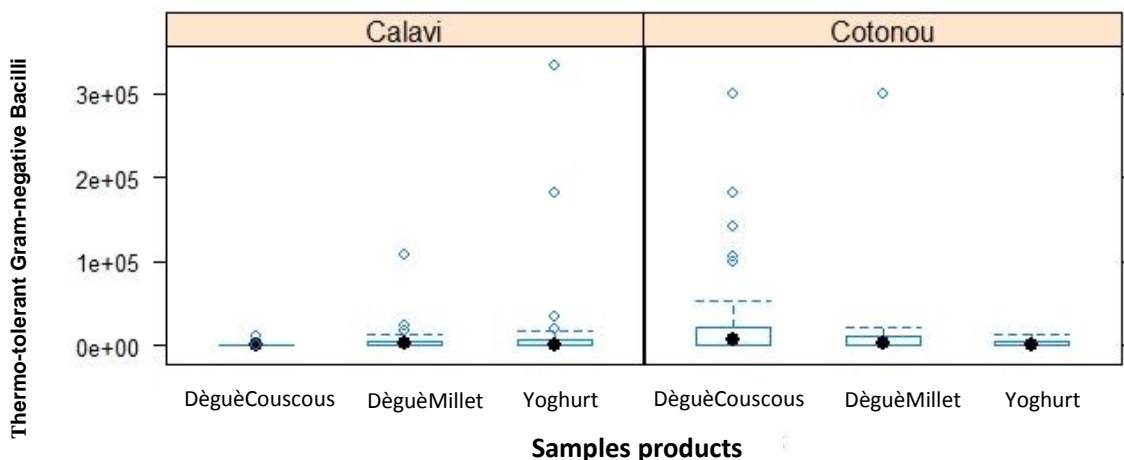


Figure 5. Average thermo-tolerant Gram-negative bacilli per product.

such as yoghurt and Dèguè millet in the two collection cities. However, these average loads are higher in the Dèguè couscous samples from Cotonou than those from Abomey-Calavi are.

DISCUSSION

A good fermented milk product must meet a number of criteria, particularly microbiological standards. These can only be achieved through the application of good hygienic practices and good manufacturing practices at all stages of product life. Thus, microbiological analyses revealed the presence of potentially pathogenic bacteria such as thermo-tolerant Gram-negative bacilli, *Staphylococcus* as well as faecal contamination index germs (total and faecal coliforms). Thus, the number of lactic acid bacteria varies according to the fermented milk product with an

average of 8.33×10^8 CFU/ml in the dèguè couscous samples, 1.14×10^9 CFU/ml for the dèguè millet samples and 1.21×10^9 CFU/ml for the yoghurt samples. These results are lower than the values (2.5×10^8 to 25×10^8 germs/g) reported by Ndiaye (2002) yoghurts in Dakar. This difference could be explained by the choice of quality and quantity of the lactic ferments used by the producers. Indeed, during investigation, it was noted that the salespersons did not always use suitable ferments when making yoghurt.

The environment of the sales sites prospected during this study allowed the observation of the presence of rubbish and open gutters draining wastewater at certain sites. It was found that fermented milk products were laid out on makeshift tables. The salespersons do not have adequate bins for garbage collection. This results in the attraction of flies, which are not only indicators of poor hygiene, but also vectors of faecal contamination germs

as mentioned by Samapundo et al. (2016). In addition, salespersons seldom change the water used to rinse service utensils. It was also noticed that the hands of the salespersons were in contact with the money and the food sold. These practices can lead to cross-contamination of the products served. All of this is detrimental to good practices in the preparation and sale of these desserts. Moussé et al. (2016) made almost the same observations in their study of street foods. The evaluation of the microbiological quality of fermented milk products reveals the presence of total coliforms, faecal coliforms, *Staphylococcus* sp. and thermo-tolerant Gram-negative Bacilli germs in fermented milk products; this illustrates a failure in hygiene and the implementation of good manufacturing practices collected in the survey results. This is in line with the work of Morou (2010) who notes that the search for these germs at the industrial level is a test of overall hygienic quality. The presence of coliforms in samples of fermented dairy products could also be explained by insufficient heat treatment or contamination during brewing, yoghurt packaging and even sale. Indeed, failure to respect the time/temperature couple during pasteurization can lead to the survival of coliforms in the yoghurt. In addition, the intake of sugar and flavourings at the end of the yoghurt manufacturing process by sealers could be a real source of contamination (Ndiaye, 2002).

Humans are the main vector of contamination during handling throughout the food chain. *Staphylococci* present in the fermented milk products studied can lead to intoxication due to the ingestion of staphylococcal enterotoxins, heat-resistant proteins performed in the food, in which *S. aureus* (or any other SE-producing *staphylococcus*) may have developed and produced its toxins. These toxins, if present in sufficient quantities in the food, can trigger nausea followed by characteristic uncontrollable vomiting (rocket vomiting), abdominal pain, diarrhoea, dizziness, chills, general weakness sometimes accompanied by moderate fever in the most severe cases, headaches, prostration and hypotension (ANSES, 2011).

Conclusion

The main objective of this study is to evaluate the microbiological quality of fermented dairy products, artisanal manufactured and sold in primary schools in Cotonou and Abomey-Calavi. This study had shown that fermented dairy products manufactured and sold by the sellers are contaminated with total coliforms, faecal coliforms, thermo-tolerant Gram-negative bacilli and *Staphylococcus* sp. Sellers do not respect the hygienic rules required during the manufacturing process of the products. Also noted are the inexistence of a quality approach, the sources of human contamination, and the inadequate framework for the sale of its products, the

contamination of the raw material used in manufacturing and poor preservation of the products, all of which are factors that influence the microbiological quality of fermented dairy products. Special attention must therefore be paid to the sanitary quality of dairy products sold in streets and schools in Benin, as microbial contamination can make the product highly perishable, unfit for human consumption following organoleptic alteration, or present a danger to young children. Thus, the monitoring of the hygienic quality of artisanal produced dairy products is essential to safeguard the health of consumers in general and children in particular.

CONFLICTS OF INTERESTS

The authors have not declared any conflicts of interests.

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Full Length Research Paper

Assessment of Arabusta coffee hybrids [*Coffea arabica* L. X Tetraploid Robusta (*Coffea canephora*)] for green bean physical properties and cup quality

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Robusta coffee is known to be high yielding than Arabica coffee, since it is more vigorous in growth. However, it is limited by the inferior cup quality. The aim of the study was to evaluate the performance of the Arabusta hybrids and its backcross derivatives for bean physical characteristics and organoleptic properties including their interrelationship. Nineteen coffee genotypes were evaluated at Siaya ATC and KALRO-Alupe, using randomized complete block design with three replications. Data on the bean grades and sensory traits was carried out on beans harvested in the year 2018. The results indicated that there was a significant variation on both the sensory and bean grade traits from the two locations. On average, Arabusta hybrids showed better performance on bean traits when compared to its backcrosses and Robusta. There was a highly significant positive correlation between the sensory traits which had highly significant positive association with aftertaste ($r=0.96$), aroma ($r=0.84$), balance ($r=0.85$), flavor ($r=0.96$) and overall standard ($r=0.96$). The positive significant correlation between the 100 bean weight and the AA bean size is an indication that weight of beans can be used in predicting the bean sizes. The interspecific hybridization enables the transfer of good cup quality attributes from Arabica coffee to Robusta coffee.

Key words: Flavor, Arabica, bean grade, quality, Arabica, Robusta, environment, variation.

INTRODUCTION

The most common popular beverages that are consumed globally are coffee and tea, with over 148 million cups of coffee being consumed on an annual basis; since it is the most preferred drink (ICO, 2019a). Coffee is the second

most traded commodity after oil and more research is being carried out on coffee due to its distinct characteristics in terms of flavor and aroma. This is in order to ensure that the consumers are supplied with

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coffee that meets the consumer preferences (Belay et al., 2016). The cup quality of Arabica coffee is superior to that of Robusta coffee; thus, Arabica coffee is usually blended with Robusta coffee on 50/50 basis to improve Robusta sensory traits and the crema formation most of the times (Folmer et al., 2017; Dias et al., 2018; Liu et al., 2019). Cup quality is termed as drinking quality or liquor quality, being one of the most important attributes of coffee (Muschler, 2001) and it is key in price determination in coffee market (Kathurima, 2013; Curzi et al., 2014; Barbosa et al., 2014). For coffee exporting countries such as Kenya, the production and supply of quality coffee is important since the prices they fetch are dependent on this factor. This is because the consumers discriminated the different coffees based on their origin, cup quality and the biochemical content (Fridell, 2014). Sunarharum et al. (2014) reported that the quality of coffee results from interaction between genotype and the environment (G x E) and consumers of high quality coffee may prefer labeling of the species and the country of origin (Cheng et al., 2016).

The mostly discussed factors influencing cup quality of coffee include temperature, altitude, soil, rainfall patterns, humidity, post-harvest practices, harvesting time (maturity) agronomic practises and genetics (Hameed et al., 2018; González et al., 2019). Assessment of coffee quality, in coffee breeding, is treated with equal importance as disease resistance and high yield. Robusta coffee is inferior in cup quality when compared to Arabica coffee; thus fetching low market prices. The interspecific hybrids between Robusta and Arabica are expected to have better cup quality than Robusta coffee. Robusta production outcompete Arabica coffee because of lower production costs; since they are tolerant to most pests and diseases and is more vigorous in growth than Arabica coffee (Mendes et al., 2001). Robusta coffee competes with Arabica in terms of sales, as it fetches lower prices (ICO, 2019b). Aroma and flavor derived from Arabica make it denser and richer, because of the infusions derived unlike Robusta (Nebesny and Budryn, 2006). A study by Michaela et al., 2013 showed that with increased quantities of Robusta in a mixture of Arabica and Robusta, there was increased fullness of extracts, yield extraction together with the astringent bitter tastes on the sensory performance; while with increase in Arabica there was an intense aroma felt in the brew.

Sensory analysis is the commonly used procedure in determining taste and flavor of coffee brews. However, being less objective than instrumental methods, it can be done in shorter times and can simultaneously be used to determine other traits (Nebesny and Budryn, 2006). The thousands of compounds emitted during roasting, determines the quality of the beverage since it affects the expression of different traits including the taste and aroma (Kathurima et al., 2009; Gichimu and Omondi, 2010). Determining the relationship to describe the effect of one of the compounds on sensory performance of

coffee containing the volatile compounds is extremely difficult (Sanz et al., 2002). Hence, sensory analysis has remained the most preferred technique in evaluating the performance of various cultivars on cup quality. The assessment of the sensory attributes of roasted coffee is carried out organoleptically, using a panel of tasters of coffee; since it is a reliable process for selection of genotypes during breeding. Acidity, fragrance, aroma, flavor, preference, body and balance are the organoleptic attributes that define the quality of coffee beans (González et al., 2019).

Bean size, which is defined as a grade in the market, is key in determining the final market price; since smaller bean sizes fetches lower prices than the larger coffee beans. The shape of the beans is critical in ensuring there is uniform roasting and the beans are sorted using color, to help identify the defective beans and this is done frequently (Batista and Chalfoun, 2014). When roasting is carried out on beans without uniform size, the smaller beans tend to roast faster than big sized beans; thus affecting the overall quality of the cup. During roasting, there is increase in weight loss and volume of beans, thus decrease in the bean density; and the non-defective beans tend to have higher volumes when compared to the defective beans (Noor-Aliah et al., 2015; Ameyu, 2016). The coffee beans may vary based on size, shape and colour depending on the coffee genotypes, availability of water during berry expansion and also the geographical origin (Yuwana et al., 2015). Formal sensory evaluation is more efficient when using a panel of judges than using the opinion of an individual, since it generates more data which is reliable during selection for quality improvement (Hampson et al., 2000). This study was to evaluate different coffee genotypes including Arabusta hybrids by subjecting them to cup quality and bean grade assessment in order to characterize their performance and to assess their relationship.

MATERIALS AND METHODS

Experimental materials

Nineteen genotypes including seven Arabusta hybrids, six different backcross derivatives of Arabica to Arabusta hybrids were evaluated alongside three Arabusta varieties, Robusta, C arabica (Batian, Ruiru 11 and SL28) (Table 1).

Description of the experimental site and experimental design

The trials were established at Siaya ATC (Siaya County) and KALRO Alupe (Busia County). Siaya lies between 0° 30' N' and 0° 45' E with an altitude that varies from 1,135 to 1,500 m above sea level. The mean annual rainfall is 1,500 mm with most parts of the county receiving between 890 and 1,900 mm; while the annual mean temperatures range from 20.9 and 22.7°C. The soils are well drained, deep to very deep (chromic/orthic acrisols and ferrasols) (Jaetzold et al., 2009). Busia county is also located in western part of Kenya between 0° 30' N' and 34° 30' SE, with an altitude that

Table 1. Description of coffee genotypes evaluated at KALRO- Alupe (Busia) and Siaya ATC.

Code	Pedigree information	Genotype description
ARH1	B11 2415 = CATURRA X B6. 1834 = (SL 28 X UT 6)	Arabusta hybrid
ARH2	B11 2554 = CATURRA X B6. 1834 = (SL 28 X UT 6))	Arabusta hybrid
ARH3	B11 2406 = CATURRA X B6. 1834 = (SL 28 X UT 6)	Arabusta hybrid
ARH4	B11 2407 = CATURRA X B6. 1757 = (SL 34 X UT 6)	Arabusta hybrid
ARH5	B11 2556 =CATURRA X B6. 1757 = (SL 34 X UT 6))	Arabusta hybrid
ARH6	B13 2271 = SL 28 X B6. 1835 = (SL 34 X UT 6)	Arabusta hybrid
ARH7	B14 1140 = SL 28 (SL 34 X UT 8)	Arabusta hybrid
BC01	B13 2400 = SL 34 X B6. 1764 = (SL 34 X UT 6)	Backcross
BC02	B13 2567 = SL 28 X B6. 1778 = (SL 28 X UT 6)	Backcross
BC03	B13 2286 = SL 28 X B6. 1836 = (SL 28 X UT 6)	Backcross
BC04	B13 2617 = SL 34 X B6. 1616 = (SL 34 X UT 6)	Backcross
BC05	B13 2806 = SL 34 X B6. 1756 = (SL 34 X UT 6)	Backcross
BC06	B14 1108 = SL 28 (SL 28 X UT 8)	Backcross
ARV1	PL 4 CONGUSTA 161 CRAMER	Arabusta variety
ARV2	PL 4 CONGENSIS 263 CRAMER	Arabusta variety
ARV3	PL 4 169, 177, 178 ARABUSTA	Arabusta variety
Robusta	Cultivar	Commercial variety
Ruiru 11	Introgressed Arabica	Commercial variety
Batian	Introgressed Arabica	Commercial variety

varies from 1241 to 1343 m above sea level. The mean annual rainfall is 1400 mm, with most parts receiving between 925 to 1900 mm and the annual maximum temperatures range from 26 and 29°C. The soils are developed on basic and intermediate rocks (dolerites and andesites) (Rachilo and Michieka, 1991). The experiment was laid out in a Randomized Complete Block Design (RCBD) with three replications at KALRO-Alupe and Siaya ATC. Five coffee trees were planted per genotype, with a spacing of 3 m by 3 m per plot measuring 855 m².

Bean processing and grading

Cherry was harvested from each of the genotypes in the year 2018 and was processed independently using the wet method. The cherry was pulped, followed by fermentation, washing and sun drying to attain 10.5 to 11% moisture content (Mburu, 2004). The coffee husks were de-hulled and 1 kilogram of coffee beans from each coffee genotype were sampled for grading into 7 grades, (AA, AB, PB, C, E, TT, T), using a hand operated hulling machine based on size, shape and density as described by Gichimu et al. (2013). The proportions (%) of each bean grade in one-kilogram sample of the hulled beans were recorded. The beans were separated based on size as determined by the size of the screens of the bean grading machine; while a pneumatic separator was used to separate light beans from grades AA and AB to obtain grade TT beans.

Sensory evaluation of coffee

The proportion (%) of each bean in grade AA and AB for each genotype were recorded and these grades were used to assess cup quality attributes by roasting. A probate laboratory roaster was used in the roasting process and the roasted beans were left to rest for at least 8 h before cupping. Green coffee beans were weighed before and after roasting to determine the roasting degree. After the 8 h rest, the roasted beans were ground into individual cups,

ensuring that whole sample was deposited into each cup. Each sample representing a specific genotype was placed into five cups (Kathurima et al., 2009). Samples were weighed to get 8.25g and 150 ml of hot water was added to each cup. The evaluation of the sensory attributes, sensory evaluation, was conducted with five trained judges forming a panel; using the procedures described by Lingle (2001). The descriptors measured included acidity, body, balance, fragrance/aroma, flavour, aftertaste, and overall standard as described by SCAA.

Statistical analysis

The bean grade and sensory data were subjected to Analysis of Variance (ANOVA) using GENSTAT statistical software version 18 and effects declared significant at 5% level. The General Linear Model (GLM) was used (Jansen, 1993). Least Significance Difference was used to separate means (Martin et al., 1978). Separate as well as combined analysis of variance was performed on data from the two sites. The sensory data was subjected to cluster analysis using the XLSTAT version 2019; using the unweight pair-group method with arithmetic average (UPGMA) to create a dendrogram based on the Euclidean distances (Hue et al., 2000). The Pearson's chi-square was used to test the similarities amongst clusters. GENSTAT statistical software was used to compute correlation and to show relationship between bean grades and sensory traits, using the Pearson Correlation Coefficient. The Principle Component Analysis of the sensory characteristics were plotted based on the important principle components (Lattin et al., 2002), using XLSTAT statistical software, version 2012.

RESULTS

Bean grading

There was a significant variation among the coffee

Table 2. Combined means of Bean grade traits for coffee genotypes at KALRO-Alupe (Busia) and Siaya ATC.

Genotype	%E	%AA	%AB	%PB	%C	%T	%TT	100 BW
ARH1	0.6	21.1	61.2	2.5	4.1	0.6	10	18.7
ARH4	0.4	14.5	75	3.6	4.3	0.3	2	16.1
ARH5	1.3	16.3	57.9	3.1	18	0.9	2.2	15.1
ARH6	1.7	24.4	57.2	4.2	8.9	0.5	3.1	16.8
ARH7	0.5	16.6	50.9	3.1	9.4	1.5	18.1	15.8
BC01	0.4	8.5	66	4.4	17	1	3.1	14.6
BC02	2.1	23.4	56.1	2.8	9.4	0.9	5.3	16.3
BC03	0.2	11.3	57	2.9	6.4	1.8	20.5	16.3
BC04	2.4	28.5	57.9	1.3	4	0.9	5	20.7
BC05	1.5	22.3	57.8	4.8	6	0.5	7.2	15.9
BC06	0.7	26.3	65.2	1.4	4.6	0.5	1.3	16.4
ARV1	0.2	4.8	74.2	2.8	6.8	0.4	10.8	14.4
ARV2	0.4	2	59.9	3.2	23	1.1	10.2	13.7
ARV3	1.8	38.4	52.6	0.6	1.1	0.3	5.2	21
Robusta	0.3	11.7	76.7	0.9	8.5	0.8	1	14.6
Ruiru 11	1.3	32.8	58	1.9	2.6	0.7	2.6	16.5
Batian	1.3	31.2	55.3	2.1	5.3	1.3	3.6	15.9
SL28	0.9	21.3	64	1	7.5	1.7	3.7	17.8
LSD	1.3	8	11.5	2.6	4.6	0.6	7.8	2.2
% CV	3.4	5.5	3.6	13	7.4	16.4	14.4	1.6
F Test	S	S	S	S	S	S	S	S

AA, % of beans retained by 7.15mm screen; AB, % of beans retained by 5.95mm screen; TT, % of beans separated from grades AA and AB by density; PB, % of beans retained by a piano wire screen with 4.43 mm spaces; C= % of beans retained by a piano wire screen with 2.90 mm spaces; T, % of very small beans and broken bits that cannot be retained by all the above screens; E, % of beans retained by a piano wire screen of the coffee bean grading machine with 8.3 mm space. 100 BW (g), 100 bean weight.

genotypes for bean grade across the two sites. The percentage of E bean grade varied among the genotypes; ranging from 0.2 to 7.2%, where genotype ARH3 recorded significantly ($P \leq 0.05$) high percentage compared to genotypes BC03 and ARV1 both of which recorded the lowest (Table 2). There was a significant ($P \leq 0.05$) difference on the percentage of AA bean grade for the coffee genotypes; where ARH3 recorded 38.4%, while ARV2 recorded the lowest percentage of 2.0%. The coffee genotypes recorded significant ($P \leq 0.05$) differences on the percentage of PB bean grade which ranged from 0.6 to 6.4%, where genotype ARH3 recorded the highest percentage while genotype ARV3 recorded the lowest. The percentage of C bean grade was also significantly ($P \leq 0.05$) different, where genotype ARV3 was highest with 23.1% and Robusta recorded the lowest with 1.1%. The variation on the 100 bean weight was significant; where genotype ARV3 scored significant ($P \leq 0.05$) heavier beans of 21 g followed closely by genotype BC04 with 20.7 g, while genotype ARV2 scored 13.7 g (Table 2).

Sensory performance

From the combined mean analysis, there was a

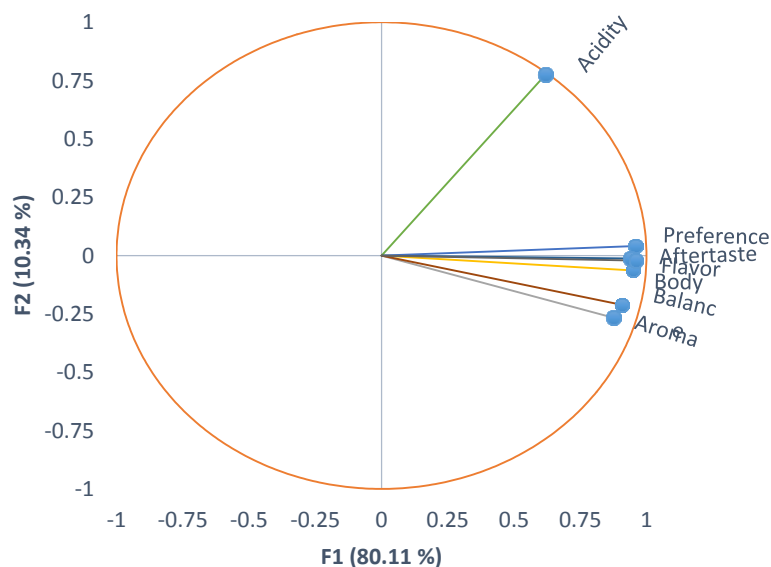
significant ($P \leq 0.05$) difference within the sensory traits among the coffee genotypes for both sites. Genotype SL 28 recorded significantly ($P \leq 0.05$) high values for all the traits except acidity, when compared to other genotypes (Table 3). Robusta recorded significant ($P \leq 0.05$) lower values for aroma and body of 6.9 and 7.2 respectively. Genotype ARV1 recorded significant ($P \leq 0.05$) low values for flavor Aftertaste and acidity when compared with other genotypes of 6.9 and 7.0 respectively; while cultivar Batian recorded high value for acidity. The genotypes that recorded significantly ($P \leq 0.05$) low values for balance included, ARH4, BC01, BC04 and BC06 of 7.2 (Table 3).

PCA and cluster analysis

The Principal Component Analysis was able to discriminate the scores of the different variables measured. PC1 was 10.34% and PC2 was 80.11%, which was sufficient to discriminate the sensory attributes. Acidity recorded low scores; while flavor and aftertaste recorded high scores respectively (Figure 1). The genotypes grouped together are similar in the sensory attributes. SL28 which was the best performer in

Table 3. Combined means of sensory traits for coffee genotypes at KALRO-Alupe (Busia) and Siaya ATC.

Genotype	Aroma	Flavor	Aftertaste	Acidity	Body	Balance	Overall standard	Total score
ARH1	7.2	7.2	7.3	7.3	7.6	7.3	7.2	81.1
ARH4	7.5	7.1	7.1	7.1	7.5	7.2	7.2	80.7
ARH5	7.5	7.4	7.4	7.3	7.6	7.3	7.4	81.9
ARH6	7.4	7.3	7.3	7.4	7.5	7.5	7.4	81.8
ARH7	7.6	7.4	7.4	7.5	7.7	7.5	7.5	82.6
BC01	7.4	7	7.1	7.1	7.5	7.2	7.1	80.4
BC02	7.5	7.3	7.2	7.1	7.5	7.2	7.2	81
BC03	7.7	7.2	7.3	7.4	7.6	7.3	7.4	81.9
BC04	7.5	7.3	7.3	7.4	7.5	7.2	7.3	81.5
BC05	7.5	7.5	7.3	7.5	7.6	7.6	7.5	82.5
BC06	7.5	7.2	7.1	7.1	7.3	7.2	7.1	80.5
ARV1	7.3	6.9	7	6.9	7.4	7.3	6.9	79.7
ARV2	7.3	7	7.1	7	7.3	7.2	7	79.9
ARV3	7.5	7.3	7.4	7.4	7.6	7.4	7.4	82
Robusta	6.9	7.1	7.1	7	7.2	7	7.1	79.4
Ruiru 11	7.3	7.1	7.1	7.2	7.5	7.4	7.4	81
Batian	7.7	7.7	7.6	7.7	7.7	7.5	7.5	83.4
SL28	8.3	8.2	7.9	7.2	8	8.1	8.1	85.8
LSD	0.3	0.3	0.3	0.3	0.3	0.4	0.3	1.5
% CV	1.7	0.9	0.9	1.1	1.6	1	0.8	0.2
F Test	S	S	S	S	S	S	S	S

**Figure 1.** The relationship between the sensory traits of coffee affecting cup quality.

quality on overall score is located on the lower right side of the quadrant on its own and it's much affected by the balance, aroma, body and flavor (Figure 1). The variation of the sensory traits is further shown using the cluster

dendrogram, by means of the seven sensory traits. The continuous broken line in the figure truncates the diagram into four classes which were generated during clustering, using the similarity index (Figure 2). Diversity of the

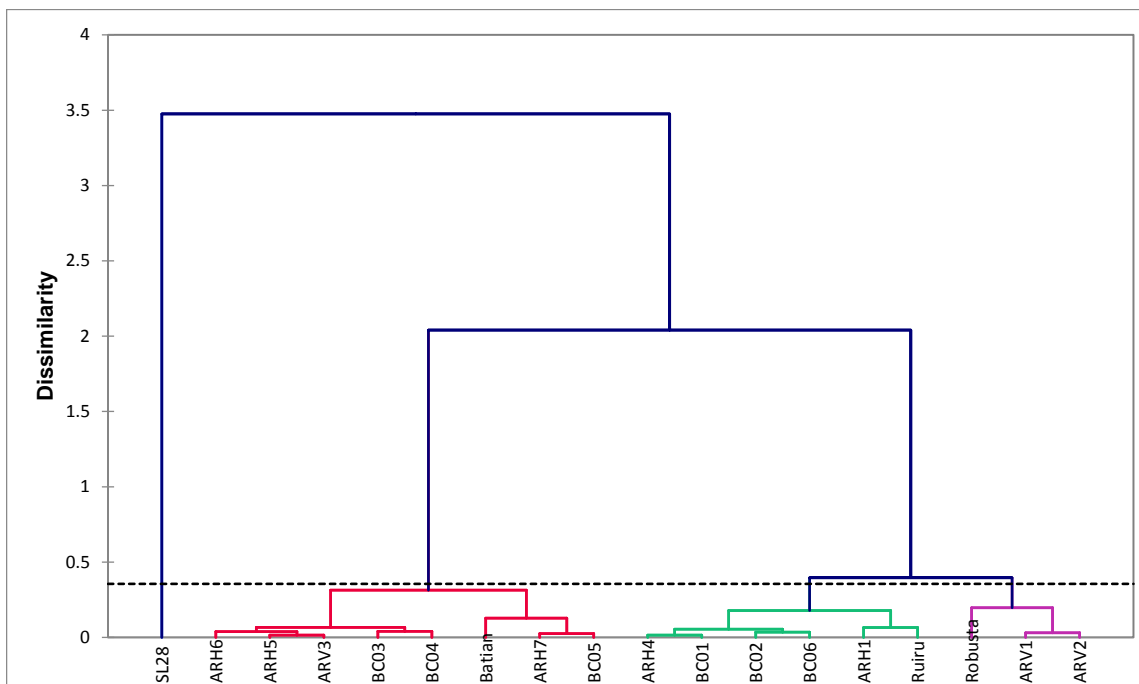


Figure 2. The diversity between the coffee genotypes based on the sensory traits.

Table 4. Pearson’s correlation analysis for sensory and bean grade traits for coffee.

	Acidity	Aftertaste	Aroma	Balance	Body	Flavor	Overall standard	AA	AB	BW
Acidity	-	0.96***	0.84***	0.85***	0.87***	0.96***	0.96***	0.43	-0.38	0.39
Aftertaste	-		0.82***	0.79***	0.89***	0.95***	0.93***	0.36	-0.4	0.39
Aroma			-	0.66**	0.71***	0.86***	0.85***	0.21	-0.24	0.19
Balance				-	0.80***	0.83***	0.88***	0.27	-0.22	0.15
Body					-	0.84***	0.91***	0.34	-0.37	0.34
Flavor						-	0.93***	0.41	-0.32	0.29
Overall standard							-	0.37	-0.35	0.3
AA								-	-0.52*	0.74***
AB									-	-0.4
BW										-

*, ** and *** significant at 0.05, 0.01 and 0.001 respectively; AA, % of beans retained by 7.15 mm screen; AB, % of beans retained by 5.95 mm screen; 100 BW(g), Weight in grams of 100 beans.

coffee genotypes within classes was 20.53%, while between class diversity was 79.47%.

Correlation between the sensory and bean grade traits

Table 4 shows the correlation coefficients for the sensory traits of coffee genotypes for KALRO-Alupe and Siaya ATC. There were positive significant associations between

all the sensory traits amongst the coffee genotypes. Acidity was positively and significantly associated with aftertaste (r=0.96), aroma (r=0.84), balance (r=0.85), body (r=0.87), flavor (r=0.95). Aroma had positive significant associations with balance (r=0.66), flavor (r=0.86). AA bean grade also had positive significant relationship with 100 berry weight (r= 0.74). AB bean grade negatively correlated with all the sensory traits. Bean weight (BW) grade had a positive significant association with acidity (r=0.39), aftertaste (r=0.39),

aroma ($r=0.19$), body ($r=0.34$), flavor ($r=0.29$) and overall standard ($r=0.3$) (Table 4). The AB bean grade was negatively correlated to all the sensory traits, while the AA bean grade was positively associated with the sensory traits.

DISCUSSION

The results of the study confirmed that there was significant variation within the coffee genotypes for bean grade traits and the sensory attributes across the two environments. Genotypes ARH2 and ARH3 were not evaluated for these two traits since most of the cherry were floats and this could be as a result of poor fertility. F1 hybrids have shown lower fertility rate and it has been reported that the number of seeds per fruit depends on ovule fertility (Leroy et al., 2006). Therefore, determination of fertility is based on the percentage of floats from the harvested cherry. Various studies have reported significant variations for the bean physical characteristics, including bean grade and the sensory attributes (Gichimu et al., 2012; Abrar et al., 2014; Gimase et al., 2014b; Tefera, 2018). Both the genotypic and environmental factors determine the size, shape and structures of beans produced.

The differences in percentage of grades across the two environments could be attributed to the different rainfall patterns since good rainfall is required during the cherry expansion stage. The plant physiological processes that occur during fruit development, affect the bean development as suggested by Adugnaw et al. (2015). The expansion of the cherry is determined by the rapid fruit expansion after pin head development, where turgor pressure developed by beans during expansion combined with high moisture availability leads to development of big sized beans (Sureshkumar et al., 2013). Photosynthesis also affects bean development with decreased leaf potential; photosynthesis is reduced leading to poor filling of the bean thus affecting the bean grades (Agwanda et al., 2003).

Cup quality is an attribute that depend on several factors for its expression, which include the genotype, environmental factors, the post-harvest processes, agronomic practices, storage, roasting, beverage preparation and also consumer preference (Kathurima, 2013; Hameed et al., 2018). The coffee markets vary and are more specific based on preference of the beverage based on performance of genotypes on sensory traits affecting the brew quality (Kahiu and Aluka, 2016). There was significant variation amongst the coffee genotypes for all the cup quality sensory characteristics.

González et al. (2019) reported the variation in sensory characteristics among the three Catimor genotypes cultivated in Mexico. The variations of the sensory traits could be attributed to both the genetics of the plant and environmental factors which include rainfall intensity and

distribution, soil characteristic, fluctuation of temperatures, humidity, and altitude (Decasy et al., 2003). These environmental factors have effect on maturity and ripening of cherry, which adversely affect the cup quality. The variation within genotypes indicates that it is possible to select genotypes that have good cup quality.

The Arabica coffee genotypes (SL28 and Batian), which are commercial varieties, showed good performance in cup quality together with the Arabusta hybrids and the backcrosses when compared to Robusta. SL28 coffee variety has always been used as a measure of standard for cup quality and therefore the results gave a good indication on the potential of the tested hybrids since on average, the Arabusta hybrids outperformed the Robusta coffee with an average score of 82%. This confirmed that through introgression, there was not only transfer of disease resistance but also other attributes including quality (Leroy et al., 2006). Based on organoleptic evaluation, introgression lines of Arabica have been found to produce good beverage quality (BQ) (Leroy et al., 2006). The sensory data has confirmed that the interspecific hybrids which were bred for improved cup quality can pass for the specialty market as defined by Lingle (2001). A score of more than 80% is regarded as specialty coffee.

Based on the Euclidean distances, four different groups were generated from the cluster analysis and the PCA plots; further confirming the variation amongst the coffee genotypes for the sensory traits (Figures 1 and 2). SL28 which has always been used as a standard measure for cup quality was grouped differently from the other genotypes. The Arabusta hybrids ARH6, ARH5, and ARH5 together with genotypes BC03, BC04 and BC05 grouped together with Batian implies that their relationship in terms of quality is very close and can be selected for good sensory traits when compared to Robusta, which clustered differently and distant from group two. These genotypes were easily differentiated using acidity, aftertaste and preference.

Correlation is key in designing an effective breeding program; thus selecting the traits that positively and significantly correlates to each other. All sensory variables which include acidity, aftertaste, aroma, balance, body, flavor and overall standard positively and significantly correlated with each other. Acidity had a highly significant positive association with aftertaste ($r=0.96$), aroma ($r=0.84$), balance ($r=0.85$), flavor ($r=0.96$) and overall ($r=0.96$). With the significant association, traits with a higher correlation such as preference, aroma and aftertaste can be used to select cup quality and this is in agreement with PCA plot results (Figure 1). The negative associations between the AB bean grade characteristics and quality indicates that bean sizes could be dependent more on the environmental factors since the berry expansion and ripening depended on the rainfall and sunlight availability for photosynthesis as

reported by Agwanda et al., (2003). Tessema et al., (2011) reported the significant positive associations within the sensory variables. The negative associations between the bean sizes and the sensory traits were reported by Kathurima et al. (2009). Dessalegn et al., (2008) found that there is a positive association between the bean grades and sensory traits. Reyes et al. (2016) and González et al. (2019) did not find any significant correlations between the beans sizes and sensory attributes which is consistent with the findings of this study.

Coffee is a beverage, where flavour is the most important quality parameter and a major motivation for consumer preference; since it is a combination of aroma, acidity and body that creates an overall impression of cup performance (Marin et al., 2008). Flavor has a strong significant association with all sensory traits, easy to determine organoleptically and has relatively high sensitivity in discriminating various genotypes; and this can be used as a single trait in the determination of coffee quality during selection (Agwanda et al., 2003 and Yigzaw, 2005).

Conclusion

The study confirmed that there was genetic variation within the coffee genotypes in both sensory and bean grade traits, since they were grouped into four clusters based on the sensory attributes. Flavor and over all standard were the most important traits in determining the organoleptic characteristics of the genotypes. All the interspecific hybrids analyzed for cup quality recorded more than 80% total score and outperformed Robusta in terms of bean size and therefore can be recommended for commercialization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Health risk assessment of Brazil nut consumption by aflatoxin biomarker in urine samples

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Aflatoxins are the main mycotoxin related to the contamination of Brazil nut and an important product extracted from the Amazon region that has a high number of consumers due to its health benefits. Considering the frequent occurrence of aflatoxins in Brazil nuts, it is necessary to study the levels of exposure to these toxins associated with its consumption using biomarkers. To do this, a study was carried out with 30 volunteers of both sexes, where each volunteer received a kit containing Brazil nuts for the ingestion of 2 (two) units / day for 30 days. Urine samples were collected from each volunteer at 0 and 30 days, and the level of Aflatoxin M1 (AFM1) was determined by ELISA using the Helica Kit®. A questionnaire was used to obtain information on weight, height, age, medication use, pathological conditions, frequency of weekly consumption of certain foods more susceptible to aflatoxin contamination, and 24-h food recall. Of the 30 samples analyzed at time 0 (zero) for exposure, AFM1 presented positive results in 3 (10%) samples with a range of 2.75-70.41 ng/mL. In the analysis of the 30 days after Brazil nut consumption, the presence of AFM1 in the urine was not detected, suggesting that Brazil nut consumption did not generate impact on the urinary levels and exposure to AFM1. In conclusion, no association was found between food consumption of food susceptible to aflatoxin contamination and concentration of AFM1 in urine.

Key words: Aflatoxin, nuts, ELISA, selenium.

INTRODUCTION

Aflatoxins (AFL) belong to the group of mycotoxins and are secondary metabolites of some fungi, carcinogenic to humans (IARC, 1993) and frequently associated with Brazil nuts. They have been reported in several stages of the production chain of the native Brazil nut of the

Amazon Forest (Baquião et al., 2012; Reis et al., 2012). This finding was observed in Brazil nuts shelled or inshell, for domestic and export markets, as well as for products derived from them (Moreira et al., 2016; Vargas et al., 2011; Pacheco and Scussel, 2009). However, in

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addition to AFL association with Brazil nut, it is important to evaluate the health risk related to frequent consumption due to the recent epidemiological evidence for the role of AFL in primary liver cancer, child growth impairment and immune suppression. In this context, risk analysis is a tool to evaluate the level of exposure to a contaminant (Jardim and Caldas, 2009) and in this field, the risk can be quantified as a function of the toxicity and exposure to which the consumer is exposed (IOM, 2001). Based on the risk analysis, it is possible to evaluate if the diet of a group of consumers is being affected by the presence of a chemical contaminant, such as the AFL, so it is possible to propose preventive measures to public health, either through the frequency/ingestion of food, or by improvements in the production chain. The risk from the exposure to AFL is also characterized by calculating the margin of exposure (MOE), which is defined as the ratio between the total intake and a toxicological reference, usually the lower bound of a benchmark dose level that caused 10% cancer incidence in rodents (EFSA, 2005). Andrade et al. (2013) evaluated the potential carcinogenic risks for the Brazilian population and observed in the Federal District, that total intakes for the total population and high consumers were 0.06-0.08 and 33.3-47.1 ng/kg bw/day, respectively, with Brazil nuts contributing the most to the intake for high consumers (54% for the upper bound level). Epidemiological studies have demonstrated a strong association between exposure to AFB₁ and increased incidence of hepatocellular carcinoma and, another way of studying the exposure of a population is the quantification of AFL metabolites through biomarkers in blood and urine (Bando et al., 2007). After oral ingestion, aflatoxin B₁ (AFB₁) is absorbed and biotransformed before urinary and fecal excretion in the form of aflatoxin M₁ (AFM₁). Biomarkers can indicate biological processes and provide information to diagnosis of individuals who may be at risk. The aflatoxin-lysine adduct in the serum has shown correlation to the AFB₁ intake, so this adduct has been used as biomarkers to quantitate exposure to aflatoxins (Benkerroum, 2019). In the United States, 1% of the population had a detectable result for aflatoxin B-lysine in a blood sample (Schleicher et al., 2013). In Malaysia, in urine samples, Redzwan et al. (2012) found a significant and positive association between milk and dairy products consumption and urinary AFM₁ level. Considering that diet and its factors are constantly associated with the occurrence of cancer, and some Brazilian regions still do not have studies involving risk assessment by biomarkers, there was the need to evaluate populations of Brazil nut production areas due to data scarcity. In the northern region of Brazil, the consumption of nuts occurs also in culinary preparations and some consumers are being considered high consumers. Therefore, the objective of the study was to study a population group and the consumption of Brazil nuts for 30 (thirty) days along with the occurrence of AFM₁ adducts in urine

biomarkers from the volunteers.

MATERIALS AND METHODS

Standards, reagents and chemicals

For AFL M₁ assay, the ELISA kits were purchased from Helica Biosystem (Santa Ana, CA, USA). The Brazil nuts supplied to the volunteer's consumption per 30 (thirty) days were purchased in the retail market of the city of Manaus-AM-Brazil from different retail lots and previously evaluated to AFL. The samples were not detected for Total AFL in the limit of detection of 0.3 µg/kg. In addition, the Brazil nut samples were evaluated for Selenium and the average was 5 µg/g.

Urine sampling and food consumption

To evaluate the AFL M₁ biomarker, urine samples were collected from volunteers, from the Faculty of Pharmaceutical Sciences of the Federal University of Amazonas, Manaus- Amazonas, Brazil. The project was approved by the Research Ethics Committee under the number: 909.195 and 60 samples were collected from students and professionals consisting of men and women. The samples were collected twice in time 0 days (*N*=30) and time 30 days (*N*=30). A total of thirty (30) volunteers participated, corresponding to 50% of the total individuals available that month in the Faculty. All volunteers were informed about the study and a consent form was given for signature prior to inclusion in this study. All the participants appeared to be in good physical health and were asked to complete a questionnaire with information about weight, height, age, medication use, pathological conditions. Urine samples from each volunteer were collected during the morning in disposable urine vials. The volunteers were given a kit containing sixty (60) Brazil nuts, for ingestion of 2 units/day (20 g- extra-large size nuts). We considered the average of the amounts described by Thomson et al. (2008) of 10 g and Strunz et al. (2008) of 45 g. The frequency of sampling was based on Teng et al. (2008) to evaluate the samples on the first day and after 30 days of consumption according to the volunteer's availability (until the end of the holidays). Volunteers were asked to complete a food frequency questionnaire to record their weekly eating habits and a 24-h food recall in which all food ingested on the day prior to urine collection was recorded. The frequency of consumption of foods that are most susceptible to AFL contamination and regulated in Brazil has been classified as 0 to 8 times per week or more. The number of male participants was 9 (30%) and female 21 (70%). Of the total volunteers, 18 (60%) were students, 4 (13.3%) were employees and 8 (26.6%) were teachers. The mean age of study volunteers was 33 ± 14 years (19-67 years). There was a difference in age between the men and women sampled in this study, with women being older than men. This difference in age was around 12 years. The men had lower age dispersion (24.5 ± 2.5 years) than the women (36.6 ± 14.4 years) and there was also a difference in BMI between the men and women sampled in this study. Women have lower BMI than men.

Aflatoxin M₁ assay

The urine sample analysis was performed using the Helica Biosystem ELISA kit for urinary AFM₁ (Santa Ana, CA, USA). All samples were analyzed according to instructions from the kit manual. A calibration curve was generated based on the absorbance values of standard solutions provided with the kit containing 0; 150; 400; 800; 1500; 4000 ng AFM₁ / ml. The

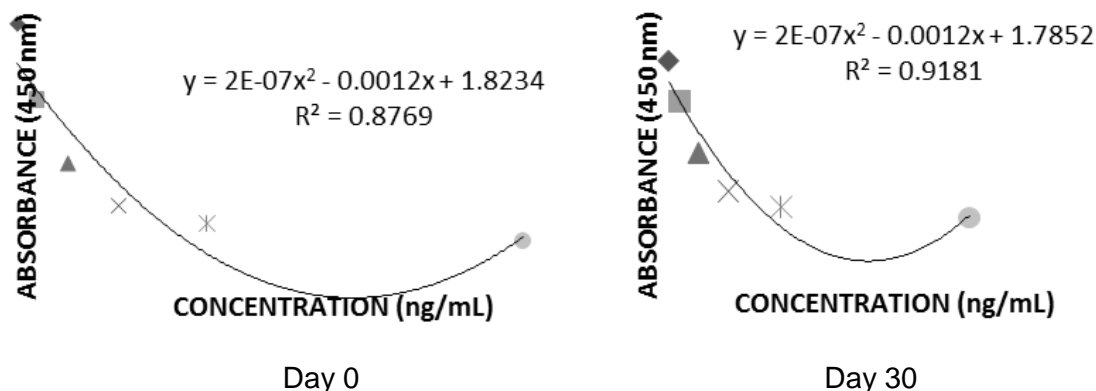


Figure 1. Calibration curve of AFM1 standard in day 0 and day 30.

absorbance was measured at 450 nm with a microplate reader. The urine samples were centrifuged at room temperature at 1500 rpm for 10 min to remove the debris and precipitates; thereafter, a 1:20 dilution (50 μ L of supernatant + 950 μ L of distilled water) was made. The validation steps were done with the samples measured against an arbitrarily chosen urine standard. They were spiked with either 0.5 or 2.0 ng/mL AFLM1 in three separate experiments and the recovery was measured. The fortified samples were covered from light and kept overnight at 2-8°C. The calibration curves and the straight equation are shown in Figure 1. The standard solutions provided by the Helica Kit AFM1 showed linearity of (a) time 0: 0-4000 ng/mL range for the analyte with a coefficient of determination (R^2) of 0.8769 and (b) time 30: range of 0-4000 ng/mL for the analyte with a coefficient of determination (R^2) of 0.9181.

Statistical analysis

The correlation between the consumption of more frequent food groups and the Brazilian nut consumption with the concentrations of AFM1 in the urine was carried out by Spearman test (Genest et al., 2013). This was also used for correlation between age, weight, height and BMI. Wilcoxon test (Fay et al., 2010) was performed to verify the statistical significance between genres while Bayesian analysis (Kruschke, 2014) was used to study the age difference and BMI between the sexes.

RESULTS AND DISCUSSION

AFM1 in urine samples

Among the samples analyzed at time 0 (zero), 3 (10%) presented positive results for the exposure of AFM1 in the urine with levels of 2.75-70.41 ng/mL, according to Figure 2.

The AFM1 values in urine observed in this study were higher than the ones previously reported in China by Qian et al. (1994), in which 317 urine samples were analyzed and levels of AFM1 detected ranged from 0.17 to 5.2 ng/ml. In another study, Zhu et al. (1987) cited the range from 0 to 3.2 ng/mL and Jonsyn-Ellis (2000) cited from 0.1 to 374 ng/mL. In Taiwan, the 250-person urinary excretion analysis evaluated by Hatch et al. (1993a, b)

resulted in AFM1 concentrations ranging from 0.003 to 0.108 ng/mL. These evidences show that most of the volunteers in this study appear not to have been exposed to toxic levels of AFL in food. In addition, AFM1 is also found in the milk of lactating cows and other mammalian species, including humans that consumed food products contaminated with AFB1. In a study conducted by Chen et al. (2018), plasma samples from 114 children less than 36 months of age from Tanzania were collected and analyzed for the presence of AFL. About 72% of the children had detectable levels of AFB1-lys, with an average level of 5.1 (95% CI: 3.5-6.6) pg/mg albumin. In Malaysia, the population appeared to be exposed to AF-B1, but at levels below enough to cause cancer (Leong et al., 2012). In Brazil, a study was conducted by Jager et al. (2016) and the authors estimated the probable daily intake (PDI) of aflatoxin by using the results from analysis of food products collected by the time of samples collection, and data from a 24-hour dietary recall questionnaire. In urine samples from volunteers of the University of São Paulo, 65% ($n=74$) presented levels of urinary AFM1 at mean levels ranging from 0.37 ± 0.23 to 1.70 ± 2.88 pg/mg creatinine. A modest but significant correlation ($r=0.45$; $p=0.03$; $N=23$) was found by the authors for the first time in Brazil between the concentration of AFM1 in urine and the PDI for total AFL (AFB1+AFM1). Urinary AFM1 was confirmed as sensitive to monitor human exposure to AFL by diet. In Baghdad, Ali et al. (2017) analyzed 218 urine samples collected from two localities and 40% of the samples from the Rajshahi District showed urinary AFM1 levels in the range of 1.7-104 pg/mL in the summer and in a range of 1.8-190 pg/mL in the winter season. In the Dhaka District, urinary AFM1 was detected in 31% of the samples in a range of 1.7 to 141.5 pg/mL. In the present study after thirty (30) days, all samples presented negative results for the presence of AFM1 in the urine. This can be explained by changes in eating habits as hepatic enzymes may form hydroxylated metabolites of AFB1 including AFM1, which can be excreted in the urine and

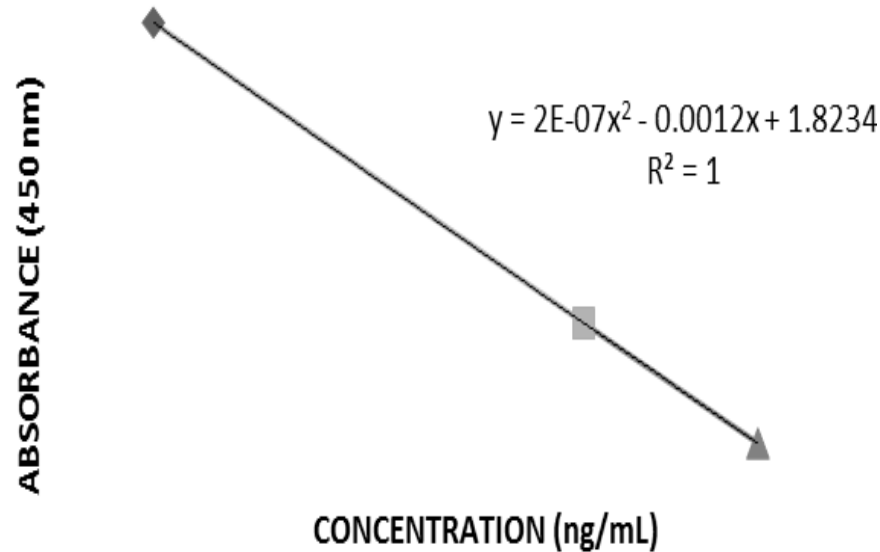


Figure 2. AFM1 in the urine samples.

used as a biomarker of short-term exposure to AFB1. Another possibility concern was the presence of protective agents and antioxidants such as selenium in the nut itself. He et al. (1993) concluded in the study that 0.4 mg/kg dietary supplement with selenium may protect the humoral immune function of the ileum mucosa of the AFB1-induced deficiency. Limaye et al. (2018) cited the ability of selenium to decrease immunosuppression, hepatic dysphonia, and apoptotic damage induced by AFB1 which is central to conferring anti-AFB1 protection through dietary supplementation. Due to the prevalence of AFB1 in animal feed and its diverse range of hosts, protective mechanisms highlighting the role of dietary selenium in relieving toxicity has been reported in many animals including swine, poultry and ducks. These works corroborate the idea that selenium may play a protective role against AFL. In the present study, the selenium level of the nuts was according to previous studies and necessary to provide the recommended daily intake, as the volunteers consumed an average of 18 g of nuts per day. If the average Selenium content of nuts was 5 ug/g, the daily dose was not toxic, although it exceeded the recommended level of 55 ug/day. Although urinary AFM1 dosage is a validated biomarker to assess AFL exposure, in this study, urinary AFM1 was a biomarker of short-term AFB1 exposure. In this context, it is suggested that more studies be conducted that correlate the selenium content and other items of the diet with the presence of AFM1 in the urine.

Consumption data versus AFM1

This study correlated the levels of urinary AFM1 with the

frequency of consumption of certain foods more susceptible to AFL contamination reported in the literature. The most consumed foods reported by all subjects were rice and milk, but there was no statistical ($p > 0.34$) significance between these foods and the level of urinary AFM1 (Table 1). The lack of association between urinary AFM1 levels and rice and milk consumption suggested that these foods do not make a significant contribution to dietary AFL exposure. This result was similar to the study by Romero et al. (2010), where the correlation analysis for maize, peanut and milk-based food intake and the detected levels of AFM1 in urine did not show a statistically significant relationship. Becker-Algeri et al. (2016) cited the AFM1 as the most frequent AFL in milk and is thermally resistant and - not completely inactivated by pasteurization, sterilization, or other milk treatment processes. Milk and dairy products are foods with a high probability of AFL contamination reported in several studies (Beltran et al., 2011; Heshmati and Milani, 2010). Concerning rice, Majeed et al. (2018) reported that in rice samples from Pakistan, 56% were contaminated with AFB1. Besides, there is the possibility that other foods not reported in the questionnaire are responsible for exposure to AFL in individuals, for example coffee, beans, corn derivatives are also associated with mycotoxins (Bui-Klimke and Wu, 2015; Nugraha et al., 2018). Individuals may present variable AFM1 excretion rates, since factors inherent to the organism may or may not interfere with the metabolism of AFB1 to AFM1 (Jager et al., 2014). Thus, the variability in the use of this metabolite by the individuals participating in this study may also have contributed to a lack of correlation between the consumption of risk products and the concentration of urinary AFM1. Only female

Table 1. Spearman's correlation test between AFM1 in urine and Rice and Milk consumption.

Group	<i>r</i>	<i>p</i>
Rice	0.12555149	0.5085629
Bovine Milk	0.17798320	0.3467183

Table 2. Spearman's correlation test between AFM1 in urine, age, weight, high and BMI.

Group	<i>R</i>	<i>P</i>
Age	0.09836534	0.6117010
Weight	0.23123550	0.2557164
Height	0.23380625	0.2311253
BMI	0.12711667	0.5360408

participants presented levels of urinary AFM1. Analysis of the statistical data did not show significant differences between the sexes ($p=0.1363$). There was also no significant ($p>0.23$) difference between age, weight, height and BMI (body mass index) (Table 2).

Conclusion

The objective of the study was to study urine samples from volunteers before and after consumption of Brazil nuts. We observed that urinary AFM1 concentrations at $t=0$ that ranged from 2.75-70.41 ng/mL, indicating a low exposure of the study population to aflatoxins. The consumption of Brazil nuts and by-products did not increase the urinary levels of AFM1. Further studies using urinary biomarkers are required to estimate AFL exposure in populations of higher food (Brazil nut) intake susceptible to AFL contamination in Brazil and Amazon region, such as vegan, elderly and health diet consumers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Variety and baking effects on injera making quality, polyphenols content and antioxidant activity of millet flours and injera

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Injera or *Biddena* commonly made from tef (*Eragrostis tef* (Zucc) Trotter) grain and a combination with other grains is a staple food of Ethiopian and neighboring countries. Millet and millet-based food products are rich in polyphenols and antioxidant activities and even so there is limited information. This study aimed to evaluate millet varieties for their injera making quality and polyphenol antioxidant activity. Five millet varieties and one tef variety called Quncho were collected, investigated and compared. A significant ($p < 0.05$) variations were noticed in rollability, adhesiveness and overall acceptance of injera. Quncho was perceived differently and rated higher in its overall acceptance (8.04). Conversely, injera made from Padet was rated lower in overall acceptance (6.21). Among millet injera's, Kola-1 rated higher whereas Padet was perceived lower in overall consumer acceptance. The result showed that the total phenols, flavonoids and anthocyanin contents of flour and injera ranged from 18.63 to 27.29 $\mu\text{g GAE/g}$, 11.99 to 15.43 $\mu\text{g catechin equivalent per g of sample}$, and 5.11 to 53.23 mg/l for flours and 22.99 to 27.25 $\mu\text{g GAE/g}$, 13.47 to 14.49 $\mu\text{g catechin/g}$ and 5.53 to 24.27 mg/l for injera. Tesema had the highest total phenols (27.29 $\mu\text{g GAE/g}$), total flavonoid content (15.43 $\mu\text{g catechin/g}$) and antioxidant activity (41.07%) against the inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH). Kola-1 showed the lowest L^* (59.76) and a^* (-0.11) and the highest b^* (4.21) flour color characteristics. Baking caused a non-significant ($p > 0.05$) reduction in total phenols, total flavonoids contents and DPPH free radical scavenging capacity. Hence, it is recommended that Tesema and Kola-1 varieties could be used for functional food development and injera making quality, respectively.

Key words: Millet varieties, tef, injera, baking, polyphenols, antioxidant effects.

INTRODUCTION

Injera or *biddena* is a staple food of Ethiopian which accounts for approximately 70% of dietary calories. It is made from quite a lot of cereal grains such as tef,

sorghum, millet, maize, barley and wheat depending on the regions and availability. However, best *injera* is made from tef (*Eragrostis tef* (Zucc) Trotter) grain. Recently,

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because of its being a whole grain product and gluten free nature (the cause for celiac disease) tef *injera* is gaining popularity in the developed countries as well (Abiyu et al., 2013). *Injera* is also considered as good sources of energy, fiber, iron, calcium and vitamins although the fermentation process during preparation results in significant reduction of most of the nutrients found in the cereals flour (Mezemir, 2015). *Injera* from white tef is most preferred due to its softer texture, preferred taste and color, and can be rolled without cracking (Boka et al., 2013). Tef is commonly grouped as a small millet along with fonio, finger millet and proso millet and it belongs to the same subfamily and tribe (Eragrostideae). As the tef price goes up, even middle income households tend to mix tef flour with cheaper cereals such as millet, sorghum maize or rice in preparing *injera* (Di Marcantonio and Demeke, 2013). Reports have shown that millet is inexpensive and nutritionally comparable or even superior to major cereals (Pathak et al., 2000).

Nowadays, agricultural research institutes have been releasing new varieties with the aim of increasing crop yield. However, grain quality and flour functionality are the most important criteria for good dough handling properties and specific health benefits. Millets and millet based food products are rich in phytochemicals which exhibit antioxidant and free-radical scavenging activity. As these are also gluten-free it could be suitable for persons suffering from celiac disease. Free radicals and reactive oxygen species are fundamentally the core cause of several disorders in humans that are generated as an imbalance between formation and neutralization of pro-oxidants resulting in oxidative stress as they cause oxidative damage to lipids, proteins, and DNA. Numerous studies and epidemiological evidences had shown that whole-grain cereal based foods are rich in plant polyphenols which could protect the body against age-related diseases such as cancer, cardiovascular ailments, diabetes, metabolic syndrome, and Parkinson's disease (Chandrasekara and Shahidi, 2012; Fardet et al., 2008; Manach et al., 2005; Scalbert et al., 2005). Antioxidants are thought to be important in reducing oxidative damage (Halliwell, 1994).

Prior phytochemical profiling of millet indicated that it contained significant amounts of antioxidants such as carotenoids, phenolics, and tocopherols (Asharani et al., 2010). Several forms of phenolics which exist in the grain have been reported to render antioxidative and antiproliferative effects and are responsible for the control of cholesterol oxidation in vitro systems (Madhujith and Shahidi, 2007, 2009; Liyana-Pathirana and Shahidi, 2006). Health benefits imparted by cereal phenolics may be a result of additive and synergistic effects of multiple compounds present in the grains. Phenolic compounds are secondary plant metabolites and their type and content in the grains may depend on a number of factors such as the type of cereal, variety, and part of the grain,

climatic conditions, and cultivation practices (Naczka and Shahidi, 2004). In addition, thermal treatments may reduce or increase the phenolic content and the antioxidant activities of cereals (Zielinski et al., 2006). Siroha and Sandhu (2017) revealed that toasting significantly increased the polyphenols content and antioxidant activity of pearl millet than cooking. Gull et al. (2018) noticed a significant reduction of antioxidant activity in cooked millet-pomace based pasta due to thermal degradation. Therefore, as *injera* is a staple food of Ethiopian, the investigation of millet varieties grown in several agro-ecological parts of the country and the effect of processing on the product making quality, and phenolic contents and antioxidant potential is unquestionable. Admassu et al. (2009) studied the chemical composition of local and improved finger millet varieties grown in Ethiopia. However, information on the polyphenol contents and antioxidant activity, and product making quality of Ethiopian millets are very limited. Thus, the objective of this study was to evaluate the polyphenol profiles, antioxidant activity and *injera* making quality of improved millet varieties grown in Ethiopia.

MATERIALS AND METHODS

In this study five samples of released millet varieties (four finger millet and one pearl millet) namely *Padet*, *Tessema*, *Tadesse*, *Aksum* and *Kola-1* grown in 2018/2019 season in moisture stress areas under similar but not the same agroecologies of Ethiopia were collected from Melkassa Agricultural Research Center, Ethiopia. One *tef* variety called *Quncho* was obtained from Debrezeit Agricultural Research Center and was used as a control. All millet varieties were grown in dry land areas and the tef grain was grown in moist and humid area. The grains were sorted, cleaned, washed, drained, sun dried and ground into flour. All the required chemicals were purchased from a company found in Can Tho City, Vietnam. The experiment was conducted at Food Science and Nutrition laboratory, Melkassa Agricultural Research center, Ethiopia and Food Technology laboratory of Can Tho University, Vietnam.

Injera processing and sensory evaluation

Injera was prepared using a standardized *injera* making procedure (Yetneberk et al., 2004). The procedure involved milling whole millet grain into a flour, preparation of a dough, and fermentation of the dough after adding yeast (a batter from a previous batch) and fermenting at room temperature for 48 h. After fermentation, 80 g of the fermented dough was thinned with 30 mL of water and cooked in 200 ml of boiling water for 1 min. The gelatinized batter was cooled to 45°C at room temperature and added back to the fermenting dough. After thorough mixing, 100 ml of water was added and the batter was fermented at room temperature for 3 h. Additional water (20 ml) was added to the fermented dough to bring to batter consistency. About 500 g of the fermented batter was poured in a circular manner on a 50 cm diameter hot clay griddle, covered, and baked for 2 min. The overall image of *injera* processing technique is shown in Figure 1.

A semi-trained panel (a panel briefed about the scoring of sensory attribute) of 40 people, who are consuming that particular product evaluated the samples. Accordingly, 20 consumer panelists

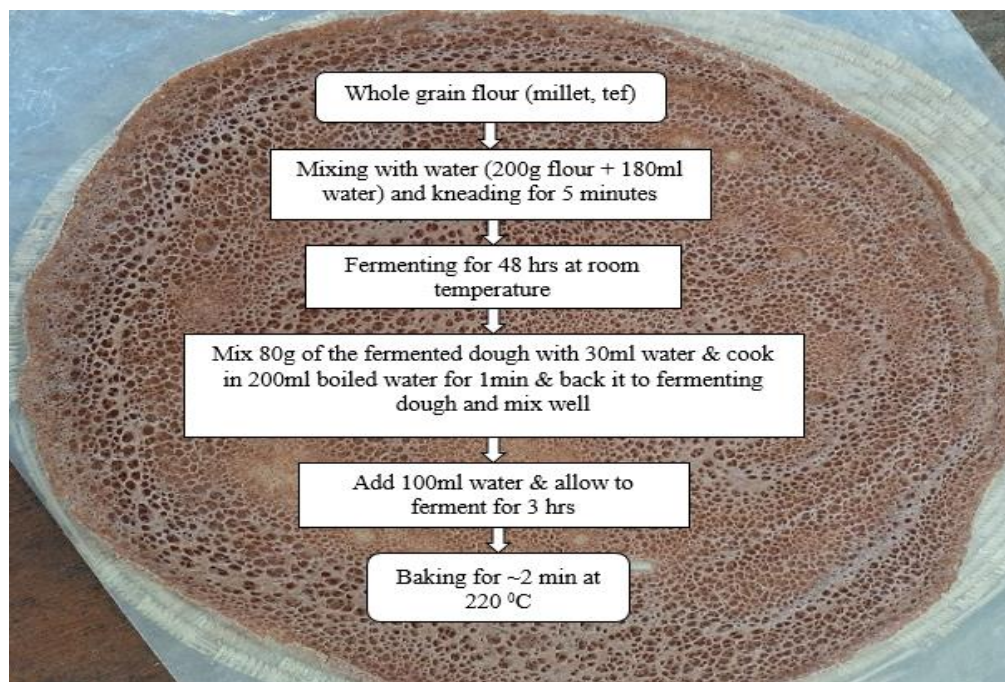


Figure 1. Injera processing flowchart (pictured: *injera* from *Tesema* variety).

were considered as replication one and the other 20 panelists as second replication. A rolled piece of *injera* was presented for panelist on a tray at room temperature within 2 h after baking. Panelists were served with a three digit coded samples on white plates in fluorescent-lighted rooms and was asked to evaluate some basic *injera* attributes (eye distribution, color, rollability, taste, adhesiveness, bitterness after taste and overall acceptability) using a nine point hedonic scale (1= extremely dislike, 9= extremely like).

Color characteristics

Color measurements of flour and *injera* samples were carried out using a Hunter colorimeter (Model, NR60CP 3NH Technology Co., LTD) optical Sensor on the basis of L^* , a^* , and b^* values as described by Kaur and Singh (2005). A glass cell containing flour was placed above the light source, covered with a white plate and L^* , a^* , and b^* color values were recorded. The instrument was calibrated against a standard red-colored reference tile ($L_s = 25.54$, $a_s = 28.89$, $b_s = 12.03$). Total color difference (ΔE) was calculated by applying the equation:

$$\Delta E = [(L_s - L)^2 + (a_s - a)^2 + (b_s - b)^2]^{1/2}$$

where, the L^* value indicates the lightness, 0–100 representing dark to light. The a^* value gives the degree of the red-green color, with a higher positive a^* value indicating redder. The b^* value indicates the degree of the yellow-blue color, with a higher positive b^* value indicating more yellow.

Polyphenol compounds and antioxidant activity

Sample preparation

Because of its high crude fat content, the flour from *Kola-1* was

defatted first by dissolving in hexane (1:5 w/v, 5 min for 3 times) to remove interfering lipids. A flour sample (2.5 g) was extracted with 25 ml acidified methanol (HCl/methanol/water, 1:80:10, v/v/v) for 2 h while shaking. The mixture was centrifuged at 3000xg for 10 min. The supernatant was used for determination of total phenols, total flavonoids and DPPH radical scavenging activity. The absorbance of the extracts was measured using Visible Spectrophotometer (Model 722, China). Note that both flour and injera were analyzed for the aforementioned parameters.

Anthocyanin content

The anthocyanin content (ACC) was determined by the pH differential method (Li et al., 2012). The diluted sample extracts (100 μ L) in 25 mmol L^{-1} potassium chloride solution (pH 1.0, 5.0 mL) and 0.4 mol L^{-1} sodium acetate buffer (pH 4.5, 5.0 mL) were measured at 510 and 700 nm, respectively, after 15 min of incubation at 23°C. Finally, absorbance (A) variation was calculated as:

$$A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$$

Total anthocyanin content of samples (mg cyanidin3- glucoside L^{-1} of sample extract) was calculated from the following equation:

$$ACC = \frac{A \times M \times DF \times 1000}{(\epsilon \times l)}$$

Where A is absorbance value, M is molecular weight (449.2 g mol^{-1}), DF is dilution factor (51), and ϵ is the molar absorptivity of cyanidin3-glucoside (26,900 $L\ mol^{-1}\ cm^{-1}$). The result was calculated in g cyanidin3-glucoside equivalents (CGE)/kg of sample wet weight.

Total phenolic content

The total phenolic content (TPC) was determined by following the Folin–Ciocalteu spectrophotometric method described by Gao et al. (2002). Aliquot of extract (250 µl) was added to 1.5 ml freshly diluted Folin–Ciocalteu reagent. The mixture was allowed to equilibrate for 5 min and then mixed with 1.5 ml of sodium carbonate solution (60 g/L). Then after incubation at 25°C for 90 min, the absorbance of the mixture was read at 725 nm. Acidified methanol was used as a blank and the result was expressed as a µg of gallic acid equivalents (GAE)/g of flour.

Total flavonoids content

The total flavonoids content (TFC) was determined following the method as described by Zhishen et al. (1999). Extract (250 µl) was diluted with 1.25 ml distilled water. Sodium nitrite (750 µl of 5% solution) was added and the mixture was allowed to stand for 6 min. Then, 150 µl of a 10% aluminum chloride was added and the mixture was allowed to stand for 5 min. Finally, 0.5 ml of 1 M sodium hydroxide was added and solution was mixed well. The absorbance was measured immediately at 510 nm using a spectrophotometer. Catechin was used as standard and the result was reported as µg of catechin equivalents (CE)/g of flour.

DPPH free radical scavenging activity

Antioxidant activity by DPPH free radical scavenging capacity was measured following a modified version of the method described by Brand-Williams et al. (1995) with slight modification. The supernatant (250 µl) was reacted with 4 ml of a 6×10^{-5} mol/L of DPPH solution. Absorbance (A) at 515 nm was read at 0 and 30 min taking a methanol as a blank. DPPH free radical scavenging activity was calculated as % discoloration.

$$\% \text{ DPPH Activity} = (1 - (A \text{ of sample } t=30 / A \text{ of control } t=0)) \times 100$$

Statistical analysis

A duplicate data was used and analyzed using Minitab 16 statistical software package and Tukey's multiple comparison tests was used to determine the significance of variation between treatments at 95% confidence level. Results were given as mean \pm standard deviation.

RESULTS AND DISCUSSION

Sensory properties of injera

In this study, a nine point hedonic scale (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) was used. Obviously *injera* with characteristics of white color, even eye distribution, less sourness and bitterness, rollable and less stick is preferred by consumers. The results of sensory characteristics of *injera* from five millet varieties and tef *injera* which was used as a control are shown in Table 1. In terms of aroma, taste and bitter aftertaste, no

significant difference ($p > 0.05$) were observed between and within millet varieties and control sample. *Padet* significantly ($p < 0.05$) differed from *Axum* and rated lower in its *injera* eye evenness (6.25) and color (5.71). *Injera* eye is a honeycomb like structure of the top surface of the product and it is formed during baking.

A significant ($p < 0.05$) variations were noticed in rollability, adhesiveness and overall acceptance of *injera*. *Quncho* was perceived differently and rated higher in its overall acceptance (8.04). Conversely, *injera* from *Padet* was rated lower in overall acceptance (6.21). In addition, among finger millet cultivars, *Padet* was perceived lower in its *injera* eye distribution and color; and a highest score of bitter aftertaste sensory attribute was for *injera* made from *Axum* (7.31) and *Tesema* (7.24) varieties with non-significant ($p > 0.05$) variation in between. Bitter aftertaste could be due to the presence of polyphenols in the grain. Rollability is one the most important *injera* sensory attribute as it describes the ability of *injera* being rolled. The result showed that *Quncho* (7.99) and *Tesema* (7.04) had the highest and the lowest rollability with a significant difference among them and more preferred from others. This difference might be due to retro gradation of starch components.

Yetneberk et al. (2004) revealed that sorghum cultivar with floury endosperm were characterized by soft and rollable *injera*. The degree of *adhesiveness* of *injera* during consumption is desirable to consumer acceptance and it is a quality of being stick to human sense organs while eating. *Tesema* showed the lowest degree of stickiness among finger millet cultivars.

Polyphenols content and antioxidant activity of flours and injera

Anthocyanin content (ACC), total phenolic content (TPC), total flavonoid content (TFC) and DPPH radical scavenging capacity of whole grain flours and *injera* from different millet varieties are shown in Table 2. Importantly, antioxidants are known to limit the amount of free radicals produced in human bodies. Phenolics are one of the major antioxidants found in millets, which chemically act by donating hydrogen atoms via hydroxyl groups on benzene rings to electron-deficient free radicals, and form a resonance-stabilized and less-reactive phenoxyl radical. Phenolics from millets have also shown their ability as reducing agents, singlet oxygen quenchers, and metal chelators (Devi et al., 2014).

Polyphenols content and antioxidant activity of flours

Anthocyanin content: The anthocyanin content of millet flours varied significantly ($p < 0.05$) among millet varieties and ranged from 5.11 to 53.23 mg/L, the lowest for

Table 1. Sensory characteristics of millet and tef injera.

Variety	Sensory attributes							
	Eye distribution	Color	Aroma	Taste	Bitterness after taste	Rollability	Adhesiveness	Overall acceptance
<i>Axum</i>	7.49±0.38 ^a	8.01±0.28 ^a	7.38±0.38 ^a	7.49±0.38 ^a	7.31±0.35 ^a	7.35±0.42 ^{ab}	6.91±0.45 ^{ab}	7.05±0.08 ^{bc}
<i>Kola-1</i>	7.28±0.09 ^{ab}	8.26±0.38 ^a	7.78±0.17 ^a	7.14±0.20 ^a	6.97±0.04 ^a	7.14±0.21 ^{ab}	6.79±0.21 ^{ab}	7.51±0.11 ^{ab}
<i>Padet</i>	6.25±0.05 ^b	5.71±1.07 ^b	7.33±0.11 ^a	7.09±0.28 ^a	6.83±0.09 ^a	7.42±0.18 ^{ab}	6.84±0.18 ^{ab}	6.21±0.11 ^d
<i>Tadese</i>	6.65±0.15 ^{ab}	7.40±0.09 ^{ab}	7.26±0.37 ^a	7.08±0.03 ^a	7.21±0.28 ^a	7.11±0.17 ^{ab}	6.495±0.12 ^{ab}	6.44±0.12 ^{cd}
<i>Tesema</i>	6.57±0.34 ^{ab}	7.41±0.57 ^{ab}	7.48±0.23 ^a	7.11±0.16 ^a	7.24±0.36 ^a	7.04±0.06 ^b	6.48±0.09 ^b	7.14±0.29 ^b
<i>Quncho</i>	7.35±0.41 ^{ab}	8.47±0.08 ^a	8.09±0.21 ^a	7.96±0.05 ^a	7.06±0.57 ^a	7.99±0.16 ^a	7.43±0.18 ^a	8.04±0.22 ^a

Mean ± SD with different superscripts in a column varied significantly ($p < 0.05$) within and between different millet cultivars and control, $n=2$.

Table 2. Polyphenols content and antioxidant activity of flour and injera.

Variety	Flour				Injera			
	TPC ($\mu\text{gGAE/g}$)	TFC, $\mu\text{gCE/g}$	ACC (gCGE/kg)	DPPH scavenging activity (%)	TPC ($\mu\text{gGAE/g}$)	TFC ($\mu\text{gCE/g}$)	ACC (gCGE/kg)	DPPH scavenging activity (%)
<i>Axum</i>	26.99±0.008 ^a	15.08±0.084 ^b	25.97±3.011 ^{bc}	39.31±1.05 ^{ab}	26.22±0.045 ^b	14.09±0.01 ^b	24.27±1.81 ^a	40.55±0.18 ^a
<i>Kola-1</i>	22.96±0.187 ^d	13.49±0.009 ^d	5.11±1.204 ^c	31.81±0.60 ^c	24.77±0.018 ^c	13.47±0.01 ^c	5.53±0.60 ^d	31.32±1.41 ^c
<i>Padet</i>	25.15±0.062 ^c	14.08±0.122 ^c	46.84±13.248 ^{ab}	38.17±0.66 ^b	23.61±0.035 ^d	13.56±0.04 ^c	16.39±0.60 ^d	36.37±0.38 ^b
<i>Tadese</i>	25.76±0.036 ^b	13.91±0.056 ^c	35.34±1.807 ^{ab}	38.15±0.00 ^b	24.89±0.045 ^c	13.57±0.00 ^c	9.79±1.81 ^{cd}	39.43±0.71 ^a
<i>Tesema</i>	27.29±0.045 ^a	15.43±0.028 ^a	53.23±1.807 ^a	41.07±0.60 ^a	27.25±0.018 ^a	14.49±0.13 ^a	20.01±1.81 ^{ab}	41.21±0.10 ^a
<i>Quncho</i>	18.63±0.098 ^e	11.99±0.075 ^e	29.81±4.818 ^{ab}	27.54±0.69 ^d	22.99±0.027 ^e	13.50±0.05 ^c	15.33±3.61 ^{bc}	32.19±1.41 ^c

Mean ± SD with different superscripts in a column varied significantly ($p < 0.05$) within and between different millet cultivars and control, $n=2$. TPC, Total phenols content; TFC, Total flavonoids content; ACC, Anthocyanin content; GAE, Gallic acid equivalent; CE, Catechin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CGE, Cyanidin3-glucoside equivalent.

Kola-1 and the highest for *Tesema* was noticed. Finger millet cultivars, *Padet*, *Tadese*, and *Tesema* were statistically similar in their anthocyanin content. Except from *Axum*, all finger millets varieties had the highest ACC than *Kola-1* and showed significant variation. *Tesema* and *Padet* was found to possess the highest ACC among all the studied varieties which had nine times higher than the ACC of *Kola-1*. *Axum* had the lowest ACC (25.97 mg/g) among finger millet varieties. *Quncho*, which is under the same family

of millets showed a significant difference with *Axum* and *Kola-1*. Anthocyanins are a group of naturally occurring and water soluble pigments that are responsible for the red-blue color of many grains, and can be found in glycosylated forms linked with sugars such as glucose, galactose, arabinose, xylose, rutinose. These compounds become increasingly popular due to their attractive colors and suggested benefits for human health (Pojer et al., 2013) and in addition, protect plants against various biotic and abiotic

stresses (Ahmed et al., 2014).

Total phenols and total flavonoids contents: The result showed that contents of total phenols and flavonoids differed significantly ($p < 0.05$) between and within millet varieties and control sample as well. The total phenols and total flavonoids contents of flours ranged from 18.63 to 27.29 μg of GAE/g and 11.99 to 15.43 μg of CE/g, with the highest value for *Tesema* and the lowest content for *Quncho*, respectively. *Kola-1* was

significantly ($p < 0.05$) different and had lower contents of total phenols (22.96 $\mu\text{gGAE/g}$) and total flavonoids (13.49 $\mu\text{gCE/g}$) compared to finger millet varieties. Among finger millet cultivars, *Padet* and *Tadese* showed the lowest TPC (25.15 $\mu\text{gCE/g}$) and TFC (13.91 $\mu\text{gGAE/g}$), respectively. The lowest TPC and TFC were observed with *Quncho*. Chandrasekara and Shahidi (2011) revealed that the phenolic content of whole pearl and kodo millet varieties was 8.63 and 32.4 ferulic acid equiv $\mu\text{mol/g}$ defatted meal, respectively. Contrarily, Ragaee et al. (2006) reported the highest total phenols content (1387 $\mu\text{g GAE/g}$) for pearl millet variety. This variation could be due to agronomic, environmental and varietal differences, and forms and level of sample processing.

DPPH free radical scavenging activity: The antioxidant activity assay by DPPH was significantly ($p < 0.05$) different among millet varieties. The highest DPPH free radical scavenging activity was observed for *Tesema* (41.07%) and the lowest for *Quncho* (27.54%). From the studied millet varieties, *Kola-1* was found to have the lowest (31.81%) antioxidant activity than finger millets and the greater antioxidant than *Quncho* with statistical difference. *Tadese* showed the lowest DPPH free radical scavenging activity (38.15%) with non-significant variation among finger millet cultivars. With the exception of *Tesema*, finger millet cultivars was not significantly ($p > 0.05$) different. The DPPH free radical scavenging ability of all finger millet varieties was nearly 1.5 times the extracts from *Quncho*. An order of a free radical scavenging capacity, *Tesema* > *Axum* > *Padet* > *Tadese* > *Kola-1* > *Quncho* was observed. DPPH radical is a synthetic organic radical that is widely used to evaluate free radical scavenging possessions of antioxidative compounds. Similar study by Siroha et al. (2016) noticed that the the antioxidant activity of pearl millet flours in the range of 31.8 to 46.7%. In addition, Ragaee et al. (2006) revealed that pearl millet had DPPH scavenging capacity of 23.83 $\mu\text{mol/g}$. Conversely, Chandrasekara et al. (2012) reported DPPH scavenging activities of 13.8 μmol ferulic acid equivalent/g for pearl millet flour. The antioxidant activity of grains could be influenced by genetic and environmental factors, and the level and method of grain processing.

Injera polyphenols content and antioxidant activity: After fermentation and baking, the polyphenol contents and antioxidant activity were ranged from 22.99 to 27.25 $\mu\text{gGAE/g}$ for TPC, 13.47 to 14.49 $\mu\text{gCE/g}$ for TFC, 5.53 to 24.27 mg/g for ACC and 31.32 to 41.21% for DPPH radical scavenging activity with a significant ($p < 0.05$) difference among all varieties (Table 2). *Tesema* had the highest TPC, TFC and DPPH free radical scavenging capacity with a values 27.25 $\mu\text{gGAE/g}$, 14.49 $\mu\text{gCE/g}$ and 41.21%, respectively. The lowest (even lower than *injera* made from *Quncho*) contents of total flavonoid, anthocyanin and antioxidant capacity was observed with

Kola-1. Among finger millet varieties, *Padet* showed the lowest content in TPC (23.61 $\mu\text{gGAE/g}$) and TFC (13.56 $\mu\text{gCE/g}$). Except for the anthocyanin content, a non-significant ($p > 0.05$) reduction or increment in total phenols, total flavonoids and DPPH free radical scavenging activity were observed in *injera* compared to flours. This could be attributed to the partial degradation of phenols by microorganisms during fermentation (Bravo, 2009) and loss of some anthocyanin, which have been reported as labile to heat. An increment in DPPH free radical scavenging activity was noticed in finger millet cultivars and tef which is likely due to the amount reducing sugars that contribute to maillard reaction. Gélinas and McKinnon (2006) also observed that the crust of white bread contained more phenolic compounds than the crumb because of the Maillard reaction.

On the other hand, Salar et al. (2017) revealed that fermentation resulted in an increased TPC with fermented pearl millet grains showing higher values than unfermented pearl millet grains. According to these authors, as fermentation increased from 0 to 6 days, the TPC increased from 6.4 to 34.1 mg GAE/g dwb . However, beyond these fermentation days a reduction in TPC was noticed. In the present study, fermentation and then baking did not result in a significant ($p > 0.05$) reduction and/or increment of DPPH radical scavenging activity of the millets extracts, except for *Quncho* which showed an increment (Figure 2). A strong correlation ($r = 0.98$) was noticed between total phenolic content and DPPH radical scavenging activity. In addition, total flavonoids content and DPPH activity were highly correlated ($r = 0.94$).

Color characteristics

Table 3 shows color characteristics (L^* , a^* , b^* and ΔE) of flours and *injera* which were evaluated using hunter color lab. The L^* value of flours ranged from 59.73 to 64.77, the more darken and light colors for *Kola-1* and *Tesema*, respectively. After baking, the L^* value of *injera* ranged from 48.04 to 57.92 and except for *Kola-1* (which showed an increment) was significantly reduced in comparison with flours. *Kola-1* and *Quncho*, and among finger millet varieties, *Axum* and *Padet* in both flours and *injera* did not show significant ($p > 0.05$) variation in their L^* value. The variation observed might be due to the chemical compositions of the grain mainly because of protein, starch and reducing sugar contents that affects Maillard reaction. The degree of a red-green color (a^*) ranged from -0.12 to 1.68 for flours and 2.14 to 4.47 for *injera*, and baking significantly increased a^* value compared to flours. The highest and the lowest a^* value with a significant difference was noticed with *Quncho* and *Kola-1* in flours, but had the lowest and exhibited insignificant variation in *injera*. *Kola-1* was statistically different and showed the minimum redness and maximum greenness

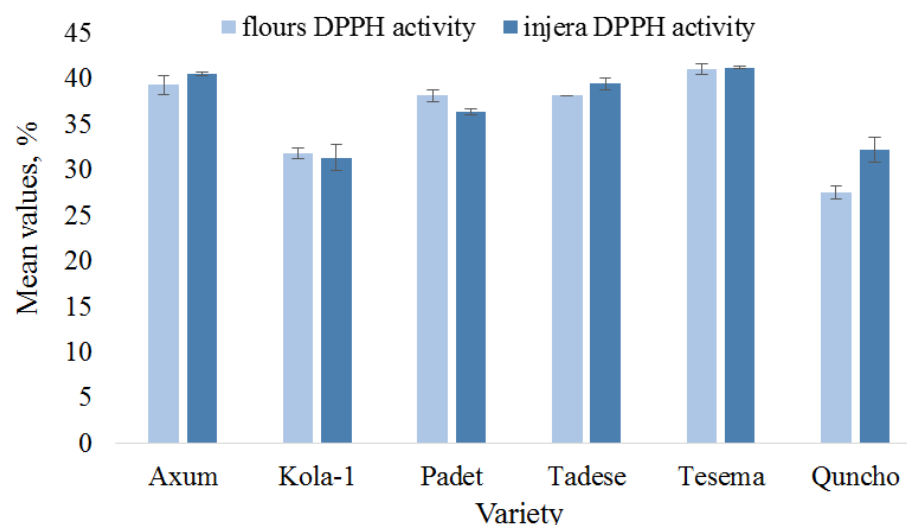


Figure 2. DPPH radical scavenging activity of flours and injera.

Table 3. Color properties of flours and injera.

Variety	Flour				Injera			
	L*	a*	b*	ΔE	L*	a*	b*	ΔE
<i>Axum</i>	61.93±0.28 ^c	1.13±0.25 ^b	2.81±0.31 ^c	46.85±0.06 ^c	49.73±0.35 ^c	3.88±0.07 ^b	4.91±0.11 ^d	35.52±0.25 ^{cd}
<i>Kola-1</i>	59.73±0.20 ^d	-0.12±0.03 ^c	4.21±0.11 ^b	45.59±0.04 ^d	57.92±0.15 ^a	2.14±0.01 ^d	8.39±0.03 ^a	42.08±0.36 ^a
<i>Padet</i>	62.16±0.41 ^c	1.05±0.06 ^b	2.53±0.46 ^{cd}	46.99±0.42 ^c	48.04±0.35 ^d	3.16±0.02 ^c	3.69±0.04 ^e	35.17±0.23 ^d
<i>Tadese</i>	63.61±0.27 ^b	0.78±0.17 ^b	2.18±0.10 ^d	48.34±0.31 ^b	50.25±0.42 ^c	3.78±0.13 ^b	4.77±0.15 ^d	35.99±0.15 ^c
<i>Tesema</i>	64.77±0.44 ^a	1.14±0.19 ^b	2.78±0.12 ^c	49.08±0.26 ^a	49.54±0.64 ^c	4.47±0.09 ^a	5.32±0.11 ^c	34.89±0.15 ^d
<i>Quncho</i>	60.05±0.13 ^d	1.68±0.03 ^a	6.85±0.12 ^a	44.25±0.09 ^e	53.14±0.35 ^b	2.08±0.02 ^d	5.88±0.07 ^b	38.88±0.22 ^b

Mean ± SD with different superscripts in a column varied significantly ($p < 0.05$) within and between different millet cultivars and control, $n=3$. L*, a*, b* and ΔE represents degree of lightness, red-green, yellow-blue and total color difference, respectively.

color in flours and injera, respectively, whereas *Tesema* exhibited the maximum a* value in injera indicating more redness. In comparison with the flour from pearl millet variety, flours from finger millet varieties showed the maximum redness with

non-significant ($p < 0.05$) difference among them. A significant difference was observed among flours of millet varieties in their b* value (yellow-blue color) which ranged from 2.18 to 4.21, the highest for *Kola-1* (which exhibited maximum blueness)

and the lowest for *Tadese* (attained maximum yellowness). *Quncho* showed the highest b* value, which indicated a less yellowness and more blueness. Baking increased the b* value in injera and ranged from 3.69 to 8.39 with the

highest and lowest for *Kola-1* and *Padet*, respectively. The total color difference (ΔE) ranged from 44.25 to 49.08 for flours and from 34.89 to 42.08 for injera, with *Tesema* having the highest and the lowest in flour and injera, respectively. Significant reduction of ΔE was noticed after baking. Among finger millet cultivars, the lowest ΔE was noticed in *Axum* and *Padet* varieties with statistically non-significant difference in between. The total color difference of flours and injera was negatively correlated ($r=-0.74$), whereas a^* value was positively correlated with the anthocyanin content in both flours ($r=0.62$) and injera's ($r=0.59$) and were statistically different. Siroha and Sandhu, (2017) revealed that toasting significantly reduced the L^* value and increased a^* and b^* values in flours from pearl millet cultivars.

Conclusion

Even though tef injera is more popular than millet, it contains less health-promoting compounds in relation to its phytochemicals content and less effective on oxidative stress and probably associated health disorders. The present study revealed that among millet varieties, injera made from *Kola-1* and *Tesema* varieties were more preferred in its overall consumer acceptance and the result was comparable to the most preferred tef injera. Finger millet varieties had the highest contents of total phenols, total flavonoids and DPPH radical scavenging activity than pearl millet variety. Among finger millet variety, *Tesema* showed the highest in polyphenols content and antioxidant activity. A lighter flour color was observed with *Tesema*. *Kola-1* had the highest total color difference both in flour and injera.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Hydrocolloids as beers foam stabilizer

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Beer foam is one of the most important parameters for consumers, affecting their purchase decision and satisfaction. Studies indicate that foam stability is positively influenced by its viscosity, and based on this fact the brewing industry uses propylene glycol alginate (PGA) as a stabilizer. However, PGA has its use restricted by Brazilian legislation to 0.07 g/L of beer. The objective of this research was to present alternatives to PGA, improving beer foam stability by adding other hydrocolloids, which does not have a maximum amount established by Brazilian legislation, and determining those with the greatest influence on the foam stability without significantly changing the colloidal stability and pH of the beverage. Colloidal stability, viscosity and foam shaking tests showed that the higher the hydrocolloid concentration in the beer, the greater the foam stability. PGA exhibited the best performance among the hydrocolloids tested, followed by Genu® Pectin type 106 HV and Genu® GUM type RL 200-Z, which had a significantly better foaming capacity than the control. Differently from the initial hypothesis, the foam stability was found to be more influenced by the chemical structure of the hydrocolloids, mainly their degree of esterification, than by foam viscosity.

Key words: Beverages, technology, viscosity.

INTRODUCTION

Beer is defined by Brazilian legislation as the beverage resulting from alcoholic fermentation, using brewer's yeast, malted barley wort or malt extract, previously submitted to a cooking process, added with hops or hop extract. A portion of the malted barley or the malt extract may be replaced in up to 45% by beer adjuncts, comprising unmalted barley and other cereals suitable for human consumption, malted or not, as well as starches and vegetable origins sugars (Brasil, 2009, 2011, 2019).

The most consumed beers in Brazil are of Pilsen type,

also called mainstream beers, which belongs to the Lager family (Mintel, 2013; AAFC, 2019). However, compared with the original Pilsen beers from the Czech Republic, these beers are lighter and more refreshing, less bitter and less full-bodied, what can be attributed to the addition of beer's adjuncts, such as corn, rice and syrups (BJCP, 2015; Justdrinks, 2018; Mega et al., 2011; Mintel, 2013). These beers are more correctly classified as American Light Lagers or American Standard Lagers and generally have original gravity content between 1.028

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and 1.050, final gravity between 0.998 and 1.010 g/cm³, the color varying from 2-4 SRM, bitterness from 8-18 IBU and alcohol by volume (ABV) from 2,8 to 5,3% (BJCP, 2015).

World beer production in 2018 was estimated in 1.90 billion hl (BarthHaas, 2019) and the market revenue in approximately USD 570 billion (Statista, 2020), in which lager beers accounted for USD 366.94 billion. In volume, Madson (2017) and Arthur (2018) estimate that lagers represent around 90% of all produced beers, a market shared mainly by standard and premium lagers (Marston's, 2018).

Although they still represent a large market, mainstream standard lagers are losing space for premium and super-premium beers, in a trend known as "drink less, but drink better". Looking for new experiences, consumers are moving to different styles, flavored beer and mixed drinks (Arthur, 2018). Also, considering the growing population with gluten-related-diseases, breweries are seeking alternatives for offering high-quality gluten-free beers, as using gluten-free cereals or enzymes treatments (Hager et al., 2014; Rubio-Flores and Serna-Saldivar, 2016). These alternatives can interfere in quality parameters appreciated by consumers, as beer foam (Hughes and Baxter, 2001; Viejo et al., 2019; Deotale et al., 2020).

Beer foam is one of the first aspects perceived by beer consumers, along with color and turbidity, after the beer is poured into a glass. It is a very appreciated attribute, affecting consumer purchase decision and satisfaction (Hughes and Baxter, 2001). The foam layer is also important to protect beer from direct oxidation, to continuously release volatile compounds into the air as bubbles pop, contributing to the perception of beer aroma, and to the beer texture (Farber and Barth, 2019).

Barley proteins, especially protein Z (Niu et al., 2018), hop acids and non-starch polysaccharides are the main factors responsible for the formation and maintenance of beer foam. However, other factors such as the pH of the beverage, solubility and diffusivity of the gas in the liquid and the viscosity of the liquid also influence its stability (Bamforth et al., 2009, Jarpa-Parra et al., 2016). Inversely, the most damaging substances for foam are lipids because they affect surface tension (Gordon et al., 2018).

Therefore, the overall composition of the raw materials has a great impact on beer foam formation and stability, and it varies with the choice of malts and grains; the use of specialty malts or adjuncts; the proportion of ingredients; the addition of unusual ingredients to achieve different tastes and flavors; the alternatives used to produce gluten-free beers; the amount of hops; and the addition of foam stabilizers.

Foam is a colloidal system formed by a continuous solid or liquid phase and a discontinuous gaseous phase. Foam stability is correlated to the presence of

foam-positive substances and absence of foam-negative negative ones (Bamforth, 2017). The amount of liquid present in the foam is also time-dependent; it leaves the foam under the influence of gravity and "plateau border suction" in a process called drainage (Evans and Sheehan, 2002). For most beer foams, drainage precedes coalescence, which is the combination of two or more small bubbles to form larger bubbles. Occurring concomitantly, the dismutation or maturation of Ostwald consists in the fact that large bubbles increase in volume through the migration of the gas from the small bubbles, generating the weakening of the film around the gas bubble, with consequent rupture of the bubble. These latter two processes are more noticeable to consumers than the drainage itself, but of equal importance in terms of overall foam stability (Hughes and Baxter, 2001; Ronteltap et al., 1991).

Ronteltap et al. (1991) concluded that the forces that counteract drainage are the viscosity of the beer and the capillary effects of the foam surface. The influence of viscosity on foam stability is consistent with observations made, which show increased foam stability at low temperatures.

Increased beer viscosity can be achieved by the addition of thickening agents, such as gums, pectin, and alginate, which may indirectly contribute to an increased film thickness between the gas bubbles, decreasing drainage rate. Furthermore, the hydrocolloids structure is composed by chemical groups that can interact with other components of the film of the gas bubbles, contributing to the maintenance of its integrity (Hughes and Baxter, 2001; Azizpour et al., 2017).

Studies have shown that foam stability is positively influenced by the increase in the beer viscosity. Therefore, it can be achieved by the addition of stabilizing agents as gums, pectins and alginates, allowed for use in Brazil in accordance with Resolution RDC 65 of November 29, 2011, which provides for the approval of the use of food additives for brewing (Brasil, 2011).

Hydrocolloids are high molecular weight polysaccharides extracted from plants, algae or produced by microbial synthesis. They are mostly water-soluble and have thickening and/or gelling properties under specific conditions. They are currently used in all areas of the food industry, with increasing application in pharmaceutical and cosmetics (Cargill, 2018; Li and Nie, 2016).

The alginates are the hydrocolloids currently used by the brewing industry; however, the propylene glycol alginate (PGA) has its use restricted by Brazilian legislation to 0.07 g per 1000 ml of beer, while other gums and pectins do not have a maximum established amount. Among the commonly used hydrocolloids in the food industry, either as gelling or as thickening agents, are xanthan gum; sodium carboxymethyl cellulose or CMC; the alginates; gellan gum; pectin; and locust bean

Table 1. Summary of the characteristics of the hydrocolloids used in the research.

Code	Commercial name	Supplier	Family
PGA	KIMILOID® BF	Kimica Vogler Ing.	Alginate
GG	Gelan Kelcogel® HF-B	CP Kelco	Gellan
CG	CMC Cekol® 30.000	CP Kelco	Carboxymethylcellulose
CGH	CMC Cekol® HVD	CP Kelco	Carboxymethylcellulose
L200	Genu® GUM RL 200-Z	CP Kelco	Locust
P106	Genu® Pectin 106-HV	CP Kelco	Pectin
P121	Genu® Pectin 121 Slow Set	CP Kelco	Pectin
P102	GENU® Pectin LM 102-AS	CP Kelco	Pectin
KG	Carragen GENUVISO® CSM-2	CP Kelco	Carrageenan
KGK	Carragen GENULACTA® K-100	CP Kelco	Carrageenan
XRD	Xantan Keltrol® RD	CP Kelco	Xanthan
X521	Xantan Keltrol® 521	CP Kelco	Xanthan

gum or LBG (Mahmood et al., 2017), which were tested in this study.

The objective of this study was to evaluate the low cost and widely available hydrocolloids on the market as an alternative to propylene glycol alginate (PGA) as a stabilizer for Pilsen type mainstream beer and others in which substitution of barley malt by other grains or adjuncts is relevant. The influence of hydrocolloids on foam stability was studied, as well as side effects on colloidal stability and beer pH.

MATERIALS AND METHODS

Twelve hydrocolloids, from 7 different families, were chosen, based on their functionality, application, and availability in the Brazilian market (Table 1). They were obtained from CP Kelco (Limeira - SP, Brazil) and Kimica Vogler Ing (São Bernardo do Campo - SP, Brazil).

For the colloidal stability, viscosity and foam stability preliminary tests, a commercial beer without any additive or adjunct was used. For the subsequent tests, 40 L of an American lager beer (Pilsen type beer) was produced in the pilot plant of the Department of Food Technology of the School of Food Engineering, with 20% of adjuncts (rice), a similar amount to those beers found in the market, using the following ingredients: Pilsen malt (Cooperativa Agrária Agroindustrial, Guarapava - PR, Brazil); rice (Pileco® Nobre Alimentos Ltda, Alegrete - RS, Brazil); drinking water; cluster hops pellets with 5.7% alfa-acids (Lamas Brew Shop, Campinas - SP, Brazil); and dehydrated lager yeast Saflager w-34/70 (Fermentis Lesaffre, Marcq-en-Barœul, France).

Palmitic, oleic and linoleic acids, all $\geq 99\%$ purity, obtained from Sigma Aldrich (São Paulo - SP, Brazil), were used to evaluate foam stability.

Preparation of hydrocolloid base solutions

The hydrocolloids were dispersed in water at 1 g/L using a homogenizer (IKA Labortechnik® T-25 Basic, with IKA Labortechnik® S 25 N-25F rod) at 8000 rpm for one minute. The samples were then placed in a water bath (Buchi® b-480) with

boiling water, coupled to a piece of variable-speed mixing equipment (Technical® TE039/1, 3.5 cm diameter naval impeller, 800 rpm), and warmed up to 85°C. For the preliminary tests, in water or commercial beer, stock solutions (1 g/L) were made in triplicate. For the final tests, in the produced beer, only one stock solution of each hydrocolloid was made, which was dissolved in the degassed beer at the concentrations to be tested, in triplicate.

Beer degasification by helium injection

The procedure was carried out by injection of 50 kPa helium as a degasser (Shimadzu® DGU-2a) into a 2 L glass beaker containing about 700 ml of beer at 8°C. To avoid excessive foaming during helium degasification, a second 5-mm diameter aperture diffuser was connected in the helium outlet channels of the apparatus to form large bubbles, to dismantle the foam by causing its rapid collapse. The beer was then transferred to a 4-L beaker partially immersed in an ultrasonic bath (Sharp® UT-204) for 10 min to remove residual gases. The degassed beer was stored in amber screw-capped glass bottles at 4°C and used as quickly as possible to avoid degradation.

Dilution of hydrocolloids in commercial beer

Samples were prepared with 90% degassed beer and 10% hydrocolloid solutions (aliquots from the base solution of hydrocolloids plus the necessary water) to obtain beers with 0.01; 0.03; 0.05; 0.07; 0.09 and 0.1 g/L of each hydrocolloid, as exemplified in Figure 1. A control was prepared to contain 10% of water. By adding the already dissolved hydrocolloid in the degassed beer, the process to be used in the industry was simulated, where the hydrocolloid solution is added to the filtered beer before the gasification and beer filling step.

Hydrocolloids pre-selection

A qualitative colloidal stability test was carried out based on Chapon's methodology (1968) using the degassed commercial beer (without adjuncts). Aliquots 10 ml of beer samples containing the different hydrocolloids at concentrations of 0.01, 0.05, and 0.1 g/L were incubated in test tubes at 8°C for 24 h to evidence precipitation

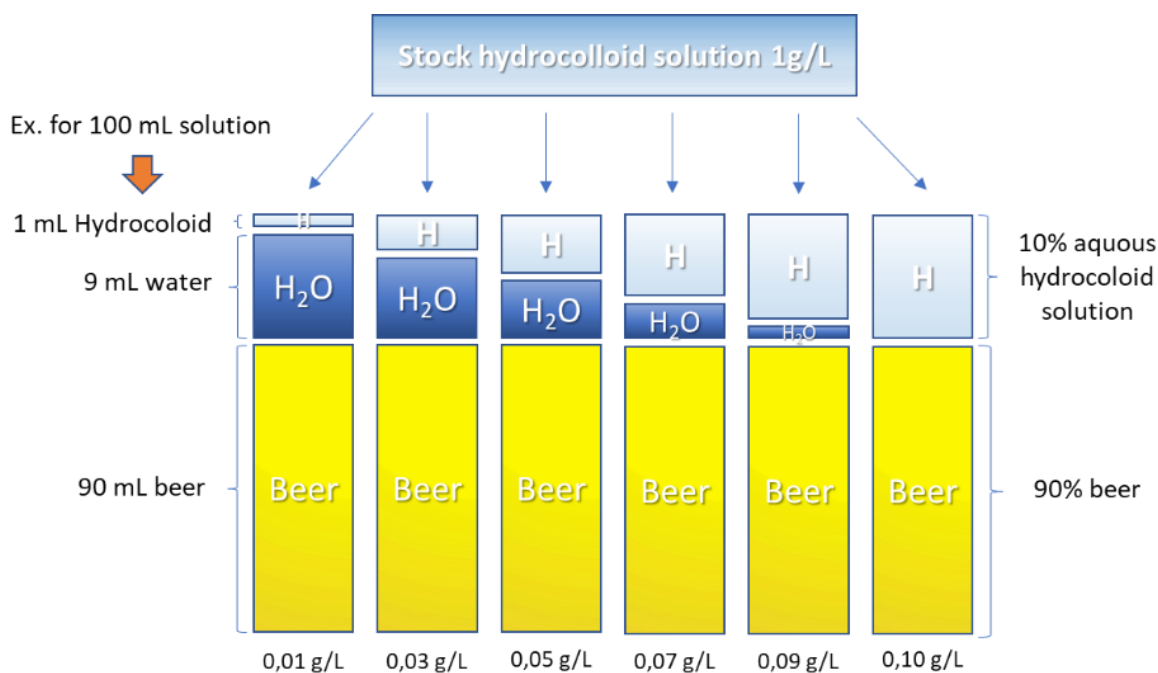


Figure 1. Hydrocolloid dilution scheme in beer.

or turbidity of the beer. After 24 h, the tubes were visually analyzed and compared to the control (beer plus distilled water). Then, 1 ml of anhydrous ethanol was added on the samples, which were incubated for another 24 h, to force the precipitation of the hydrocolloids in those samples which, in the first moment, had no turbidity neither precipitate. Images were recorded with a Canon® 6D camera.

pH

The pH of the hydrocolloid solutions in beer was measured using a pHmeter (Digimed DM 20) according to AOAC (2010).

Viscosity test

The assays were carried out at 20°C in a viscometer (Brookfield® LVDV-IIT with spindle and Brookfield ULA cup camera) coupled to a bath (Brookfield® TC-550). The tests were performed using 16 ml samples, at 60 rpm, according to the manufacturer's specifications. The solutions' viscosity was evaluated in triplicate, in solutions of distilled water and degassed beer in the concentrations 0.01; 0.03; 0.05; 0.07; 0.09 and 0.10 g/L.

Shaking test

The shaking test was based on the method developed by Knapp and Bamforth (2002). The test tubes were placed in a rack and images were recorded at times 0 and 30 min, respectively with a camera (Canon® SX150IS) attached to a tripod, then, analyzed with the help of the software program Charten software® Bitruler. Assays were carried out with samples of degassed beer containing hydrocolloids at concentrations of 0.01, 0.05 and 0.10 g/L, in

triplicate. The percentage of residual foam was calculated by difference with the initial and final foam data. Only the best performance hydrocolloids were chosen to be evaluated in the following foam stability tests using a produced beer with 20% of adjunct.

Beer preparation

For the final tests, an American Lager beer was prepared. The ground rice (Quadrimat Senior Brabender® roller mill) was mixed with water and boiled in a jacketed kettle until complete gelatinization of the starch, resulting in 2 h of processing. The gelatinization was verified by polarized light microscopy (Olympus BX51) with a magnification of 10x and 100x in slides, with an aqueous dispersion of starch (0.1 g of starch with 5 ml of water) (Zambrano et al., 2001).

The previously milled malt (Guzzo mill cod. 2508) was mixed with the adjunct and mashing was conducted at 63-64°C for 1 h. The end of the process was verified through the 0.01 N Iodine test, in which the absence of purple color in the wort indicates the complete hydrolysis of the starch into smaller sugars.

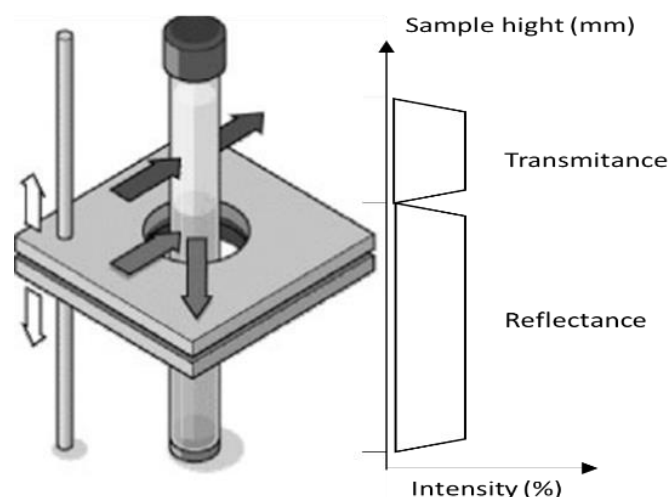
The wort was separated from the malt husks and other insoluble materials by centrifugation. The clarified wort was boiled for 1 h and hopping was conducted in two steps: 70% at the beginning of the boil and 30% at the last 15 min.

The cooling was carried out in a plate heat exchanger (0.7 m² of exchange surface), in counterflow, until a temperature < 27°C was reached. The soluble solids concentration of the wort was 11 °Brix. It was placed in 15 L fermenters and inoculated with 11.5 g of the dehydrated yeast.

The fermentation was carried out at 8°C for 18 days. On the 19th day, temperature was decreased to 4°C, initiating maturation. On the 28th day, maturation was finished, and beer was stored at 2°C. For the analysis, the beer was filtered at 4°C on filter paper

Table 2. Production parameters of Pilsen clear lager with 20 % adjunct.

Adjunct	20% w/w
Apparent fermentation	92.7%
Original extract	11 °Brix
Alcohol by volume	6.45%v/v
Bitterness	8.2 IBU
pH	4.32

**Figure 2.** Turbiscan measurement principle. Source: Buron et al. (2004).

(Whatman 1 Cat No 1001 110) with the aid of a vacuum pump, then degassed as described opportunely. Table 2 presents the process parameters.

Foam stability test by light scattering

The foam stability was also evaluated through light scattering using a vertical scanner (Turbiscan™ LAB expert), which produces a series of backscattering (BS %) and transmittance (T %) profiles as a function of time and tube length (Pasin et al., 2014). For this research, only the transmittance value was used. To measure foam stability, 10 ml of the sample at 20°C was added in the equipment's tube and manually shaken in the same way as in the shaking test described above. Immediately after that, the tube was disposed of in the apparatus for the starting of the measurements, as illustrated in Figure 2. This procedure was performed 3 times for each sample and read at every 30 s over 30 min, as suggested by Knapp and Bamforth (2002).

For the result analysis, the peak thickness measurement performed by the device software, TurbSoft 2.0.0.28 (Formulation SAS, 2013), was used. The two peaks identified as correspondent to the beginning and the end of the foam in the tube length were selected in the Delta Transmission ($\Delta T\%$) chart, as described in Figure 3, which uses as the reference curve the scan transmittance

reading performed in 30 s. The software measured the width of each peak at $\Delta T = 5\%$, the value chosen to avoid noise readings. The peak width represents how much each reading is different from the reference reading ($t = 30$ s), that is, the width of the set of the two peaks represents the collapse of the foam over time. Thereafter, the smaller the peak width, the greater the hydrocolloids stabilizing power.

In addition to the analysis with every single hydrocolloid, to investigate synergetic effects between PGA and the other products, light scattering analysis was also performed for samples containing 0.05 g/L of PGA and 0.05 g/L of the other hydrocolloids.

From these data, a graph of foam collapse by time was built, which represents what happened to the foam height in the Turbiscan tube for 30 min, and it was possible to evaluate which hydrocolloid was able to improve the foam stability compared to the control.

Shaking test with free fatty acids (FFA) using the produced beer

Based on the work of Knapp and Bamforth (2002), a shaking test, similar as described previously, was carried out, using an intermediate hydrocolloid concentration of 0.05 g/L and an aliquot of 10 μ L of free fatty acids (FFA) dissolved in ethanol PA to reach a

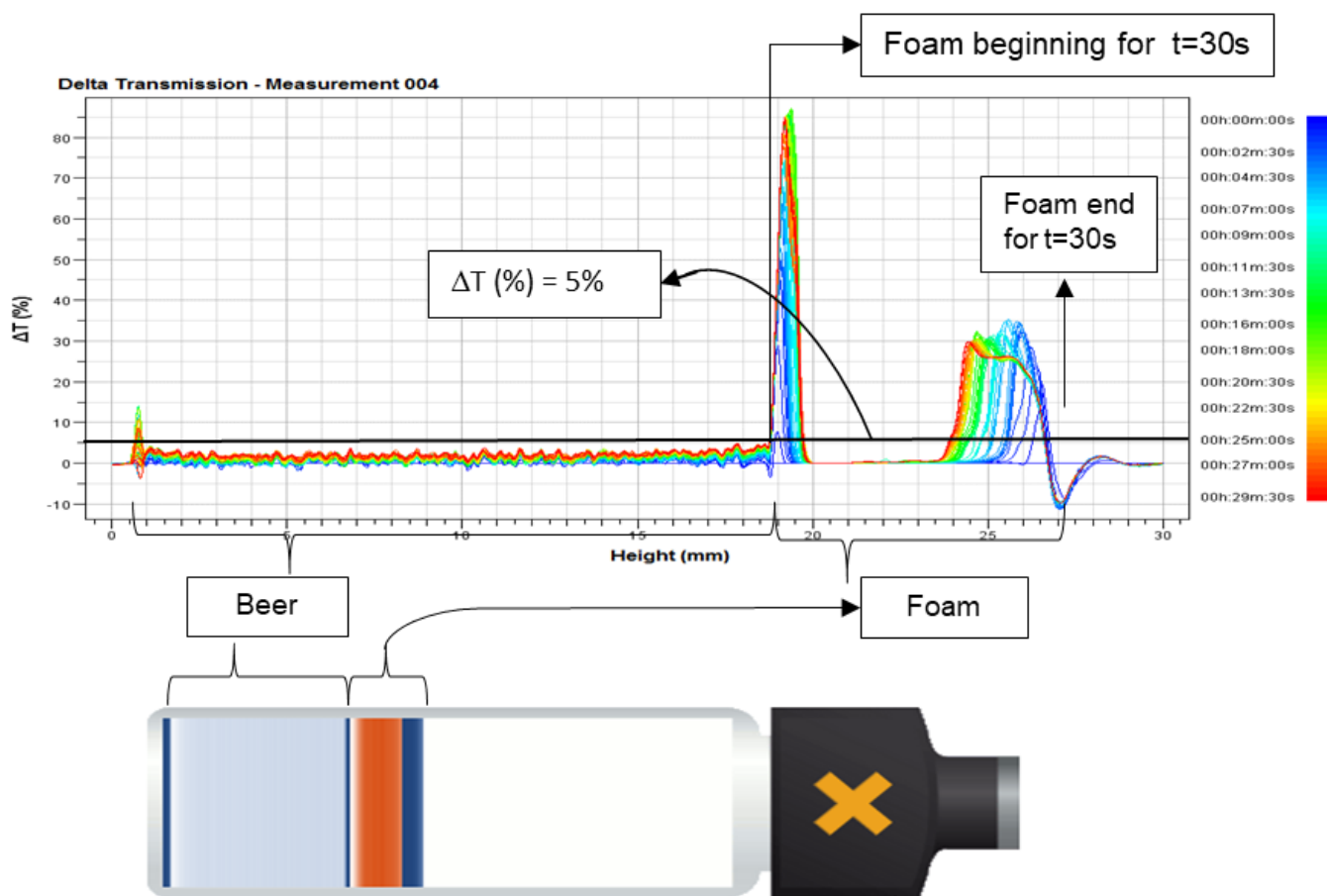


Figure 3. Transmittance (%) by height (mm) of the White sample. The darker blue line represents the first reading at $t = 30$ s (00 h: 00 min: 00 s in the graph). The red line represents the last reading at $t = 30$ min (00 h: 29 min: 30 s on the graph).

concentration of 1 mg/L of palmitic, oleic or linoleic acids. The agitation was done immediately after the introduction of the FFA, evaluating the damage generated in the beer just after the contact with the lipids, simulating problems related to beer service and consumption (Robert et al., 1977).

The foam height in the test tubes was recorded using a Canon® 6D camera with a Canon® 24-105 f4 L lens, mounted on a tripod, so that the videos were digitally treated (Adobe® Premiere Pro CC) to insert a time counter, and used to measure the foam height as function of the time, for the different hydrocolloids.

Statistical analysis

Data was analyzed with SAS statistical software (SAS, 2002), using Tuckey Tests ($p \leq 0.05$) for mean comparison.

RESULTS AND DISCUSSION

Hydrocolloids pre-selection

Concerning the preliminary tests, the hydrocolloids KG,

CG, CGH, XRD, and X521 were withdrawn from the study after 24 h incubation, because they caused haze, while KGK caused precipitation in the commercial beer, making their use impossible at the conditions tested. In addition, during the hydrocolloid dissolution and dilution stage, GG formed a visible gel or high viscosity solutions, even at low concentrations, making it difficult to handle, so it was also disqualified. After the addition of anhydrous alcohol, P102 formed a suspension gel and it was also removed.

Through the preliminary visual analysis, the hydrocolloids PGA, L200, P106, and P121 were chosen to remaining assays, at concentrations 0.01; 0.03; 0.05; 0.07; 0.09 and 0.10 g/L.

pH

The pH value of beer solutions, independent of the hydrocolloid used and its concentration, remained between 4.57 (P106 – 0.01 g/L) and 4.65 (control), with

Table 3. Viscosity (mPa.s) at 60 rpm of solutions of distilled water or beer with hydrocolloid at different concentrations.

g/L	PGA	L200	P106	P121
In distilled water				
0.01	1.13±0.01 ⁱ	1.14±0.01 ^{hi}	1.13±0.01 ⁱ	1.14±0.01 ^{hi}
0.03	1.15±0.01 ^{ghi}	1.16±0.01 ^{fghi}	1.16±0.01 ^{fghi}	1.18±0.02 ^{fghi}
0.05	1.20±0.01 ^{defgh}	1.19±0.02 ^{efghi}	1.20±0.02 ^{defgh}	1.24±0.05 ^{bcde}
0.07	1.22±0.02 ^{bcddef}	1.21±0.05 ^{cdefg}	1.26±0.01 ^{abcd}	1.25±0.03 ^{abcde}
0.09	1.27±0.03 ^{abc}	1.28±0.03 ^{abc}	1.29±0.02 ^{abc}	1.27±0.00 ^{abc}
0.10	1.26±0.01 ^{abcd}	1.28±0.02 ^{ab}	1.29±0.02 ^a	1.29±0.01 ^a
In beer				
0.01	1.64±0.01 ^d	1.68±0.03 ^d	1.65±0.03 ^d	1.64±0.01 ^d
0.03	1.66±0.02 ^d	1.71±0.01 ^{cd}	1.65±0.02 ^d	1.66±0.02 ^d
0.05	1.67±0.02 ^d	1.75±0.01 ^{bc}	1.68±0.03 ^{cd}	1.66±0.02 ^d
0.07	1.67±0.02 ^d	1.79±0.02 ^{ab}	1.68±0.02 ^{cd}	1.68±0.01 ^{cd}
0.09	1.71±0.05 ^{cd}	1.82±0.03 ^a	1.67±0.02 ^d	1.69±0.01 ^{cd}
0.10	1.68±0.03 ^{cd}	1.83±0.02 ^a	1.69±0.01 ^{cd}	1.69±0.01 ^{cd}
Control	1.64±0.01 ^d	1.64±0.01 ^d	1.64±0.01 ^d	1.64±0.01 ^d

Means with different letters differ significantly by the Tukey test ($p < 0.05$).

no significant difference between the samples ($p < 0.05$). Despite this small difference, this is an important result, since pH influences hydrocolloid performance and solubility (CP Kelco, 2001; 2009; Ngouémazong et al., 2015). Jarpa-Parra et al. (2016) investigated the stability mechanisms of lentil legumin-like protein and polysaccharide foams at different environmental pH conditions (3.0 to 7.0), and the best foam stabilization occurred at pH 5.0, followed by pH 3.0, while pH 7.0 led to phase separation.

Viscosity tests

As expected (Li and Nie, 2016), the viscosity of the hydrocolloid solutions in water increased with the concentration (Table 3); however, there was no significant difference between the samples of the different hydrocolloids at the same concentration. Regarding the formulations with beer, viscosities did not differ statistically from the control either, except for the formulation with hydrocolloid L200, where from the concentration 0.05 g/L, the viscosity increased significantly. However, it was noted that hydrocolloids have a slight tendency to increase viscosity as their concentration increases. In addition, it is important to note that the hydrocolloid used in industry, PGA, at the maximum concentration allowed by legislation, was not able to provide a significant increase ($p < 0.05$) in beer viscosity.

The difference in the viscosity profile of the

hydrocolloids when in distilled water or beer solutions probably occurs due to the small differences in pH and the complexity in the beer matrix, whose components can interact with hydrocolloid molecules, altering their performance. In addition, beer contains mono and divalent metal ions that interfere with the viscosity of solutions containing hydrocolloids.

When beers were produced using mixtures of hydrocolloids PGA/P106, or PGA/L200, at 0.05 g/L (total 0.10 g/L), there was no significant difference ($p < 0.05$) in their respective viscosities, when compared to beers formulated alone with 0.10 g/L of P106 or PGA, respectively. As the effect on beer viscosity by the addition of hydrocolloids (Table 3) was statistically similar ($p < 0.05$) at all the concentrations tested, except for L200, we chose to continue with only three concentrations, two extremes, 0.01 and 0.10 g/L, and an intermediate, 0.05 g/L.

Foam stabilization

In the shaking tests (Table 4), the hydrocolloids showed a tendency to increase foam stability with increasing concentration, the higher the percentage of residual foam, the greater the foam stability provided by the hydrocolloids. Azizpour et al. (2017) showed a similar effect on the foaming properties of shrimp puree using different hydrocolloids (xanthan gum, tragacanth methylcellulose, and Arabic gum). Jarpa-Parra et al. (2016) revealed that guar, xanthan, and pectin improved

Table 4. Percentage of residual foam in hydrocolloid beer solutions after shaking test.

Solution (g/L)	PGA	L200	P106	P121
0.01	63.15±4.63 ^{bA}	58.98±3.44 ^{bcA}	63.96±6.19 ^{abA}	60.52±2.97 ^{abA}
0.05	76.32±5.45 ^{aA}	69.00±2.38 ^{abAB}	75.65±5.35 ^{aAB}	61.09±8.05 ^{abB}
0.10	76.27±0.24 ^{aA}	72.36±3.87 ^{aAB}	72.17±3.10 ^{aAB}	67.09±0.82 ^{abB}
Control	52.92±5.38 ^b	52.92±5.38 ^c	52.92±5.38 ^b	52.92±5.38 ^b

Means followed by the same letters (uppercase in the row and lowercase in the column) do not differ significantly by the Tukey test ($p < 0.05$).

Table 5. Variation in the foam height (%) of beers when fatty acids are added, in relation to the control with each FFA.

Solution (g/L)	Palmitic		Oleic		Linoleic	
	0	30 min	0	30 min	0	30 min
Control*	-39.33	-90.05	-36.95	-56.12	-56.93	-74.95
PGA 0.05	25.42	25	58.45	57.89	99.75	100
L200 0.05	-38.90	-39.28	-2.29	-2.02	73.55	73.75
P106 0.05	-8.87	-8.92	12.40	12.55	57.21	57.44
P121 0.05	-9.21	-8.90	64.69	65.18	103.36	103.54

*Variation in relation to the control without FFA.

the stability of lentil legumin-like protein foams at mildly acidic pH, by the formation of aggregates and a dense network, which helped mitigate drainage and avoid coarsening.

There was no significant difference ($p < 0.05$) between 0.05 and 0.10 g/L for all hydrocolloids, and, as the concentration of 0.01 g/L was insufficient for foam stabilization, we concluded that the concentration of 0.05 g/L was optimal.

According to Table 4, P121 showed the lowest residual foam averages by concentration, while PGA, L200, and P106 were similar at concentrations of 0.05 and 0.10 g/L. Thus, only the latter hydrocolloids were evaluated in the light scattering assays.

When comparing Tables 3 and 4, it was noted that the increase in viscosity is not necessarily related to the increase in the foam stabilizing property conferred by the hydrocolloids, as initially assumed. The hydrocolloid stabilizing power can then be attributed to their chemical nature and interactions with the different foam-forming chemical compounds (Hughes and Baxter, 2001), as proteins (Wijaya et al., 2015).

This hypothesis is reinforced when comparing the two hydrocolloids of the same family, but different in degrees of esterification: P106 has a better performance as a foam stabilizer than P121 and presents a 10% higher degree of esterification (69.2 and 59.4%, respectively) (CP Kelco, 2009). Freitas et al. (2017) also demonstrated high-methoxyl pectin shows a higher solubility in a greater pH range, increasing the stabilization of protein-pectin

complexes. It is known that the protein content is significantly correlated to parameters representative of foam stability (Condé et al., 2017), especially protein Z (Niu et al., 2018). Thus, it is confirmed that the higher the degree of esterification, the greater its foam stabilizing capacity, which has already been described in the literature for alginates (Hughes and Baxter, 2001).

Shaking test with free fatty acids

Lipids and high ethanol concentrations are the main foam-negative substances (Bamforth, 2017). Kosin et al. (2018) considered foam stability dependent on both foam-stabilizing and foam-damaging compounds and Kosin et al. (2017) stated the importance of the presence of foam-negative compounds in the beer foam study since they can be present in many recipes. When studying model beer-foam solutions, Kosin et al. (2017) demonstrated the importance of the presence of linoleic acid in the model foam due to its interactions with specific components and its impact on the foam structure and behavior.

In this study, the influence of palmitic, oleic and linoleic acids on the beer-hydrocolloids solutions was determined. Table 5 shows the reduction (- signal) or increase (+ signal) in the foam height (%) when fatty acids are added in the beer with or without the addition of hydrocolloids.

Only PGA at 0.05 g/L showed a good performance

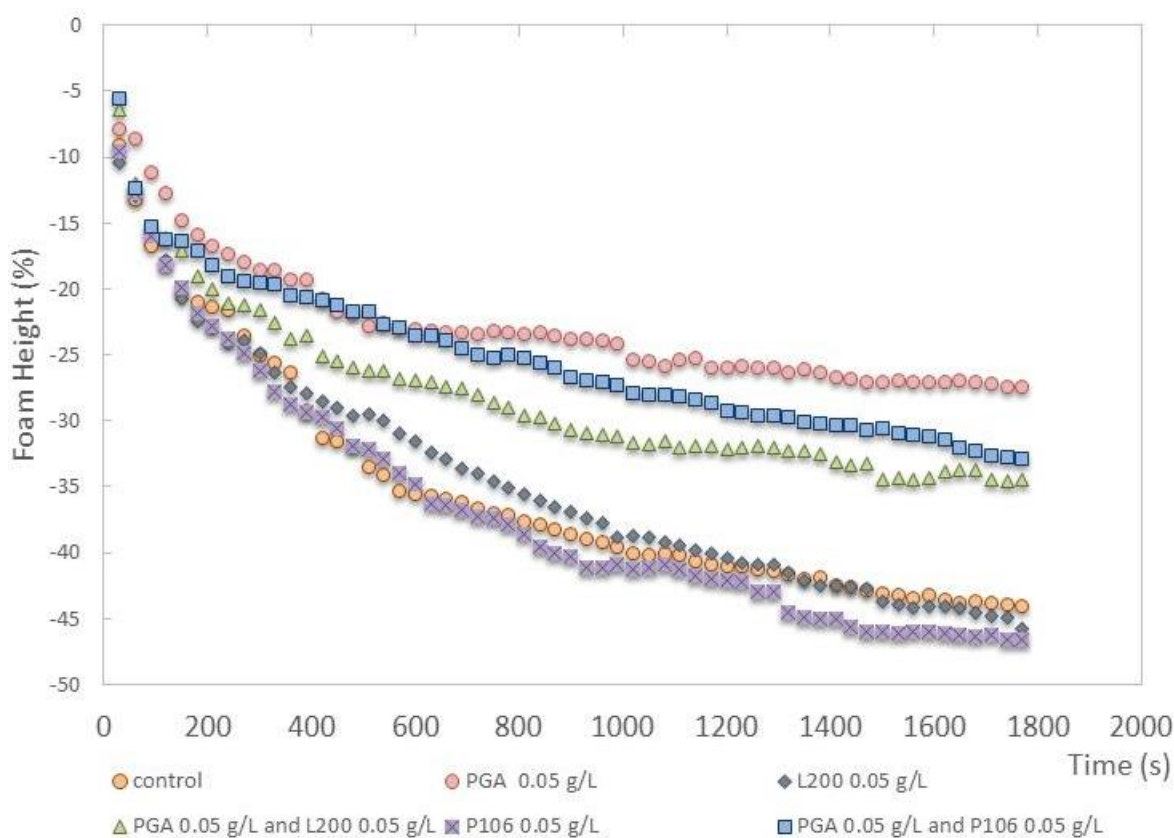


Figure 4. Foam collapse (%) by time (s). The mean height of the foam of the replicates at each time, relative to the initial height, is plotted.

when fatty acids were added, increasing the foam height in the presence of all interfering agents. Hydrocolloids P106 and especially P121, both at 0.05 g/L, also showed good results for oleic and linoleic acids. According to Qin et al. (2018), PGA shows emulsification-stabilizing characteristics which make it highly efficient in acid-protein beverages.

Foam stability test by light scattering

For their higher performance, L200 and P106 were chosen to be compared to PGA and the control, when applied to the beer produced in our Pilot Plant, at the concentration of 0.05 g/L. Figure 4 shows the foam collapse of the samples containing the three hydrocolloids evaluated at this stage (PGA, L200, and P106) and the mixtures of PGA with each of the other two in equal concentrations by the light scattering test. The 0% value of foam height at time zero represents the initial sample foam, which, as the analysis proceeds, collapses, acquiring a negative height value, as a percentage of the initial foam.

PGA was the only hydrocolloid capable of significantly decreasing foam collapse. It was verified that, from 660 s onward, PGA presents a significantly ($p < 0.05$) higher foam retention than the other samples. However, it should be noted that foam stabilization for 5 min (300 s) already represents sufficient time for a suitable consumption of the product. In this aspect, again PGA and its mixtures showed good results.

Conclusion

Although the viscosity of the hydrocolloid solutions in water increased significantly with the increase of its concentrations, the same did not occur when the hydrocolloids were added to beer, which can be attributed to the complexity of the beer matrix and possible interactions of the hydrocolloids with its components.

It was noted that the increase in the beer viscosity is not necessarily related to the increase in the foam stabilization conferred by the hydrocolloids at the concentrations tested, as originally supposed, but

probably to their chemical nature, mainly to their degree of esterification, and interactions with the different foam-forming chemical compounds.

The shaking test indicated that the ideal concentration of hydrocolloid to be used is 0.05 g/L since there was no significant difference ($p < 0.05$) between 0.05 and 0.10 g/L, and neither between 0.01 and control. In addition, in this test, the PGA presented the best foam stability indexes, followed by hydrocolloids L200 and P106.

PGA at 0.05 g/L showed the best performance when fatty acids were added, but P106 and especially P121, both at 0.05 g/L, also showed good results for oleic and linoleic acids.

When assessing the height of the foam for 30 min, it was noted that the PGA hydrocolloid was the only one able to significantly decrease ($p < 0.05$) its collapse. When foam stability was tested for PGA blends with L200 and P106, there was observed no synergy in terms of viscosity increase in the tested formulations; also, no positive synergistic effect when evaluating the foam height over the 30 min in the formulations tested.

Therefore, it is concluded that to improve the stability of industrialized beer foam, the addition of P121 and P106 are good alternatives, although PGA still shows the best results.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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