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Full Length Research Paper

Sensory, microbiological and physico-chemical characterization of *Klila*, a traditional cheese made in the south-west of Algeria

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***Klila,* an Algerian cheese produced in steppe and mountainous areas, is proving increasingly popular with consumers. It is traditionally made with ewe, goat or cow milk, leading to a product with high dry matter content (> 90%). In this work, we have characterized three different *Klila* cheeses made with the three different milks using physico-chemical and microbiological parameters. A triangle test was also performed on naïve consumers, and the three types of *Klila* were clearly distinguished by sensory analysis. They exhibited distinct features, in particular very low Aw (< 0.5) and pH values (< 4.5) preserving them from pathogens. Lactobacilli and enterococci were counted at low levels (< 4 log (cfu)/g) as well as some spore-forming bacteria (< 3 log (cfu)/g). Colonies were picked from MRS and BEA media. They were identified by sequencing and characterized on their ability to produce lactic acid and using REP-PCR. *Lb plantarum* was the main species isolated, followed by *Pediococcus pentosaceus*, *Leuconostoc pseudomesenteroïdes* and *Lactobacillus fermentum*. The *Enterococcus* genus was dominated by *Ec durans*, *Ec faecium* and *Ec hirae*. Among these two main populations, different subgroups were observed by means of the REP-PCR profiles and the lactic acid production of the isolates. Some strains were found in two and even three cheeses. We suppose that these microbes are representative of the environmental context in which *Klila* is produced.**

Key words: *Klila*, lactic acid bacteria, Algerian traditional cheese, *Lactobacillus plantarum, Enterococcus.*

INTRODUCTION

The cheeses were developed to preserve and even improve the biochemical properties of milk over time: vitamins, essential fatty acids, amino acids, minerals (Hill and Kethireddipalli, 2013). Although most cheeses are made with cow milk, in some regions such as Mediterranean countries, ewe, goat and even camel and

buffalo milks are used (Vivar-Qüintana et al., 2009). Today, consumers' appetite for raw milk cheeses seems to be on the increase. The flavour of these products is more intense and varied compared with cheeses made with pasteurised milk (Yohan et al., 2016). Many cheeses produced at the industrial scale derive from traditional farmhouse know-how (Demarigny and Gerber, 2014). In Mediterranean countries, traditional milk products are a major component of the daily diet in many small communities, especially in rural areas. The specificities of the climate (frequently dry and warm) means adapted processes have been developed to store milk over long periods. This is all the more true if we refer to rural areas found in the north of Africa. In these regions, people have developed many fermented milks and cheeses (Christelle et al., 2016).

In Algeria, traditional cheeses are almost always produced at a small, local scale, which explains why they are too frequently unknown to consumers further afield. Consequently, they are less popular than industrial cheeses. At the present time, around 10 traditional cheeses have been identified in this country (Aissaoui et al., 2006), but many others still have to be characterised.

The cheese *Klila* has been consumed by Algerian people for many centuries, probably from as far back as the Antiquity until now (Duval, 1855). This is a traditional handmade North African cheese with good nutritional values. Its high dry matter (more than 80%) allows it to be stored over long periods without any risk of microbial spoilage.

Klila is obtained from a fermented churned milk called *Lben*. *Lben* is heated, drained and pressed to obtain *Klila* (Harrati, 1974). This popular cheese is still based on a traditional farmhouse production method which contributes to the pleasant sensory attributes and nutritional properties, It enjoys and partly explains the increasing consumer demand for *Klila* (Lahsaoui, 2012). Unfortunately, *Klila* has never been seriously studied and only few microbiological, biochemical and technological data are available (Benamara and Megaiz, 1998; Leksir and Chemmam, 2015). On the contrary, *Jameed*, a similar Middle-East cheese or *Chhana* a cheese made in India, are well characterized (Mazahreh et al., 2008). These studies laid the groundwork for the development of specific technologies such as atomization or lyophilisation to produce these traditional cheeses at the industrial scale.

In this study, we compared three different *Klila* cheeses made from ewe, goat and cow milks at the farmhouse scale to establish the interesting sensory, microbiological and physico-chemical properties of these products. In a later work, the nutritional properties will also be characterized. This work aims to provide solid arguments for developing *Klila* production at the industrial scale.

MATERIALS AND METHODS

Sampling

Three different *Klila* cheeses made respectively with ewe, goat and cow milk were sampled in February and March 2015 in the Sfissifa daira region from Ain Sefra Wilaya Naama – around 730 km from the capital, Algiers, in the south-west of Algeria. The cheeses were made by the local tribes from this steppe and mountainous area (mean altitude: around 1, 200 m; Figure 1).

Cheese making

The cheeses were made according to the process summarized on Figure 2, using the three following milks:

i) The ewe milk was obtained from the local breed *El Hamra*,
ii) The cow milk was obtained from the selected breed BLA, a cross between the local breed BLL and the Holstein breed.
iii) The goat milk used to make the cheeses was a mix of two local breeds, *Arabia* and *Kabiles*.

Sensory analysis

To compare the cheeses, three triangle tests were performed. This methodology was chosen so as to evaluate the ability of untrained panellists to discern differences between similar products. Twenty-four persons were hired to test the cheeses in pairs in a controlled environment (in terms of light, smell and sound). Three sessions were necessary to cover the three possible comparisons: goat vs cow, goat vs ewe and ewe vs cow. Each time, the normalized procedure, as described in (AFNOR, 2007), was followed: sample randomization and coding, use of bread and still water, etc.

On the result sheet, panellists could also express how difficult they found the discrimination test to be (simple, hard, very hard). And they were also asked to qualify the type of sensation which allowed them to differentiate one product from the other two. The descriptors encompassed the following terms: colour, aspect, odour, aroma, rancid, bad taste, “other”.

Physico-chemical analyses

pH analysis: Cheeses were first ground and mixed with distilled water to obtain a diluted slurry (1/10), the pH of which was measured. pH evaluations were made in triplicate with a Hanna instrument pH meter (PHM210 standard, MeterLab®), immediately after grinding, and after a 20 min delay.

Water availability (Aw): Aw was measured in triplicate with an Aw-meter apparatus (Sprint TH 500, Novasina) on a 1.5 g cheese sample.

Dry matter (DM, %): To determine the dry matter, 5 g of cheese were dried in a halogen desiccator (Mettler). The result was directly expressed in percentages of dry matter.

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Figure 1. Location of the *Klila* production area, the Sfissifa daira region from Ain Sefra Wilaya Naama. Source: Google map.

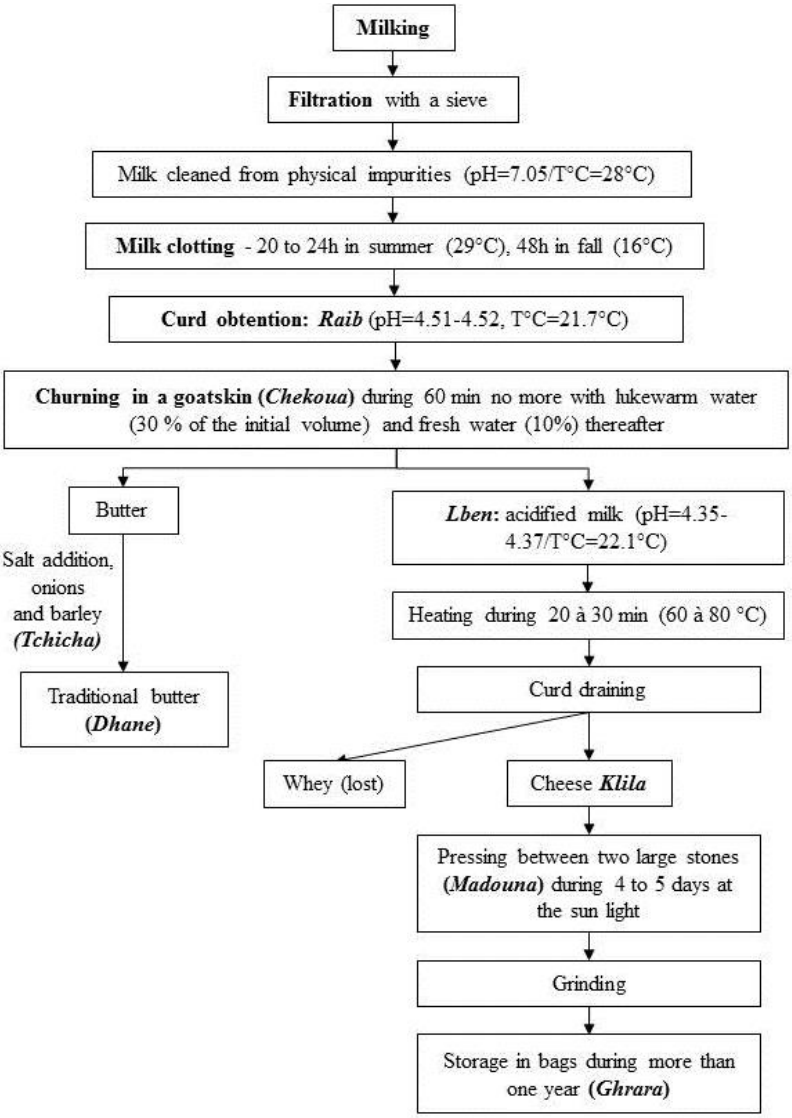

Figure 2. *Klila* cheese making.

Lactic acid concentration: Ten grams of cheese were first ground with 50 mL of distilled water. After centrifugation (6,000 g, 5 min), the supernatant was sampled for analysis. D- and L-lactic acids were measured with an enzymatic kit (D/L lactic acid Enzytec™ from R-Biopharm).

Fat rate (g/100 g of cheese): The evaluation of the fat rate followed the methodology described in the ISO 3433-2002 standard. The fat on dry matter (F/DM) ratio was then calculated.

Microbiological enumerations

Ten grams of cheese were diluted in a mix of trisodium citrate (4% w/v, 50 mL) and sterilised distilled water at 50°C (around 40 mL to adjust the final weight to 100 g). The resulting slurry (1/10) was used as the mother solution, and submitted to subsequent dilutions.

The following microbial populations were looked for:

i) Total count was performed on Plate Count Agar (30°C, 48 to 72 h, aerobic conditions, Biokar), according to the NF EN ISO 4833-1:10-2013 standard.

ii) The presumed *Bacillus* population was checked according to the methodology developed by Rosenkvist and Hansen (1995): Aerobic incubation at 30°C during 24 to 48 h.

iii) Bacillus cereus was specifically counted on Mossel agar (Biokar): aerobic incubation at 30°C during 24 to 48 h.

iv) Spores of butyric acid bacteria (BAB) were enumerated in the Bryant-Burkey broth modified by Bergère (Biokar) according to the most probable number method: anaerobic incubation during 7 days at 37°C.

v) Mesophilic presumed *Lactobacillus* were searched for on MRS agar (De Man et al*.,* 1960): anaerobic incubation at 30°C during 48 to 72 h.

vi) Positive β-glucuronidase *Escherichia coli* were counted on PTX agar (Biokar): aerobic incubation at 44°C during 18 to 24 h.

vii) Listeria monocytogenes was looked for according to the ISO 16140 standard. Twenty-five grams of grated cheese were incubated in semi-Fraser broth (225 mL) during 24 h at 30°C. Suspect colonies were then observed on Compas Listeria agar

(Biokar) after a 24 to 48 h delay at 37°C.
viii) Salmonella spp were searched for according to the ISO 6579 standard. This method involves successive cultures and a final check on XLD agar and Hektoen agar.
ix) Staphylococcus aureus was counted on Baird-Parker agar (Biokar): Aerobic incubation at 37°C during 24 h. In case of presumed *S. aureus* colonies, the presence of the coagulase was checked by exposing a 24 h culture (BHI, Biokar) to rabbit plasma.
x) Enterococci were enumerated on BEA (Biokar): Aerobic incubation during 24 h at 37°C.
xi) Moulds and yeasts were counted on GGC (Biokar): Aerobic incubation at 25°C for 5 days.

Phenotypic identification of presumed *Lactobacillus* and *Enterococcus* colonies

For each cheese, a maximum of 5 colonies of presumed enterococci were sampled from BEA to be cultured in M17 broth (Biokar) during 24 h at 37°C. After purification on M17 agar, isolates were frozen at -80°C in a mixture of glycerol 30% and M17 broth until further analyses. For colonies sampled on MRS, the same operation was performed (10 colonies per cheese) except that purifications, broth cultures and freezing were all performed on MRS. The culture temperature was 30°C.

Identifications relied on microscopic observations (after Gram staining) and catalase tests.

i) Metabolic affiliation: This test allowed the *Lactobacillus* strains to be separated following their preferred metabolic pathway: homofermentative, facultatively or strictly heterofermentative. The procedure described by Demarigny et al. (1997) was applied.
ii) The type of lactic acid produced was checked on a 24 h bacterial culture (M17 or MRS) with an enzymatic kit (D-/L-Lactic acid ENZYTEC^{TM}, R-Biopharm).

Genotypic characterizations

All the isolates were characterized on the basis of their REP-PCR profile. REP-PCR was performed with primers Rep1R-Dt and Rep2-D according to the methodology followed by Gemelas et al. (2013). The final identification relied on the 16S DNA sequencing. This was carried out by the LGC Genomics Company (Berlin, Germany). Sequences were analysed thanks to the NCBI data base (http://www.ncbi.nlm.nih.gov/BLAST/).

Statistical analyses

All the statistical analyses were performed using the XLSTAT software (Microsoft, 2010).

RESULTS

Sensory analyses

The three triangle tests led to the same results: 15 panellists out of 24 gave the correct answer. According to the binomial test (1/3), the probability is highly significant (< 1%). From a sensory point of view, the cheeses were dissimilar, even if the differences were scarce. In general, tasters found it difficult to separate goat milk from ewe milk cheeses (hard + very hard: 91%) and goat milk from cow milk cheeses (hard + very hard: 80%). However, 50% of the tasters found it easy to differentiate ewe milk from cow milk cheeses. The reasons for the difference observed between cheeses were not systematically recorded. Generally, colour and odour were given as the main discriminatory factors, and less frequently aroma and aspect. In a few rare cases, the rancid sensations (for goat milk vs ewe milk cheeses) and bad taste were also reported.

Analytical results

If we refer to Table 1, we can make two observations. Firstly, the cheeses were very dry (DM > 90%) and the available water inside was low (Aw < 0.500). Secondly, dry matter is the sole parameter which remains almost identical in all the cheeses (between 93.18 and 94.97%). The other parameters changed from cheese to cheese.

If we look specifically at the microbial populations, they were quite low. Except for the total count and the level of yeasts and moulds, the other populations were all inferior to 6 log (cfu)/g. No trace of *Samonella*, *E. coli*, *S. aureus* and *L. monocytogenes* was found. Spore forming bacteria (BAB and *B. cereus*, *Bacillus* spp) were also undetectable or at very low levels. Moreover, the lower the level of presumed lactobacilli, the higher the total count. And the same tendency opposed the level of spores of BAB and the presumed *Enterococcus* population.

All the physicochemical and microbial data were pooled together through a Principal Component Analysis (results not shown). Each cheese was clearly separated from the other. For instance, ewe milk cheeses were characterized by a low pH (4.25) and a low lactate rate (694 mg/100 g), but rather high levels of enterococci and lactobacilli (respectively, 4.41 and 5.43 log (cfu)/g).

From a general point of view, we could observe a strong positive correlation between total counts and Aw (r = 0.938) and a negative correlation between presumed lactobacilli, *Enterococcus*, *Bacillus* spp levels (r > 0.980). The incidence of pH on the microbial counts was less evident except with those of enterococci (r = -0.963).

Microbial characterizations

A total of 41 colonies were isolated from the analysis of the three cheeses, 26 on MRS agar (presumed lactobacilli) and 15 on BEA (presumed enterococci). All the isolates proved to be Gram positive and catalase negative. The isolates from BEA were all round (10) or tapered (5) cocci. Five out of the 26 isolates from the MRS sampling presented a shape between coccus and rods. The aspect of the colonies (the morphotype) was nearly the same whatever the origin of the sampling. Generally, they appeared round, smooth, convex and white.

Table 1. Physico-chemical and microbiological characterization of goat, ewe and cow milk *Klila*.

Characteristics	Ewe milk *Klila*	Goat milk *Klila*	Cow milk *Klila*
Total count	5.19	6.48	5.24
Yeasts and moulds	> 5.7	> 5.7	> 5.7
Bacillus	< 0.30	< 0.30	2.05
Bacillus cereus	< 0.70	< 0.70	3.63
Butyric acid bacteria *(Clostridium)*	1.00	2.30	2.60
Lactobacillus	5.43	3.69	6.15
Enterococcus	4.41	3.43	3.44
pH	4.25	4.46	4.40
Aw	0.368	0.467	0.320
Dry matter	94.51	94.97	93.18
Lactic acid	0.694	1.008	1.210
Fat	29.33	20.33	25.33
Fat on dry matter	31.03	21.41	27.18

Data are expressed in log(cfu)/g (bacterial populations), g/100 g of cheese (dry matter, lactic acid, fat) and % (fat on dry matter).

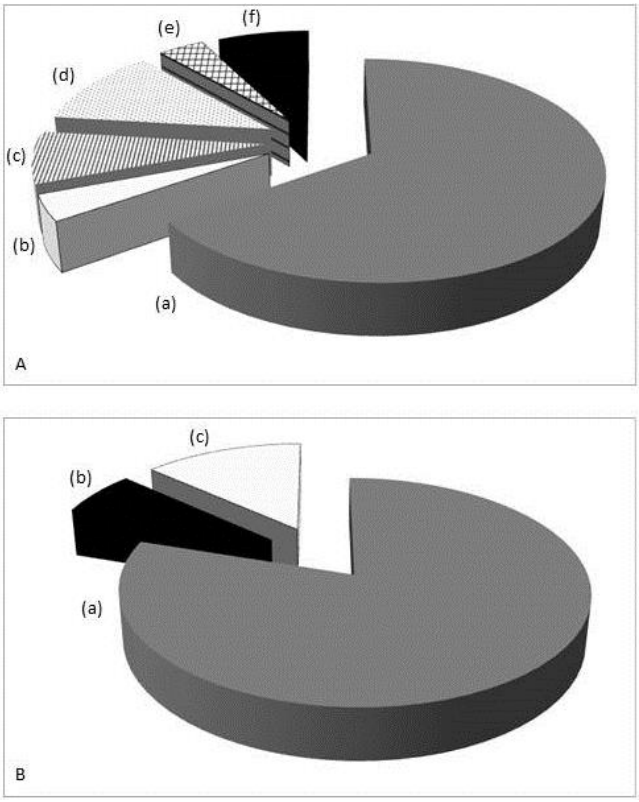

Figure 3. Distribution of the isolates according to the DNA 16S sequencing in respect of the culture medium used. (A)-MRS with a) *Lactobacillus plantarum* b) *Enterococcus durans* c) *Leuconostoc pseudomesenteroides* d) *Pediococcus pentosaceus* e) *Lactobacillus fermentum* f) unidentified; (B)-BEA with a) *Enterococcus durans* b) *Enterococcus hirae* c) *Enterococcus faecium*.

The DNA 16S sequencing of the isolates led to the results presented on Figure 3. On MRS, nearly 70% of the isolates belonged to the genus *Lactobacillus*, and more precisely to the species *Lb plantarum*. On BEA,

Table 2. Classification of the strains according to their lactate production.

Group 1 Low lactate production	Group 2 Mid lactate production	Group 3 High lactate production
Ec durans (a)	*Ec hirae* (a)	*Lb plantarum* (a)
Ec durans (a)	*Ec durans* (a)	*Lb plantarum* (a)
Ec durans (b)	*Ec faecium* (a)	*Lb plantarum* (a)
Ec durans (b)	*Ec durans* (b)	*Lb plantarum* (b)
Ec durans (c)	*Ec durans* (b)	*Lb plantarum* (b)
Ec durans (c)	*Ec durans* (b)	*Lb plantarum* (b)
Ec durans (c)	*Lb plantarum* (a)	*Ln pseudomesenteroides* (b)
Ec durans (c)	*Lb plantarum* (a)	*Lb plantarum* (b)
Ec faecium (c)	*Lb plantarum* (a)	*Lb plantarum* (b)
	Ec durans (c)	*Pc pentosaceus* (b)
	Ln pseudomesenteroides (c)	*Lb plantarum* (b)
		Unidentified
		Lb plantarum (b)
		Lb plantarum (b)
		Lb plantarum (b)
		Pc pentosaceus (c)
		Pc pentosaceus (c)
		Lb plantarum (b)
		Lb plantarum (a)
		Unidentified
		Lb plantarum (a)

Ec: *Enterococcus*, Lb: *Lactobacillus*, Ln: *Leuconostoc*. Strains from ewe milk cheeses, goat milk cheeses, and cow milk cheeses are noted respectively (a), (b), and (c).

80% of the isolates belonged to the species *Enterococcus durans*. The other species (*Lb fermentum*, *Pediococcus pentosaceus*, *Leuconostoc pseudomesenteroïdes*, *Ec hirae* and *Ec faecium*) were less frequently observed. No link was detected between the species identified and the type of cheese.

Lactobacilli were separated according to the test proposed by Kandler and Weiss (1986), that is, the production of CO_2 in presence of glucose and/ or gluconate. However, this test proved to be inappropriate when we crossed the results with those obtained from the sequencing. Only 6 *Lb plantarum* were identified as facultatively heterofermentative and 2 leuconostocs as strictly heterofermentative. Although the lactate test was pertinent for the lactobacilli, it led to strange results for some isolates of enterococci: some strains produced L- and D-lactate instead of L-lactate solely.

Strains were grouped by hierarchical classification according to the concentration of total lactate produced. Three groups were obtained (Table 2). The first group (low lactate producers; around 630 ppm of lactate) only included *Enterococcus* strains. In the third group (high producers; around 10 000ppm), the only isolates present belonged to the genus *Lactobacillus*, *Pediococcus* and *Leuconostoc*. The second group (around 4 900 ppm) mixed *Lactobacillus*, *Enterococcus* and *Leuconostoc* strains.

Strains diversity

Isolates were characterized on the basis of their REP PCR profile (Figure 4). *Lb plantarum* strains were divided into 7 clusters, containing respectively 10, 5, 4, 3, 1, 1, 1 isolates (Figure 4A). It is interesting to note that in each cluster containing more than one isolate, we systematically found strains coming from two cheeses or even all three cheese types. This was also observed for *Enterococcus* strains – 4 clusters (Figure 4B).

DISCUSSION

Klila cheeses are produced in a semi mountainous area of Algeria. People of this region have developed specific know-how to optimize milk storage for long periods in harsh climatic conditions. Whatever the type of milk used, they obtain a cheese with high dry matter, low water availability, and low pH which allows it to be kept for many months in harsh conditions. As such, it appears interesting to characterize *Klila* more precisely than had previously been done in view of considering industrial scale production (Leksir and Chemmam, 2015).

In this work, we studied the physico-chemical and microbiological characteristics of three *Klila* cheeses made either with cow, goat or ewe milk. Data obtained

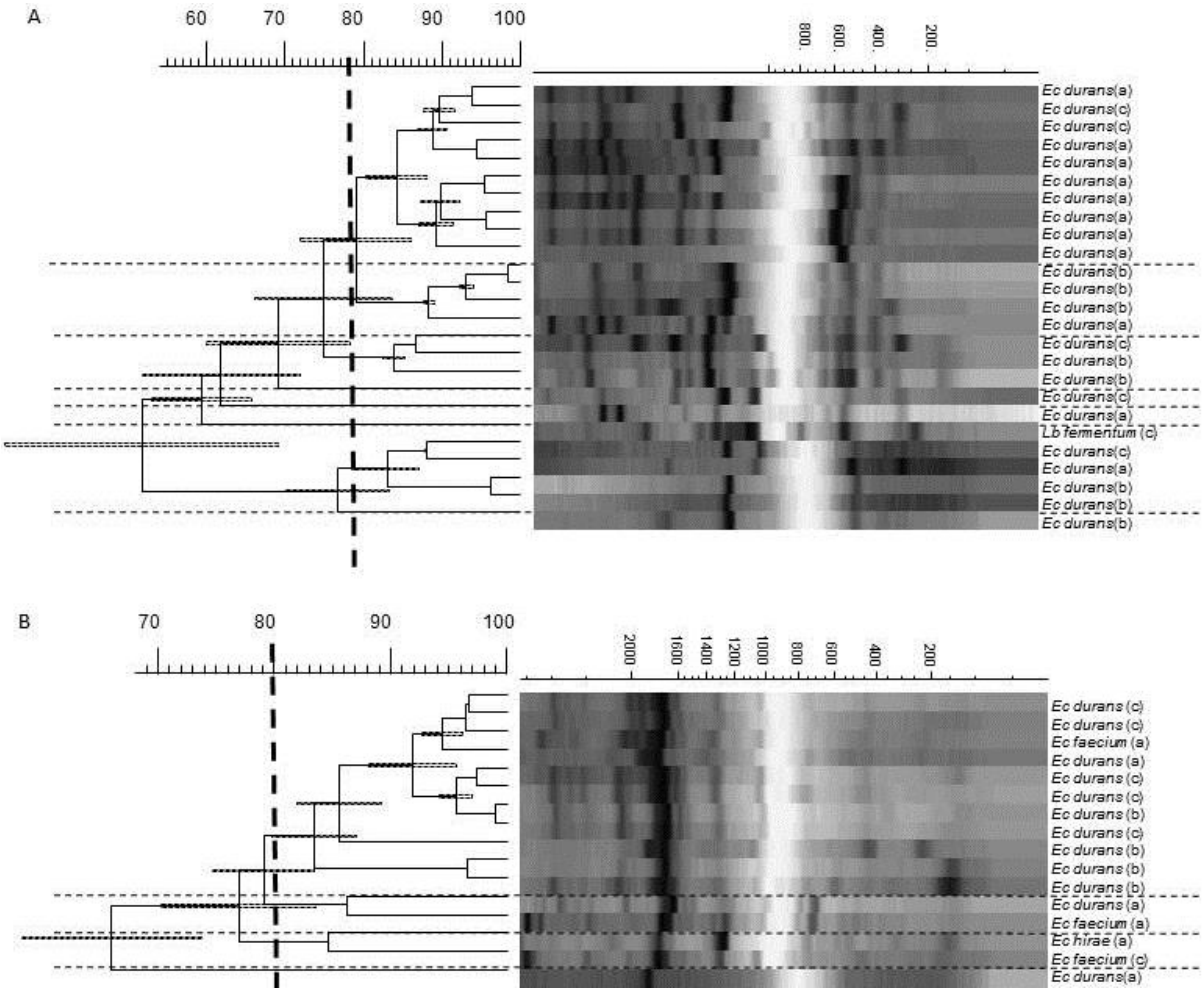

Figure 4. Dendrograms drawn by UPGMA of correlation value of normalized REP-PCR patterns from A) *Lactobacillus plantarum* and B) *Enterococcus* spp obtained with primers Rep1R-Dt and Rep2-D. Strains from ewe milk cheeses, goat milk cheeses, and cow milk cheeses are noted in a), b) and c) respectively. Horizontal dotted lines indicate the separations between clusters whereas the vertical dotted lines correspond with the coefficient of similarity (80%).

showed that, except for the dry matter, the three types of cheeses were clearly distinct. These observations appeared congruent with those obtained by Leksir and Chemmam (2015) and Guetouache and Guessas (2015) - for instance for the pH which ranged between 4.2 and 4.5 - but slightly higher than those reported by Rhiat et al. (2013). Concerning the dry matter, our measurements were higher than the values reported before, probably as a consequence of differences in the cheese making process (Claps and Morone, 2011). As a result of this high dry matter, the Aw of *Klila* was very low (< 0.467). The decrease of Aw below 0.6 is a guarantee against spoiling; it is explained by the heating, pressing, and drying of the curd (Cuvillier, 2005).

The fat rate of Klila can vary a lot depending on the season, the breed and the feed; but also from one region to another. This explains the differences between our results and those reported by Harrati (1974) (between 20 and 29 g/100 g vs 17 g/100) and Boubekri and Ohta (1996), collected in two Algerian regions (Batna and Sétif). If we consider the fat on dry matter ratio (between 21 and 31 g/100 g), we can argue that *Klila* is a low-fat hard cheese with high protein content (Lahsaoui, 2012).

The sensory tests demonstrated that the three types of cheeses could be differentiated beyond any doubt on the basis of their colour and odour and to a lesser extent of their aroma and aspect. If the colour was mainly due to the cooking of the acidified milk *Lben*, the intense aroma was a further consequence of the high dry matter. Aroma compounds and sapid substances were concentrated in the residual moisture of the cheese, thereby making them more perceptible (Walther et al*.,* 2008). Mallatou et al*.* (2003) compared the degree of lipolysis in three Greek fresh *Teleme* cheeses made either with ewe, cow or goat milks. Although they noticed some differences between the concentrations of short and long chain fatty acids, the

flavour of the three cheeses was not affected. This is congruent with our idea that the high dry matter of *Klila* explains sensory differences rather than other factors.

The physico-chemical characteristics of *Klila* are not favourable for microbial growth. As indicated above, the pH and the Aw are too low. As a consequence, ripening mechanisms are nearly inexistent. The presence of microbes inside the cheeses can, then, be surprising, especially the high total counts (between 5.0 and 6.5 log (cfu)/g). For instance, Leksir and Chemmam (2015) and Guetouache and Guessas (2015) reported lower values: between 3.1 and 3.8 log (cfu)/g. We can suppose with Cuvillier (2005) and Jakob (2011) that hygienic practices, initial contamination of the milk and storage duration explain these variations between studies.

We observed strong correlations between physico-chemical parameters and some microbial populations, especially Aw. A low Aw was associated with high levels of lactobacilli, enterococci, and spore-forming bacteria. These populations were not necessarily favoured by the decrease of Aw, but they were certainly less affected than other microbes. *Bacillus* spp and butyric acid bacteria (*Clostridium butyricum and C. tyrobutyricum*) were dormant, since the technic we used was specifically aimed at the detection of spores. *C. tyrobutyricum* is responsible for late blowing. Its specific metabolism leads to the transformation of lactate into butyric acid, CO_2 and H_2 (Jakob, 2011).

The numbers of yeasts and moulds detected in our Klila were very high (> 5.7 log (cfu)/g) compared with the results obtained by Guetouache and Guessas (2015): around 2.0 log (cfu)/g. Many explanations can be put forward to explain this discrepancy: hygiene conditions and air contamination for instance (Cuvillier, 2005). We can argue that these spore forming populations explained partially and even totally the high levels of total counts.

Salmonella, *E. coli*, *S. aureus* and *L. monocytogenes* were not found. This can be explained by the double effect of low pH and low Aw (Yohan et al*.,* 2016).

Enterococci are commensal with many Mediterranean cheeses, in which their level varies in general from 5 to 6 log (cfu)/g. They are known to contribute significantly to the ripening of cheeses, being able to survive in drastic conditions: wide pH range (4.6-9.9), high salt rates (< 6.5%), wide temperature range (5-50°C), bile resistance (Franz et al*.,* 2003; Fisher and Phillips, 2009). They are generally brought by the raw materials but also result from poor hygienic conditions. Among species frequently reported, *Enterococcus faecalis* and *Ec faecium* are the most recurrent, followed by *Ec durans* and seldom *Ec casseliflavus* (Giraffa, 2003). Enterococci are generally involved in the ripening process as a consequence of their proteolytic and lipolytic activities, but also, for some strains, their ability to produce diacetyl (Martín-Platero et al*.,* 2009; Aguilar-Galvez et al*.,* 2012). In our case, we found significant (although low) levels of enterococci (between 3 and 4 log (cfu)/g). And we can suppose with Oliver (2005) that these levels under-estimated the “real” level of enterococci in *Klila* since this bacteria can enter the viable but non cultivable state (VBNC). This is all the more true for *Ec faecium* and *Ec hirae*. The dominant presence of *Ec durans* in our cheeses is difficult to discuss since no preceding work indicated that this species was particularly xerotolerant. The majority of the strains isolated in our work were classified as low acid producers. This is in conformity with previous results reported by Cogan et al*.* (1997) which indicated that the majority of *Enterococcus* strains they isolated were unable to lower the pH bellow 5.3 in milk. We can suppose that the *Enterococcus* population played a part in the cheese making, especially during the acidification step. And they were partly inactivated by the heat treatment.

The levels of lactobacilli varied a lot from one cheese to another, between 3.69 and 6.15 log (cfu)/g. This observation confirms the results obtained by Boubekri and Ohta (1996). These authors noted that the microbial composition of *Klila* cheeses could change from region to region, the levels of *Lactobacillus, Enterococcus, Pediococcus* and *Leuconostoc* varying a lot. Among the strains isolated during our studies, the majority (65%) belonged to the *Lb plantarum* species, followed by *Pediococcus pentosaceus* (12%), *Leuconostoc pseudomesenteroïdes* (8%) and *Lactobacillus fermentum* (4%). The remaining strains were unidentified or identified as *Enterococcus*. The use of the test proposed by Kandler and Weiss (1986) to discriminate lactobacilli on the basis of their fermentative aptitude proved to be less relevant and led to strange results. We also observed on mesophilic lactobacilli originating from other cheeses that this test could fail in some cases (Demarigny, personal communication).

Compared to the *Enterococcus* isolates, the strains isolated on MRS medium were all high lactic acid producers. This is a characteristic feature of *Lb plantarum* (and to a lesser extent of *Lb fermentum*), this species being one of the most acidifying germs among lactic acid bacteria (Vescovo et al*.,* 1993; Todorov and Gombossi de Melo, 2010). *Lb plantarum* is frequently found in spontaneous fermented plant products in relation with other heterofermentative microbes such as *Leuconostoc* (Demarigny et al*.,* 2012). This bacterium is also a major actor in the ripening step during cheese ageing. Our results were therefore as expected. However, in the literature, *Lb plantarum* is not usually referred to as xerotolerant, contrary to *Pediococcus*, a germ frequently found in old hard cheeses (that is, Grana cheeses). Recently Bouton et al*.* (2016) indicated that they found *Lb plantarum* strains on dry hay. It would indicate that this species can contribute to the biopreservation of a fermented food even in harsh conditions (low pH, low Aw, etc.) and can present xerotolerant aptitudes.

Concerning the diversity of the *Lactobacillus* and *Enterococcus* isolates evaluated by REP PCR, we

observed the presence of different clusters: 4 for *Enterococcus* spp and 7 for *Lb plantarum*. It indicates that inside each population different sub-populations could be found and consequently, a relative diversity even in extreme environmental conditions. More interestingly, we observed that the different strains could be found in two or three cheeses simultaneously. Two hypotheses can thus be put forward: 1) these strains are naturally present in the region from which the cheeses were made; 2) and/or, the physico-chemical conditions that prevail in the cheeses tend to select the best adapted strains which consequently exhibit very similar patterns.

Conclusion

Klila is a traditional cheese which has been produced in Algeria for many centuries. The adaptation of the cheese making to the environmental context allowed people of these regions to produce a cheese which can be stored over time without running any sanitary hazard. The type of milk used gives rise to specific products, clearly identified by naïve consumers. The aromatic balance is probably influenced mainly by the different steps of the process, which results in a concentration of the aroma compounds. Indeed, the microbes inside the cheese – especially, *L. plantarum* and *Enterococcus* – probably have no influence on the ageing process. However, they contribute to the preservation of the cheeses over many months (“hurdle concept”) and seem to represent a part of the typicality of the region from where the cheeses are produced. They also possess interesting physiological features (xerotolerance) which could be studied in order, for instance, to develop specific starters dedicated to the making of *Klila*. It could be the first step towards the industrialisation of this cheese. Right now, it would also be interesting to characterise the nutritional virtues of *Klila* more precisely and to correlate them with the technological, physico-chemical and microbiological characteristics of this cheese. This is intended in a future study.

Conflict of Interests

The authors have not declared any conflict of interest.

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Physiochemical evaluation and liability of dromedary camel's milk in combating various pathogens

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Dromedary camel's milk is a natural source of probiotics; enzymes and secondary metabolites that have the ability to combat many pathogens. The aim of this study was to compare the effectiveness of filtered and non-filtered dromedary camel's milk activity against various pathogens. Filtered and non-filtered (raw and boiled) dromedary milk was assessed against different pathogens by using agar well diffusion on Muller Hinton agar (MHA) and Blood agar assays. The sensitivity pattern against all pathogens was determined on MHA plate, by incubating for 24 h at 37°C. *Streptococcus and Lactobacillus* have antagonistic activity against various pathogens. The results showed that effectiveness of non-filtered milk was about 40 and 60% of boiled and raw milk respectively. Filtered milk had a 50% of effectiveness for both raw and boil milk. The antibacterial activity of filtered milk indicates the presence of such Immunoglobulins and enzymes that help in providing immunity. The streptococci inhibit 64% of the test organisms, while Lactobacillus suppresses 54% of pathogens. *Acinetobacter baumannii* is more susceptible to 37mm zone while Lactobacillus suppressed the growth of *Micrococcus luteus* with 45 mm zone.

Key words: Dromedary milk, probiotics, Alzheimer's, agar well diffusion method, disk diffusion method, Immunoglobulins.

INTRODUCTION

Camel milk is beneficial for all health purposes in treating various diseases from hereditary to bacterial like diarrhea, diabetes, tuberculosis and autism. The fact is that camel milk does not coagulate even in an acidic environment like in stomach so it is easily available for absorption in the intestine. According to Sunni Islamic tradition, camel's milk has medical properties - (Hadith Sahih al-Bukhari) and according to the FAO organization camel's milk is the healthiest milk produced by animals. In Pakistan 0.8 million Camels breeds is leading mostly in the desert areas, particularly in the areas of Sindh province, Cholistan (Punjab) and hilly areas of Balochistan (Khaskheli et al., 2005). Camel milk is comprised of proteins that have the ability to combat many bacterial infections and boosting up immunity. Proteins are divided into two; Casein and Whey proteins

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(Brezovečki et al., 2015). Casiens are found in the highest fraction of 52 to 82% (Brezovečki et al., 2015; Al Kanhal, 2010). In total about 65% of β-CN, 21% αs1-CN (Kappeler et al., 2003) makes it easily digestible, less allergic to newborn because it decomposes in low time (Brezovečki et al., 2015). K-casiens is 3.47% (Kappeler et al., 2003) while 13% in bovine milk (Seher and Hifsa, 2013). On the other hand a whey protein includes a variety of proteins, α-lactalbumin, c serum albumin, lysozyme, lactoferrin, peptidoglycan recognition proteins, lactoperoxidase and Immunoglobulins (Brezovečkil et al., 2015; Seher and Hifsa, 2013). β-lactoglobulin from 50 percent, makes up a major portion of whey protein (Kappeler et al., 2003). There is a beneficial combination observe between fats and proteins (Seher and Hifsa, 2013). During starvation, the level of fat content is decreasing due to hydrolysis of fats (Konuspayeva et al., 2010). Dromedary camel's milk has decreased the level of carotene with lower concentrations of short chain fatty acids as compared to milk of bovine (Seher and Hifsa, 2013) which have pH of about 6.5 to 6.75 (Al-Saleh et al., 1992). Camel milk contains about 2.40 to 5.80% of lactose (Seher et al., 2013) and consume plants which overcome salt and mineral requirements (Yagil et al. 1980). ,

Furthermore, it contains mineral contents, vitamins, bioactive native proteins which includes Immunoglobulins, Lactoferrin and Indigenous enzymes which also includes Lysozymes and Lactoperoxidase and provide ability to combat against many of the life threatening diseases (Brezovečkil et al., 2015) (Seher and Hifsa, 2013) (El-Agamy et al., 1998). There is variation in nutritional composition in camel milk due to some changes over a specified period of time (Brezovečkil et al., 2015; Yagil et al., 1980), due to analytical procedures (Mehaia et al., 1995).

Probiotics are gram positive group of live microbes which exists as a single or colonized form; playing a role in improving immunity by maintaining the normal flora (Joshi et al., 2015; Nelson et al., 1995). These organisms produce secondary metabolites that carry antimicrobial activity against many of the pathogenic organisms (Yateem et al., 2008), use in food and aquaculture (Joshi et al., 2015). These gram positive bacteria are categorized as LAB (lactic acid bacteria) (Yateem et al., 2008), which are usually use as a starter culture in the production of dairy items. Today, LAB is classified into thirteen main groups Lactobacillus, Leuconostoc, Lactococcus, Streptococcus, Enterococcus, Pediococcus, Bifidobacterium, Carnobacterium, Oenococcus, Weissella, Aerococcus, Tetragenococcus and Vagococcus (Fatma et al., 2013). These can be identified on the basis of bile tolerance, pH NaCl tolerance, catalog production, and motility (Nelson et al., 1995). Further identification was made according to Bergey's manual of determinative of bacteriology (Holt, 1994). These organisms play role in maintaining pH and reducing the lactose intolerance and cholesterol level, antitumor activity and activation of the immune system (Eva et al., 2002). The taxonomic tool for their identification is fructose-6-phosphate phosphoketolase (F6PPK) (Eva et al., 2002). The identification of these species can be possible at molecular level by PCR.

A recent study has shown a beneficial effect of a prebiotic and probiotic association highlighting the growing interest of symbiotic in digestive health (Picard et al., 2005). S. salivarius backbone of these bacteria is joined together by ether linkages which separates it's from other bacterial species, and are virulent streptococcus species due to absence of surface proteins (lipoproteins) (Francesca et al., 1999). Pseudo genes epsE, epsF, epsG, and epsI are responsible for the production of exopolysaccharide (EPS) in Streptococcus thermophiles (Francesca et al., 1999) that play role in attachment and production of reduced fat cheddar cheese and other dairy products (Awad et al., 2005). They boost up human immunity (Wollowski et al., 2001), present as intestinal flora (increase digestion) (Wollowski et al., 2001), use in replacement of chemotherapy (Whitford et al., 2009) and in treatment of antibiotic associated diarrhea (Nopchinda et al., 2002). Now days, this has gained most of the researches because S. thermophilus' genome is shorter than most genomes, having 1.8 MB (Rao et al., 1977).

MATERIALS AND METHODS

Milk sample was collected from the camel in sterile container brought to the microbiological laboratory of Jinnah University for women Karachi; to check the antibacterial activity and Phytochemical analysis. pH, acidity, total solids, ash, total solids non fat, Fat proportion and total proteins was done by the formal method, titration.

Phytochemical Analysis

pH

This was Observed using the digital pH meter by placing 20 ml of raw and heated milk in two separate beakers, and immersing an electrode in the beakers.

Acidity

Take 10ml of milk separately in two flasks for raw and heat milk. Add two to three drops of phenolphthalein. Titrate with 0.1N NaOH. Note when pink color appears

Total solids

Heat 5 ml sample at 100°C for three hours.

Fat detection

This was carried out by Babock Method**.** 10.94ml of raw and boiled milk was place in two separate test tubes labeled, raw and boiled. Add 10 ml of H_2SO_4 and 1 ml of isoamyl alcohol**.** Mixture is then centrifuge at 1100 rpm for 5 min at 65°C. Result is noted by measuring the fat layer at the surface of tubes. Solid non-fat (SNF) was determined by SNF = Total solid% - Fat%.

Measurement of total proteins

This was carried out by the formal method which is done by titrating all the chemical and reagents required are phenolphthalein (prepared in laboratory by adding 0.5 g of phenolphthalein powder in 50% ethanol, 0.1 NaOH, 40% Formalin and 28% potassium oxalate from the (department of chemistry Jinnah university for women). 10 ml of sample was pipette in 50ml flask, 0.4 ml of saturated potassium oxalate and 0.5 ml of phenolphthalein was added and set for two minutes. Milk was then neutralize by NaOH to end point (note the reading). 2 ml of 40% formalin was added to it, stand for two minutes and titrated with 0.1 N NaOH till, endpoint is attain. Blank was run by titrating it with 2 ml of 40% formalin.

Ash detection

This was done by formal method;

1. Weight of crucible was noted (a)
2. Add sample in crucible and measure the weight again (b)
3. Sample was then put in oven for 4 h
4. Note the weight again (d)

Formula;
Weight of sample (c) = a – b
Original weight of sample (e) = a – d

Isolation of lactic acid bacteria

10ml quantity of milk sample was stomached with 90ml of peptone water, by using same diluents sample which is serially diluted. Diluted sample was inoculated on De Man, Rogosa and Sharpe (MRS) agar prepared medium (Merk), plates were incubated at 37°C for 48 to 72 h to analyze the colony morphology. Gram staining was performed for microscopic analysis. It has been sub cultured further to get pure colonies by inoculating single colony on MRS agar for lactobacillus spp, MRS supplemented with L-cystien for the streptococcus (fat lowering bacteria) (Lim et al. 2004), incubate at 37°C for 24 and 48 h, respectively, colonies were inoculated on heart infusion agar supplemented with 5% glucose (HIAG) and heart infusion agar supplemented with 5% Sucrose, 0.5% glucose, and 0.02% sodium azide (HIAS), colonies appeared on this are inoculated on Mayeux Sandine Elliker agar (MSE) prepared by adding (tryptone 10 g/l, Gelatine 2.5 g/l, yeast extract 5 g/l, sucrose 100 g/l, glucose 5 g/l, sodium azide 75 mg/l, sodium citrate 1 g/l, agar 15g/l) which is elective medium for Leuconostoc species and was incubated at 30°C for three days.

Spot tests

All the LABs were determined on the basis of spot testes like motility, catalase and oxidase by picking up colonies from their respective medium plates. Motility of isolated cultures was determined by cavity slide, catalase by picking up a colony and inoculates on the drop of H_2O_2.

Antibacterial activity of dromedary camel's milk (non-filtered milk)

This is checked out by both agar well diffusion method.

Preparation of inoculums

Inoculums of pathogenic organisms were prepared by standardizing it according to the turbidity of 0.5 McFarland tube (as per given by Kirby-Bauer, standard method) which mean 150 million cells per ml of bacterial suspension.

For antibacterial activity

Lawn of eight clinical isolates (*Escherichia coli, Listeria monocytogenes, Klebsiella pneumonia, Pseudomonas aeruginosa, Micrococcus luteus, Staphylococcus aureus (MRSA), Acinetobacter baumannii, Shigella burnetiiwere* prepared on MHA and TSA, *Haemophilus influenzae* and *Moraxella catarrhalis)* was isolated on chocolate agar and blood agar streptococcus pneumonia and allow the plates to stand for 5 min. Two wells on each plate were prepared by the help of borer (6.5 mm), thickness of the medium was about 25 mm. Load 100µl of raw and boil milk sample in each respective well (Yassin et al., 2015) and placed prepared disks of sample on each plate. Incubate it for 24 to 48 h at 37°C and observe zone of inhibition in millimeters. The same protocol was repeated for the antibacterial activity of Filtered milk.

Antagonistic activity of LAB isolated from milk

Isolated LABs cultures were inoculated in respective broth mediums of MRS broth for *Lactobacillus*, MRS+ L-Cystine for *Streptococcus* while MRS was supplemented with 6.9% NaCl for *Lecuconostoc* spp. Antagonistic activity was checked against *Escherihia Cali, Acinitobacter baumannii, Listeria monocytogenes, Pseudomonas originals, Staphylococcus. aureus, Micrococcus luteus* and *Klebsiella pnumoniae;* by agar well diffusion method. All test organisms were inoculated on MHA. Leave the plates for 5 min. Two wells on each plate were prepared by the help of borer (6.5 mm) and the thickness of the medium was about 25mm. Load 100 µl of cultured broth of streptococcus and lactobacillus in each respective well. Incubate it for 24 to 48 at 37°C and observe zone of inhibition in millimeters. Zone larger than 2mm was considers as sensitive (Lim et al., 2004).

RESULTS

Fresh raw dromedary camel's milk was purchased from the camel herd in street of Karachi Pakistan. Milk was kept in an airtight container at 4°C for 15 days and was used for compositional analysis of dromedary camel's milk in the Microbiology Department of Jinnah University for Women, Pakistan. The process of composition analysis was carried out within 3 weeks. (Table 1) shows the observed values for the components of dromedary camel's milk both raw and heat to check the effect of heat

Table 1. Phytochemical analysis of dromedary milk.

Components of milk	Raw milk	Boiled mlik
pH	7	6
Fat %	2.42%	3.32%
Acid	2.76 g/ml	o.13 g/ml
Total proteins %	0.05%	0.03%
Total solids	5.2 g	5.4 g
Total solid non fat	0.046%	0.06%
Ash*	0.6%	-
Microbial count	$73x10^5$ cfu/ml	-

Table 2. Antibacterial activity of non-filter camel milk.

Test organism	Media	Sensitivity or resistivity pattern	
		Agar – well diffusion	
		Raw milk (R)	Boiled milk (B)
Himophilus influenza	Chocolate agar	25 mm	15 mm
Morexilla catarrhalis	Chocolate agar	28 mm	R
Streptococcus pnumoniae	Blood agar	35 mm	25 mm
Lister monocytogenes	MHA	36 mm	35 mm
Klebsiella pneumonia	MHA	10 mm	30 mm
Escherichia coli	TSA	R	R
Pseudomonas aureginosa	MHA	27 mm	R

Ash* **=** the crumbly scum left after the burning of a substance, MHA*= Muller Hinton agar, TSA*= tryptic soy agar.

on the components of milk sample. The observed pH value of raw and heat milk sample was 7 and 6 respectively. Fat was observed to be 2.42 and 3.32 in raw and boil respectively. Total proteins, acids, total solids and non-fats were 0.05 and 0.03%, 2.76 and 0.13 g/ml, 5.2 and 5.5 g, and 0.046 and 0.06% in raw and boil milk respectively.

Total ash content in milk sample was 0.6 and microbial count was$73X10^5$ cfu/ml. Table 2 indicates sensitivity patterns of various pathogens againstnon-filter camel's milk (milk does not filter by filter assembly and contain probiotics). Table 3 indicates sensitivity patterns of various pathogens against Filtered Dromedary camel's Milk (that is filtered by passing through filter assembly that is free of probiotics, to check the efficiency of non microbe particles that are various proteins and Immunoglobulins; in Camel's milk.

DISCUSSION

According to (Abbasiliasi et al., 2012) total microbial count obtained was 155,000 colonies. The values of fat%, fat, proteins and ash are 3.6, 3.2, 0.8 and 0.7% (Mayeux et al., 1962) while the pH is 6.5 to 6.75 (M.H Yassin et al., 2015). Total solid contents in camel milk vary from 9.8 ± 0.59 to 11.9 ± 0.71%, in comparison with our results that are mentioned in (Table 1). The variation in total solids of camel milk is mainly due to the changes in fat, lactose, minerals and protein content of camel milk. The total amount of minerals is generally presented as total ash and in case of dromedary camel milk this value ranged between 0.60 to 0.90% (Choct, 2009). Camel milk protein contents vary from 2.15 to 4.90%. The amount of non-protein nitrogen varies with total protein (Choct, 2009).

All obtained data revealed the variations in different components of camel milk. It was observed that the composition of camel milk depends on various factors like fluctuations in mineral level which were proposed to be due to the differences in breeding and water intake (Choct, 2009; Lim et al., 2004). Changes in the atmosphere also brought fluctuation in almost all the parameters (Choct, 2009). The fat content was decreased in dromedary milk at the time of malnourishment (Lim et al., 2004). Dromedary camel's milk is a possible source of Probiotics (Fatma et al., 2013). It contains a different variety of Lactic acid bacteria (LAB) main groups according to (Fatma et al., 2013) which are *Lactobacillus, Leuconostoc, Lactococcus, Streptococcus, Enterococcus, Pediococcus, Bifidobacterium, Carnobacterium,*

Table 3. antibacterial activity of camel milk (filtered-milk).

Test Organisms	Media	Sensitivity or resistivity pattern	
		Agar – well diffusion	
		Raw milk	Boiled milk
Escherichia.coli	MHA	30 mm	35 mm
Acinetobacter. baumannii	MH	R	15 mm
Listeria.monocytogenes	MHA	10 mm	30 mm
Pseudomonas.aureginosa	MHA	35 mm	35 mm
Staphylococcus. aureus	MHA	25 mm	R
Micrococcus. luteus	MHA	20 mm	22 mm
Klebsiella. pnumoniae	MHA	10 mm	19 mm

Oenococcus, Weissella, Aerococcus, Tetragenococcus and Vagococcus; while from our sample we isolate *Lactobacillus, Streptococcus, Leuconostoc, Pediococcus* and yeast cells from raw dromedary camel's milk.

Raw milk when inoculated on MRS media, mix culture of LABs were isolated, which were further isolated when supplemented with different nutrients and using selective and elective mediums for the isolates. Lactobacillus was grown on the MRS agar, Streptococcus and pideococcus on MRS+L-Cystien, Leuconostoc on MSE agar after 2 to 4 days incubation at temperature 37°C except Leuconostoc 6.9% NaCl at 30°C. Microscopy of mix LAB culture revealed the presence of gram positive, long to short rods, cocci and coco bacillus arranges in chains and tetrads. Lactobacilli are long to short rods, Streptococcus is cocci in chains, Leuconostoc are coco bacillus, pediococcus are tetrads of cocci while yeasts are ovoid in shape. All isolates were further confirmed on the basis of spot tests catalase, motility, spore formation, and oxides; all cultures are catalase negative (Podrabsky 1992; Mehaia et al., 1995) except Lactobacillus species that was pseudo positive and *L. planetarium* that was isolated by Whittenbury (1964). Oxides are spore negative (Whitford et al., 2009), and are non-motile (Yagil et al., 1980) except leuconostoc which was confirmed by the growth on 6.9% NaCl concentration, growth on heart infusion agar was supplemented with 5% glucose and 5% sucrose, 0.5% glucose and 0.02% sodium azide. Leuconostoc are motile (Podrabsk, 1992) while, rest of all isolated species were non-motile. Non-filter (that contain probiotics and not pass through filter assembly) raw and boil Dromedary camel's milk was then checked for the antibacterial activity against many of the virulent bacterial strains by the agar well diffusion method that is about 60% and 40% of raw and boil respectively against various pathogens mention in (Table 2). Results from Whittenbury (1965) also revealed that isolates had inhibitory activity against pathogenic bacteria, because the inhibition was scored positive, if the diameter of the clear zone around the colonies was 0.5 mm or larger, according to this study camel milk inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* at greater extend. Thus, Whittenbury (1965) suggests that camel milk is a possible source for the isolation of probiotic LAB strains and can be considered good for health with antibacterial properties against pathogenic bacteria because of the presence of bacteriocin producing strains. Filtered raw and boil was also applied against pathogenic organisms to check the antimicrobial activity of milk portions and enzymes as, it is a good source of Vitamin C, Insulin, Lysozymes, Lactoferrin and Immunoglobulins that make it healthier to human. The susceptibility pattern by agar well diffusion method is about 50% of raw and boils milk against various pathogens that are mentioned in (Table 3). This indicates the presence of certain other components that may have the ability to combat a variety of pathogens. These components include Lactoperoxidases, Lactoferrin and Lysozymes that play essential role in killing of pathogens (Khedida 2009; Choct, 2009; Mutlag et al., 2013).

Furthermore, lactoperoxidase work as anti-tumor agents and play functional role in degradation of Catecholamines. Our results also suggest that boiled camel milk has anti-bacterial activity, but demolish various nanoparticles and stopped some of biological functions especially in the treatment of diabetes. This carried out essential nutrients with specific properties, particularly anti-infectious action; which should be replace with other milks (Hickman, 2007). In the present Study, camel milk supplementation decreased the oxidative stress biomarker malondialdehyde and decreased the activity of antioxidant enzymes (catalase, SOD, and glutathione reductase). Alteration in oxidative stress was induced by reactive oxygen species (ROS) and impairments of the antioxidant system play a critical role in the pathogenesis of *E. coli* and *S. aureus* challenge (Hickman, 2007).

Metabolic by-products such as bacteriocins are loosely defined as biologically active protein moieties, with a bactericidal mode of action. These bacteriocin producing strains have natural immunity to their own bacteriocins. LAB has ability to inhibit the growth of other bacteria. So we apply it in our industries to minimize food spoilage and inhibiting pathogenic organisms (Yateem et al., 2008). Streptococcus and Lactobacillus are considered

Table 4. Antagonistic activity of LAB against various pathogens.

Test organism	Media	Zones produce by labs (mm)	
		Lactobacillus	Streptococcus
Escherichia coli	MHA	40	30
Acinetobacter baumannii	MHA	20	37
Listeria monocytogenes	MHA	20	30
Pseudomonas aureginosa	MHA	45	7
Staphylococcus aureus	MHA	R	30
Micrococcus luteus	MHA	45	25
Klebsiella pnumoniae	MHA	28	25

as gut flora that provide a healthy environment by combating all pathogenic organisms (Nopchinda et al., 2002). Among six LAB isolates antagonistic activity of only two were determined against various pathogens. These two species were grown in their respective medium cultures. Lactobacillus antagonistically acts on almost all pathogens and kill 54% of all organisms, while Streptococcus is 64% active against all pathogens mention in Table 4. The one of the study (Pritchard et al., 1993) reveals that lactobacillus shows weak inhibition against *P. aeruginosa* and *K.pneumoniae* and could not inhibit *S. ausres* and *E.coli*.

The study (Hu et al., 2007) showed that *S.thermophilus* had broad spectrum activity against Gram-positive bacteria which showed that the concentrated supernatant of *S. thermophilus* had inhibitory activity against pathogenic bacteria; *Pseudomonas aeruginosa, Klebsiella spp*, *Staphylococcus aureus* and *Escherichia coli*. LABs have the ability to produce acetic acid, lactic acid, formic and benzoic acids, hydrogen peroxide, diacetylacetoien and bacteriocin as a secondary metabolite. The level of these metabolites depends on the medium and physical parameters (osmanağaoğlu et al., 2001). Camel milk contains peptidoglycan recognition protein (PGRP) that provides passive immunity to the body (Makarova et al., 2006). *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are more effective in deactivating etiologic risk factors of colon carcinogenesis than being cellular components of microorganisms (Papagianni et al., 2009).

According to (Van et al., 1969) *S.thermophiles* may be helpful during chemotherapy by protecting the intestinal tissues from irritation caused by chemotherapy drugs. Another study (Vashist et al., 2013) shows that *S.thermophilus* correlates with better growth in children antibiotic-associated diarrhea (AAD) which is a growing issue today thereby making people to seek natural methods for relief. Since antibiotics kill good bacteria and sometimes allow harmful bacteria to grow, diarrhea is often the cause of the result. Certain strains of S.thermophilus have been shown to reduce AAD (Cowan et al., 2004). This is not surprising, considering that many other probiotic strains also provide similar benefits.

Conclusion

Today, the use of antibiotics is increasing day by day that makes pathogen more resistant to treat. This may lead the world towards 'Pre-Antibiotic Era' where we need a replacement of antibiotics; due to the continuous use of antibiotics and self medication, making many organisms mutated and pathogens more resistant by using the natural sources of antibiotics. We can overcome this mechanism of resistance and save our surroundings from many of hazardous upcoming emerging superbugs.

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Production of fermented sweet potato flour using indigenous starter cultures

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The present study develop various microorganisms for starter culture purpose from fermented sweet potato and used the developed starters in the fermentation of sweet potato and products for consistent quality product with improved hygiene. Sweet potato was purchased from different markets and microorganisms were isolated and developed as starters. Comparative studies on different starters for the fermentation of sweet potato for 48 h were investigated. The microbial count of the starter cultures increased as fermentation period increased from 6.06 to 9.14 cfu/ml, the pH reduced from 5.49 to 3.28 while the total titrable acid increased from 0.001 to 0.04. Amongst the various starters used, the use of the mixed cultures (combination of lactic acid bacteria L201 and yeast 601A) as starter cultures in the fermentation of sweet potato for 48 h had the best attributes in terms of texture, flavour, rate of fermentation and consistency when prepared as meal (bolus). The mineral analysis showed that most of the mineral content in the sample increased after fermentation for calcium (Ca) 2043.9 to 2177.5 ppm and Mn 6.29 to 26.32 ppm. The functional properties of the fermented sweet potato flour produced showed moisture content of 10.42%, water absorption capacity of 1.77 g/g and swelling capacity of 86.67%.

Key words: Starter culture, fermentation, sweet potato, mineral analysis, functional properties.

INTRODUCTION

Fermentation is the conversion of carbohydrates to alcohol and carbon dioxide or organic acids using yeasts, bacteria or a combination under anaerobic conditions. The primary benefit of fermentation is the conversion of sugars and other carbohydrates to usable end products. According to Steinkraus (1995), the fermentation of foods

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improve flavour, aroma, and texture in food substrates, preservation and shelf-life extension through lactic acid, alcohol, acetic acid and alkaline fermentation, enhancement of food quality with protein, essential amino acids, essential fatty acids and vitamins, improving digestibility and nutrient availability, detoxification of anti-nutrient through food fermentation processes.

Starter cultures are living microorganisms of defined combination used for fermentation purposes. They help to ellicit specific changes in the chemical composition, nutritional value and sensorial properties of the substrate (Opere et al., 2012) and they are generally recognised as safe (Augirre and Collins, 1993). Moreover, their properties are as follows: They are harmless, initiate and control the fermentation process, typical for product, help in rapid acid formation, and help protect against spoilage organisms. Starter cultures are cheaply reproducible in large amount, they also help provide desirable sensory properties and also assists in reducing fermentation period. Work has been done on the fermentation of sweet potato into flour suitable as meal for home consumption by Oluwole et al. (2012). This study therefore investigates the possibility of developing indigenous starter culture for the fermentation of sweet potato into flour and this will help in promoting food and nutrition security in developing countries. According to Sanni (1993) and Kimaryo et al. (2000), the use of the starter cultures will assists in optimizing the fermentation process and also help in alleviating the organoleptic and microbiological instability problems.

Lactic acid bacteria (LAB) are Gram positive acid tolerant, generally non-sporulating, either rod or cocci shaped bacteria that produce lactic acid as the major metabolic end product of carbohydrate fermentation. Lactic acid bacteria have been reported to be predominant microorganisms in most of the African indigenous fermented foods (Nout, 1991; Halm et al., 1993; Hounhouigan et al., 1993; Sanni, 1993; Steinkraus, 1996; Olasupo et al., 1997; Nago et al., 1998, Kunene et al., 2000; Duhan et al., 2013). Yeasts are eukaryotic microorganism, unicellular, diverse in nature found in a wide variety of habitat. Yeast converts carbohydrates to carbon dioxide ($CO_{2)}$ and alcohol. They are used in baking, wine making, brewing and a large variety of industrial products. Stable co-metabolism between LAB and yeasts is common in many foods and this enables the utilization of substances that are otherwise non fermentable (for example starch) and thus increasing the microbial adaptability to complex food ecosystems (Gobbetti et al., 1994; Stolz et al., 1995 and Gobbetti and Corsetti, 1997). Sweet potato (*Ipomea batatas* L.) is a dicotyledonous plant belonging to the family Convolvulaceae. The plant, which is native to tropical America, is cultivated on sandy or loamy soils throughout many warm regions of the world. (Zhang et al., 1998; Ikeorgu et al., 2000). It is a starchy, sweet tasting, tuberous root vegetable with smooth skin and comes in different colors: yellow, purple, orange and beige (Antonio et al., 2011). Sweet potato can be considered as low and medium glycemic index food, it is also an excellent source of vitamin A (14187 IU /100 g) and it is highly nutritious in terms of vitamin C (2.4 mg / 100 g). It is also rich in calcium (30 mg /100 g), iron required for adequate energy (0.61 mg /100 g) and also a good source of magnesium (25 mg/100 g). USDA National Nutrition database (2009-2015).Sweet potato is readily available, inexpensive and delicious. Nigeria is the largest producer of sweet potato in Africa with 3.46 metric tonnes annually (NRCRI, 2012), Also the second largest producer globally (FAO, 2008).In the tropics, sweet potatoes are consumed or marketed soon after harvesting because of their shelf life that can be as short as one week (Ravindran et al., 1995).

Raw sweet potato contains anti nutritive factors such as raffinose and trypsin inhibitory activity. Raffinose is one of the sugars responsible for flatulence, it is not digested in the upper respiratory tract. Tryspin inhibitory activity (TIA) is a serine protease inhibitor that reduces the biological activity of trypsin, they interfere with its protease activity. The fermentation of sweet potato helps reduce these anti nutritive factors and also make the nutrients more bioavailable. The use of starter cultures provides consistency and reliability of performance (Mc Feeters, 2004).This a pioneer investigation into the fermentative ecology of sweet potato and this study reports the use of indigenous starter cultures in the fermentation of sweet potato.

This study will add value to sweet potato and this will enhance industrialization, job will be creation through manufacture of value added food, moreover, wealth will be generated for farmers through diversification of the use of sweet potato and a lot more. About 805 million people have been reported to be hungry worldwide and 23.8% from sub-sahara Africa and the hunger index has been reported to range from 15 to 16.3 in 2013 (International Food Policy Research Report, 2013) this study will also lead in the production of novel food products from sweet potato crops thus assisting in minimizing food insecurity problems particularly in most developing African countries where hunger, poverty and starvation are highly prevalent.

MATERIALS AND METHODS

Sourcing of raw materials

Yellow-fleshed sweet potatoes were obtained from Dalemo market (Alakuko), Aguda market (Surulere) and Oshodi market (Oshodi), Lagos-Nigeria.

Isolation of microorganisms from fermentation broth

This was carried out using the method by Hedges et al. (2002). Sweet potato was washed, peeled and sliced into potable water

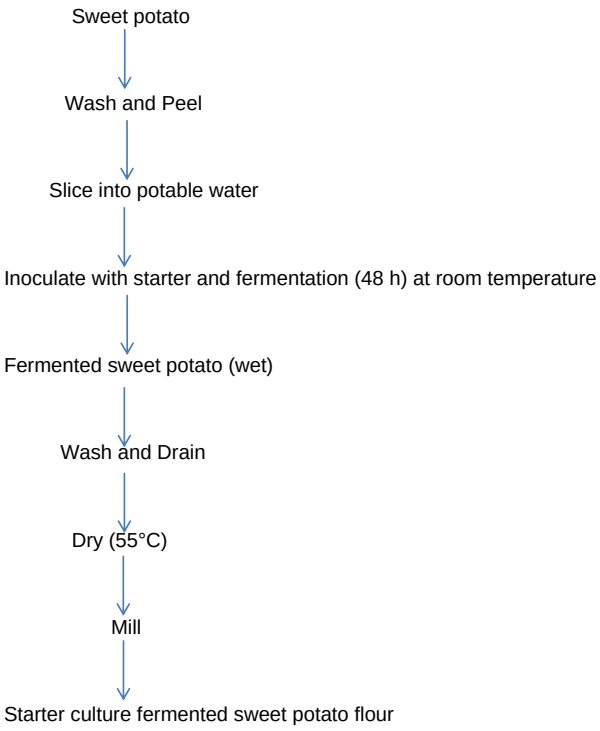

Figure 1. Starter culture fermented sweet potato flour flowchart.

and allowed to ferment for 72 h. Samples were collected at 24 h interval. This was then serially diluted using tenfold serial dilution.1 ml of different dilution were plated out into well labelled petri dishes. PDA (Potato Dextrose agar with streptomycin) MRS (De Man Rogosa Sharpe agar).PDA was incubated for 3 to 5 days at 30°C, MRS was incubated anaerobically at 37°C using the anaerobic jar for 18 to 24 h. After incubation the plates were brought out and evaluated, it was read and the organisms were isolated. The organisms that were successive throughout the fermentation period were identified as proposed starters.

Screening of isolates

The isolates were screened for their abilities to produce lactic acid and hydrolyse starch and these were carried out using modified methods of Coulibaly et al. (2008) and Collins and Lynes (1989). The organisms were inoculated onto the surface of agar containing 1% $CaCO_3$ MRS agar (which contain glucose, yeast and peptone). The culture was incubated anaerobically at 30°C. Acid producing bacteria were recognised by the clear zones around the colonies
Each of the isolates was first tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Immediate formation of bubbles indicated the presence of catalase in the cells. Only those isolates which were catalase negative were Gram-stained, and only those which were Gram positive were put through to determine whether the isolate produced carbon from glucose during fermentation. An isolate was deemed to be a homo fermentative lactic acid producer if no gas was produced.

Identification of isolates

These isolates were identified by the API 50 CHL identification kit (BioMérieux, Marcy-l'Etoile, France). The 50 CHL API Kit was used for Lactobacillus related genera and ID 32 C was used for the identification of yeast related genera. A suspension is made in the medium with the organisms to be tested and each tube strip is then inoculated with the suspension.it was incubated for 24 to 48 h at 37°C and 30°C for 24 to 48 h.The apiweb™ identification software with data base (V5.1) was used for identification of 50CHL API Kit results and ATB Expression™ identification software with database (V3.0) was used for the identification of the ID 32C API Kit results.

Preparation of innoculum

This was carried out using the method by Asmahan et al. (2009).Lactic acid bacteria cultures were cultivated by streaking on MRS agar (Oxoid) and incubated anaerobically (BBL, Gas Pak, Becton Dickinson) at 37°C for 24 h. A colony was picked from each pure culture plate, grown successively in MRS broth before centrifugation at 5000 rpm for 15 min. The pellet was washed in sterile distilled water centrifuged again and redistributed in distilled water. This procedure achieved a culture preparation containing 10^9 colony forming units cfu/ml, checked as viable count on MRS agar. Pure cultures of yeast were cultivated by streaking on Potato dextrose agar (Oxoid), incubated at 37°C for 24 h and the picked colony was inoculated into 10 ml of yeast extract peptone dextrose broth (Oxoid) and incubated at 28°C for 24 h. These cultures were centrifuged and washed as described above. This procedure achieved a culture preparation containing 10^7 cfu/ml, as viable count on malt extract agar. Yeast cultures had been stored on malt extract agar slants at 4°C until required. The number of LAB and yeasts was monitored during fermentation by serial dilution of the samples, using the media described above.

Production of fermented sweet potato using starter culture

The starter culture fermented sweet potato flour was produced using the flow chart in Figure 1.

Production of fermented sweet potato flour (control)

The starter culture fermented sweet potato flour was produced using the flow chart (Figure 2).

Preparation of starter culture fermented sweet potato flour

The sweet potatoes were washed to remove adhering soil particles and peeled. Then peeled tubers were chipped into slices (4 to 5 mm) (Figure 3). Starter cultures were prepared as shown in Figure 4 and inoculated into the sweet potato it was then left to ferment for a period of two days (48 h) as shown in Figure 5.

After this period has elapsed, the fermented chips were drained and dried in a cabinet drier (Mitchel, Model SM220H) at 55°C for 9 h and milled into flour (≤ 250 µm) as prepared by Oluwole et al. (2012) and the dried starter culture fermented sweet potato flour was shown in Figure 6.

Preparation of fermented sweet potato flour

The sweet potatoes were washed to remove adhering soil particles and peeled. The peeled tubers were chipped into slices (4-5 mm) and soaked in potable water for a period of two days (48 h). After this period elapsed, the fermented chips were drained and dried in a cabinet drier (Mitchel, Model SM220H) at 55°C for 9 h and milled into flour (≤ 250 µm) (Oluwole et al., 2012).

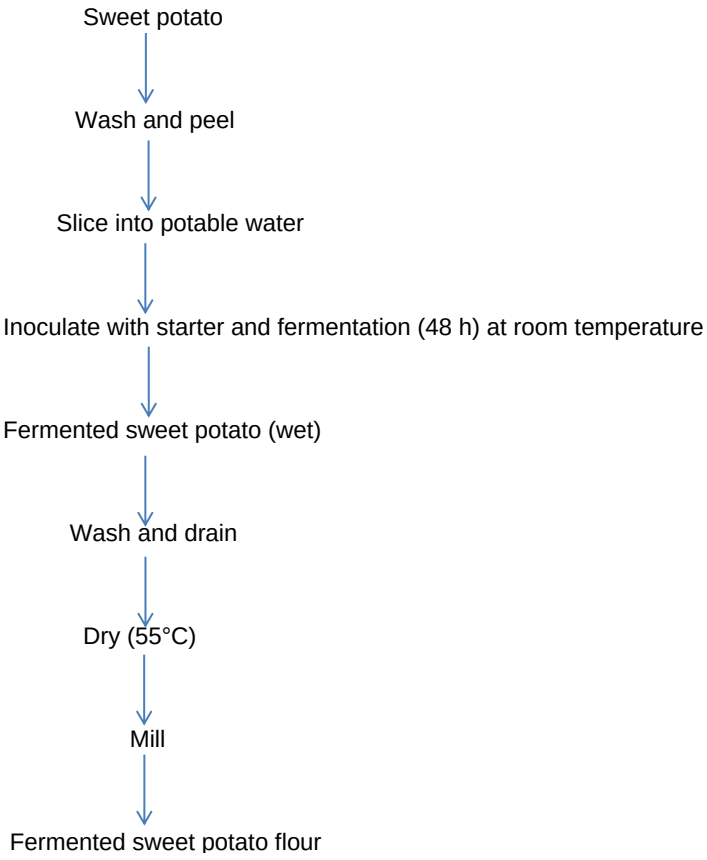

Figure 2. Fermented sweet potato flour (control) flowchart.

Figure 3. Processing of sweet potato

pH determination

The pH of the fermenting sweet potato was determined using a pH meter (OAKTON, pH 700 pH/ Mv/°C/°F) which has been previously adjusted with buffer solutions of pH 4 and 9.

Total titrable acidity

The production of lactic acid was determined by titrating 10 ml of the homogenized sample against 0.1 M NaOH using 2 drops of phenolphthalein indicator (0.5 in 50% alcohol). The titrable acidity was calculated as percentage lactic acid (v/v). Each millilitre of 0.1 M NaOH is equivalent to 0.9008 mg of lactic acid (AOAC, 1990).

Microbiological analyses of products

One gram of the fermented sweet potato flour samples were weighed serially diluted using tenfold serial dilution.one ml of different dilution were plated out into well labelled petri dishes and Mac broth containing Durham tube. Different media NA (Nutrient agar), PDA, EMB (Eosin Methylene Blue agar), VRBA (Violet Red Bile agar), S/S (Salmonella Shigella agar) was added to the inoculums in the petri dishes and mix thoroughly. It was then left to solidify before incubating at various temperatures for various duration. NA, PDA, VRBA, EMB,S/S was incubated at 37°C for 18 to 24 hours, PDA was incubated for 3 to 5 days at 30°C ,and Mac broth were incubated at 37°C and 44°C for 24 to 48 h. Moreover MRS plates were incubated anaerobically at 37°C (Harrigance and McCance, 1976).

Proximate composition of raw sweet potato and fermented flour

This was determined in the raw sweet potato and the fermenting sweet potato flour as described by AOAC (2010). Parameters such as moisture content crude protein, crude fibre, ash, fat and carbohydrate were determined in the sample.

Mineral analyses

This was determined using Shimadzu AA7000 Atomic absorption spectrophotometer (AAS); samples were ashed and 3 drops of concentrated HNO_3 were added to the ash and was dissolved in 100 ml distilled water. The filtered ashed sample was then aspirated into flame and atomise. Atoms of elements present will absorb radiation from light source and it will be detected.

Determination of functional properties of products

This was done by using the method by Onwuka (2005). Moisture content- moisture was determined in fermented sweet potato samples using the rapid moisture analyser as described by AOAC (1990).

Figure 4. Preparation of inoculum.

Figure 5**.** Fermentation of sweet potato using starter cultures.

Figure 6. Starter culture fermented sweet potato flour.

Swelling index

100 ml of graduated cylinder was filled with the sample to 10 ml mark. Distilled water was added to give a total volume of 50 ml, the top of the graduated cylinder was tightly covered and mixed by inverting the cylinder, the suspension was inverted again after 2 min and left to stand for a further 8 min and the volume occupied by the sample was taken after 8 min.

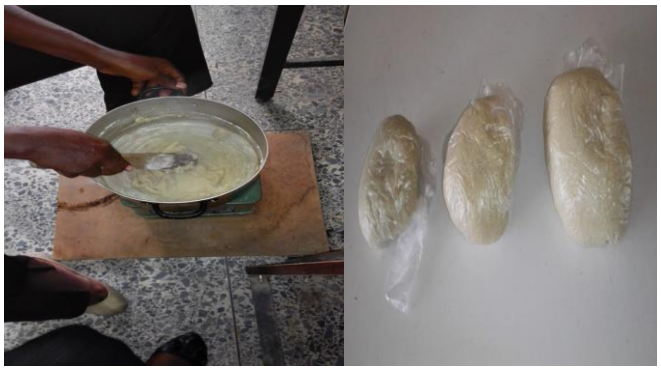

Figure 7. Production of stiff porridge from starter culture fermented sweet potato flour.

Water absorption capacity

One gram of sample was mixed with 10 ml of distilled water and allowed to stand at ambient temperature (30± 2°C) for 30 min, centrifuged for 30 min at 3000 rpm.

Preparation of stiff porridge from fermented sweet potato flour

Dried fermented sweet potato flour was poured into a clean bowl and mixed with potable water to form a paste it was then added to boiling water and stirred until a smooth consistency was obtained as shown in Figure 7.

Preservation of isolates

Bacteria were purified by several isolations and fresh cultures of these isolates were conserved at -80°C with glycerol (30%) as cryoprotective agents (Williams et al., 2009).The isolate were preserved using the method by Williams et al. (2009) but it was not conserved at -80°C. MRS (broth and agar) and PDA were used for the lactic acid bacteria and yeast isolates respectively.

Data analysis

The data generated were subjected to analysis of variance (ANOVA). All determinations were performed in triplicate. All statistical analysis were conducted with non-parametric one-way ANOVA procedures, differences were reported at a significance level of 0.05.

RESULTS

Lactic acid bacteria and yeast were isolated from sweet potato. Oluwole et al. (2012) also reported that lactic acid bacteria and yeast were isolated from sweet potato. Smith et al. (2007) and Panda et al.(2007). The screening of lactic acid bacteria for lactic acid production in Table 1 showed that all the micro-organism screened where able to produce lactic acid and L201 had the highest zone of inhibition of about 1.8 mm and L205 was 1.5 mm. The ability of the yeast isolates to hydrolyse starch was also

Table 1. Lactic acid production potential of lactic acid bacteria and starch hydrolysis potential of yeast isolated from fermented sweet potato.

Lactic acid bacteria		Yeast	
Isolates	**Halo (mm),$CaCO_3$ ± S.D**	**Isolates**	**Halo (mm)starch± S.D**
L201	1.8 ± 1.00	601A	2.0 ± 0.50
L202	1.6 ± 0.50	602B	1.0 ± 0.50
L203	1.7 ± 0.50	603C	2.0 ± 1.00
L204	1.6 ± 0.50	604D	1.5 ± 0.50
L205	1.5 ± 0.50	605E	1.8 ± 0.50
L206	1.7 ± 0.50	607F	1.3 ±1.00
L207	1.5		
L208	1.6		

S.D., Standard deviation.

Table 2. Biochemical characteristics of lactic acid bacteria and yeasts isolates.

Biochemical characterisation	Lactic acid bacteria (L201)	Yeast (601A)
Gram's staining status	+ rods	+oval
Homo fermentative	-	ND
Hetero fermentative	+	ND
Catalase production	-	+
Lactic acid production	+	ND
Starch hydrolysis	-	+
Growth in NaCl 6.5%	+	ND
Growth at temperature 15 and 45°C	+	ND

-, Negative reaction; +, Positive reaction; ND, not detected.

investigated and yeast isolate 601A, 2.0 mm and isolate 607F, 1.3 mm.

The tolerance of each of the selected isolates to the environmental conditions was tested in Table 2. The strains of the *Lactobacillus* sp. grew at 15 to 45°C, the ability to grow at high temperature is a desirable trait as it could translate to increased rate of growth and lactic acid production. At the same time, a high fermentation temperature reduces contamination by other micro-organisms. During industrial fermentation, as lactic acid is being produced by the cells, alkali would be pumped into the broth to prevent excessive reduction in pH. Thus, the free acid would be converted to its salt form which would in turn increase the osmotic pressure on the cells. Therefore, a LAB strain with high osmotolerance would be desirable as an industrial strain and the LAB strain used in the study is osmotolerant.The fermentation pattern among carbohydrates was determined by using the API 50CHL gallery with the API 50 CHL medium (Bio Merieux, Marcy, France).

Anaerobiosis in the inoculated tubes was obtained by overlaying with sterile paraffin oil for the lactic acid bacteria while the API ID 32 C was used for the identification of the yeast. The strip consists of dehydrated carbohydrate substrate, a mineral medium is inoculated with the suspension of the yeast organisms to be tested. The inoculated galleries were incubated at 37°C and the observations were made after 24 and 48 h. The identification of isolates was facilitated by the use of a computer programme.

The fermentation patterns among the carbohydrates were determined using API Kit gallery. The differential characteristics of the lactic acid bacteria and yeast isolate used for the fermentation were showed in Table 3.

The lactic acid starter and yeast starter used had different effects on the fermentation of sweet potato. The fermentation of sweet potato with the combined starters yeast and lactic acid bacteria gave the best attributes. The association of Lactic acid bateria and yeasts during fermentation may also contribute secondary metabolites, which could impact on the taste and flavour of foods (Akinrele, 1970; Halm et al., 1993; Brauman et al., 1996; Hansen and Hansen, 1996) and this was evident in Table 4.

Values in the same row not followed by the same superscript are significantly different ($P < 0.05$). Reduction in pH was observed during the fermentation process in Table 5. Acids are produced during fermentation, thereby

Table 3. Differential characteristics of lactic acid bacteria and yeast isolates based on Analytical Profile Index (API) kits analysis.

API kit	50 CHL	API Kit	ID 32 C
Isolate code	L201	Isolate code	601A
Glycerol	-	D-galactose	+
Erythritiol	-	Cycloheximide (ACT idione)	+
D-arabinose	-	D-saccharose	+
L-arabinose	-	N-acetyl-glucosamine	+
D-ribose	+	Acide lactique	-
D-xylose	+	L-arabinose	+
L-xylose	+	D-celiobiose	+
D-adonitol	-	D-raffinose	+
Methyl-βd-xylopyranoside	-	D-maltose	+
D-galactose	-	D-trenalose	+
D-glucose	+	Potassium2- keto gluconate	+
D-fructose	+	Methyl-αD-glucopyranoside	+
D-mannose	+	D-mannitol	+
L- sorbose	+	D-lactose (origine bovine)	+
L-rhamnose	-	Innositol	-
Dulcitol	-	Pas de substrat	+
Inositol	-	D-sorbitol	-
D-mannitol	-	D-xylose	+
D-sorbitol	-	D-ribose	+
Methyl- α D-mannopyranoside	-	Glycerol	+
Methyl-α D-glucopyranoside	-	L-rhamnose	-
N-acetylglucosamine	-	Palatinose	+
Amygdaline	+	Erythritol	+
Arbutine	+	D-melibiose	-
Esculin citrate de fer	+	Sodium glucuronate	+
Salicine	+	D-melezitose	+
D-celioboise	+	Potassium gluconate	-
D-maltose	+	Acid levulinique(le veulina te)	+
D-lactose (origine bovine)	+	D-glucose	+
D-melibiose	+	L-sorbose	+
D-sacchrose	+	Glucosamine	+
D-trehalose	+	Esculine citrate de fer	
Inuline	+	Identified microorganisms	*Debaromyces polymorphus*
D-melezitose	+		
D-raffinose	-		
Amidon	+		
Glycogene	-		
Xylitol	-		
Gentioboise	-		
D-turanose	+		
D-lyxose	-		
D-tagatose	-		
L-frucose	-		
D-frucose	-		
D-arabitol	-		
L-arabitol	-		
Potassium gluconate	-		
Potassium 2-cetogluconate	+		
Potassium5-cetogluconoate	-		
Identified microorganism	*Lactobacillus brevies*		

-, Negative reaction; +, Positive reaction. API, Analytical profile index kit.

Table 4. Physiological observation during the fermentation of sweet potato using starter culture and spontaneous fermentation.

Characteristics		Sample A	Sample B	Sample C	Sample D
Texture	24H	Hard	Hard	Soft	Hard
	48H	Soft	Soft	Very soft	Slightly soft
Water	24H	Light cream	Light cream	Light cream	colourless
	48H	Thick cream	Thick cream	Thick cream	Light cream
Aroma	24H	No aroma	Little aroma	Little aroma	No aroma
	48H	Aroma	Full aroma	Full aroma	Little aroma

Sample A, Lactic acid Starter; Sample B, Yeast starter; Sample C, combination of yeast and Lactic acid; sample D, Control.

Table 5. Changes in pH during fermentation.

Sample	Time duration in hours		
	0	24	48
A	$5.34^{a} \pm 0.005$	$3.47^{b} \pm 0.005$	$3.28^{c} \pm 0.005$
B	$5.02^{a} \pm 0.005$	$4.20^{b} \pm 0.005$	$3.51^{c} \pm 0.005$
C	$5.46^{a} \pm 0.005$	$4.09^{b} \pm 0.005$	$3.66^{c} \pm 0.005$
D	$5.49^{a} \pm 0.005$	$3.83^{b} \pm 0.005$	$3.44^{c} \pm 0.005$

S.D, Standard deviation. Sample A, lactic acid starter; Sample B, Yeast starter; Sample C, combination of lactic acid and yeast; Sample D, Control. Values are average of two determinations.

Table 6. Changes in % total titrable acidity (lactic acid) during fermentation.

Samples	Time duration in hours		
	0	24	48
A	0.001	0.022	0.040
B	0.001	0.002	0.023
C	0.001	0.005	0.033
D	0.001	0.014	0.036

Sample A, Lactic acid starter; Sample B, Yeast starter; Sample C, combination of lactic acid and yeast; Sample D, Control.

making the environment acidic and this results to reduction in pH. All the samples fermented had a reduction in their pH value. The fermentation that used lactic acid bacteria starter as a mono culture had the lowest pH and this was observed in similar findings by Farahat (1998) and Asmahan et al. (2009).

During fermentation, acid is produced and it increases as the fermentation period increases. The increase in lactic acid in Table 6 followed the same trend as reported for some fermented foods (Mohammed et al., 1991; Choi et al., 1994; Dziedzoaze et al., 1996) as the pH reduces the production of lactic acid increases.

There was increase in the population of the some starters as fermentation period increased while some experienced a decrease. Sample C which had the combination of lactic acid starter and yeast starter had the population of starters increase simultaneously and this follows the trend reported by (Nout, 1991) that the proliferation of yeasts in foods is favoured by the acidic environment created by lactic acid bacteria while the growth of bacteria is simulated by the presence of yeasts, which may provide growth factors such as, vitamins and soluble nitrogen compounds. Co-metabolism of yeast and lactic acid bacteria was also reported by Nout (1991), Gobbetti et al. (1994) and Steinkraus (1996).

Reduction in microbial count observed in the control as shown in Table 7 was in line with earlier works by Melaku and Faulks (1998). The results are in agreement with those reported by other authors (Mbugua, 1984; Odunfa and Adeyele, 1985; Mohammed et al., 1991; Nche et al., 1994). Melaku and Faulks (1988) also indicated that numbers of lactic acid bacteria increased during the first stages of the natural fermentation with a slight reduction in number during the later stages of fermentation. This may be as a result of the antagonistic nature of other microorganisms present.

The microbial analysis of the fermented sweet potato samples and raw sweet potato was carried out to

Table 7. Changes in microbial count (log_{10} cfu/ g) during fermentation.

Sample	Physiological group	Time duration in hours		
		0	24	48
A	L201	6.06	7.02	7.39
B	601A	6.07	7.58	8.02
C	L201	6.67	8.92	9.14
	601A	6.40	7.99	8.02
D	Microbes	5.18	6.50	6.07
	Yeast	5.20	6.45	6.01

LAB, Lactic acid bacteria. Sample A , lactic acid starter; Sample B, yeast starter; Sample C, combination of yeast and lactic acid starter; Sample D, control. cfu/g, colony forming unit per gram.

Table 8. Microbial qualities of fermented sweet potato flour.

Parameter	Sample (cfu/g)		
	C	D	E
Total bacteria count	3.56×10^2	3.9×10^2	5.3×10^2
Total yeast and mould count	6.6×10^2	3.0×10^1	8.2×10^2
Total Lactic acid bacteria recovered	1.6×10^2	Nil	8.0×10^1
Total coliform count	Nil	Nil	Nil
Total Salmonella Shigella count	Nil	Nil	Nil
Presence of *Escherichia coli*	Nil	Nil	Nil

Sample C, Combination lactic acid and yeast; Sample D, control; sample E, Raw sweet potato; cfu /g, colony forming unit per gram.

Table 9. Mineral analysis of samples (mg/kg).

Element	Sample (ppm)		
	C ± S.D	D ± S.D	E ± S.D
Ca	2177.51^a± 0.0238	2432.68^b ± 0.1269	2043.99^c ± 0.0235
Mg	28.31^a± 0.1266	29.12^b ± 0.1173	28.53^c ± 0.1229
Mn	26.32^a ± 0.0013	32.94^b ± 0.0008	6.29^c ± 0.0012
Fe	16.91^a ± 0.0007	15.89^b ± 0.0009	17.82^c ± 0.0007
Zn	41.58^a± 0.3365	11.01^b ± 0.2045	6.70^c ± 0.1353
Na	1058.27^a ± 0.1157	1040.09^b ± 0.1169	672.98^c ± 0.1134
Cu	5.02^a ± 0.0006	5.90^b ± 0.0002	6.42^c ± 0.0008
K	380.31^a ± 0.0081	2390.02^b ± 0.0078	1488.36^c ± 0.0319

Sample C, Combination of lactic acid and yeast; sample D, control; sample E, Raw sweet potato. The values are average of three determinations. Values in the same column followed by the same superscript are not significantly different ($P < 0.05$).

determine the total bacteria count, total yeast and mould count, presence of Salmonella, Shigella, Coliforms and *Eschericia coil*. The microbial qualities of the fermented sweet potato showed that the fermented sweet potato flour is save for consumption, no Coliform, no Salmonella, no Shigella was detected (Table 8).

Table 9 shows the mineral analysis carried out on the fermented sweet potato flour and the raw sweet potato. The mineral content of some of the sample increased while some decreased. The increase observed could be as a result of the solubility of some minerals by fermentation. Nevertheless, microorganisms require nutrients and minerals for growth and development and this may be responsible for the reduction in some mineral after fermentation Destrosier (2004) and Talaro (2002). Heat produced during the drying operation (that is, drying the

Table 10. Proximate analyses of samples (%).

Parameter	Sample		
	C	D	E
Moisture	10.70[a] ± 0.18	10.52[b] ± 0.05	62.36[c] ± 0.06
Ash	0.86[a] ±0.09	0.77[b] ± 0.03	1.25[c] ± 0.35
Fat	0.56[a] ± 0.00	0.64[b] ± 0.0	0.79[c] ± 0.0
Crude fibre	2.66[a] ± 0.28	2.64[b] ± 0.20	1.98[c] ± 0.01
Total Carbohydrate	82.72[a] ± 0.00	81.85[b] ± 0.01	31.09[c] ± 0.01

Sample C, Combination of lactic acid and yeast, sample D, control, sample E, Raw sweet potato. The values are average of three determinations. Values in the same column followed by the same superscript are significantly different ($P < 0.05$).

Table 11. Functional properties of starter culture fermented sweet potato flour (%).

Functional property	Samples	
	C	D
Moisture content (%)	10.42[a] ± 0.50	9.56[a] ± 0.21
Water absorption capacity (g/g)	1.77[b] ± 0.03	1.84[b] ± 0.01
Swelling capacity (%)	86.67[c] ± 5.80	96.67[c] ± 5.80

Sample C, Combination of lactic acid bacteria and yeast; Sample D, control. Values are average of three determinations. Values in the same row with the same superscript are not significantly different ($p < 0.05$).

fermented mash) could also bring about reducing effect on some minerals such as calcium, phosphorus and iron, which are strongly adversely affected by heat.

Decrease in some minerals was also observed in findings by Talaro et al. (2002) and reducing effect on some minerals such as phosphorus and iron, could be as a result of heat and the affinity for some of these elements by microorganisms.

Table 10 shows the proximate composition of raw sweet potato (sample E), starter culture fermented sweet potato flour (sample C) and spontaneous fermented sweet potato flour (sample D).

The moisture content was high in the raw sweet potato with a value of 62.36% and 10.70 and 10.52% in samples C and D respectively. There was a reduction in the ash content in the raw tubers (sample A) from 1.25% to 0.86 in sample C and 0.77% in sample D. The crude fibre content was lower in the raw tuber, which was 1.98% than 2.66 in sample B and 2.64 in sample D. The fat content in the raw tuber was 0.79% while in the fermented sweet potato it was 0.56 in sample C and 0.64% in sample D.

The carbohydrate content in the raw tubers was 31.09% as against 82.72% in sample C and 81.85% in sample D. The results of the proximate composition of the raw sweet potato and fermented sweet potato flour showed that the level of water has been removed as the sweet potato was processed from the raw tuber into fermented flour. Consequently, the moisture content in the fermented sweet potato flour was low enough for proper storage (Destrosier, 2004; Ihekeronye et al., 1985). In addition, there was a reduction in the ash content of the fermented sweet potato flour as compared with the starting raw sweet potato. Fermentation and drying may have contributed to reduced level of ash content in the fermented flour. It is however possible that some of the available minerals in the raw sweet potato as shown in Table 9 were utilized by the microorganisms during the fermentation of sweet potato (Talaro, 2002). The crude fibre content of the fermented sweet potato flour sample C and sample D was higher than that in the raw tuber. The fat content in the raw sweet potato was higher than the starter culture fermented sweet potato flour and the spontaneously fermented sweet potato flour (sample C and D). Talaro (2002) stated that some microorganisms may require some level of fat to thrive hence the reduction in the fat content after fermentation.

Table 11 shows functional properties such as moisture content, water absorption capacity and swelling capacity of starter culture fermented sweet potato flour. The moisture content values of the fermented sweet potato flour sample C and D are 9.56 ± 0.21 and 10.42% respectively and this falls within acceptable limit for sweet potato flour (ISO 712). The moisture content in the starter culture fermented sweet potato flour and spontaneously fermented sweet potato flour was low enough for proper storage (Destrosier, 2004; Ihekeronye et al., 1985).

Onimawo and Egbekun (1998) reported that the water absorption capacity gives an indication of the amount of water available for gelatinization during the heat processing. The water absorption capacity for the values of the fermented sweet potato flour samples C and D are

1.84 ± 0.01 and 1.77 ± 0.03g/g respectively, the result reveals that there is no significant difference in the values for both samples. Shimles et al. (2006) also reported that water absorption of flours are influenced by a number of factors such as hydrophilic hydrophobic balance of amino acids, molecule size and shape.

The swelling capacity values 86.67± 3.33 and 96.67 ± 3.33% for samples C and D respectively, the swelling capacity of flour depends on particle size paricles, types of variety and types of processing methods and unit operations (Adebowale et al., 2005).

DISCUSSION

Lactic acid bacteria and yeast were isolated from sweet potato. Oluwole et al. (2012) also reported that lactic acid bacteria and yeast were isolated from sweet potato. Smith et al. (2007) and Panda et al. (2007). The screening of lactic acid bacteria for lactic acid production in Table 1 showed that all the micro-organism screened were able to produce lactic acid and L201 had the highest zone of inhibition of about 1.8 mm and L205 was 1.5 mm. The ability of the yeast isolates to hydrolyse starch was also investigated and yeast isolate 601A, 2.0 mm and isolate 607F, 1.3 mm. The tolerance of each of the selected isolates to the environmental conditions was tested in Table 2. The strains of the *Lactobacillus* sp. grew at 15 to 45°C, the ability to grow at high temperature is a desirable trait as it could translate to increased rate of growth and lactic acid production. At the same time, a high fermentation temperature reduces contamination by other microorganisms. During industrial fermentation, as lactic acid is being produced by the cells, alkali would be pumped into the broth to prevent excessive reduction in pH. Thus, the free acid would be converted to its salt form which would in turn increase the osmotic pressure on the cells. Therefore, a LAB strain with high osmotolerance would be desirable as an industrial strain and the LAB strain used in the study is osmo tolerant. Reduction in pH was observed during fermentation process in Table 4. The fermentation that used lactic acid bacteria growth as a mono culture had the lowest pH and this was observed in similar findings by Farahat (1998) and Asmahan et al. (2009).

The decrease in pH and increase in lactic acid followed the same trend as reported for some fermented foods (Mohammed et al., 1991; Choi et al.,1994; Dziedzoaze et al., 1996).Co-metabolism of yeast and lactic acid bacteria was also reported by Nout (1991), Gobbetti et al. (1994) and Steinkraus (1996) . It has been suggested that the proliferation of yeasts in foods is favoured by the acidic environment created by LAB while the growth of bacteria is simulated by the presence of yeasts, which may provide growth factors such as, vitamins and soluble nitrogen compounds (Nout, 1991). The association of LAB and yeasts during fermentation may also contribute secondary metabolites, which could impact on the taste and flavour of foods (Akinrele, 1970; Halm et al., 1993; Brauman et al., 1996; Hansen and Hansen, 1996).

Reduction in microbial count observed in the control as shown in Table 7 was in line with earlier works by Melaku and Faulks (1998). The results are in agreement with those reported by other authors (Mbugua, 1984; Odunfa and Adeyele, 1985; Mohammed et al., 1991; Nche et al., 1994). Melaku and Faulks (1988) also indicated that numbers of LAB increased during the first stages of the natural fermentation with a slight reduction in number during the later stages of fermentation.

The decrease in pH and increase in lactic acid followed the same trend as reported for other traditionally fermented foods (Mohammed et al., 1991; Choi et al., 1994; Dziedzoaze et al., 1996). Table 10 shows the proximate composition of raw sweet potato (sample E) and starter culture fermented sweet potato flour (sample C) and spontaneous fermented sweet potato flour (sample D).

The moisture content was high in the raw sweet potato with a value of 62.36% and 10.70 and 10.52% in samples C and D respectively. There was a reduction in the ash content in the raw tubers (sample A) from 1.25% to 0.86 in sample C and 0.77% in sample D. The crude fibre content was lower in the raw tuber, which was 1.98% than 2.66 in sample B and 2.64 in sample D. The fat content in the raw tuber was 0.79% while in the fermented sweet potato it was 0.56 in sample C and 0.64% in sample D.

The carbohydrate content in the raw tubers was 31.09% as against 82.72% in sample C and 81.85% in sample D. The results of the proximate composition of the raw sweet potato and fermented sweet potato flour showed that the level of water has been removed as the sweet potato was processed from the raw tuber into fermented flour. Consequently, the moisture content in the fermented sweet potato flour was low enough for proper storage (Destrosier, 2004; Ihekeronye et al., 1985). In addition, there was a reduction in the ash content of the fermented sweet potato flour as compared with the starting raw sweet potato crop. It is believed that the processes of fermentation as well as drying may have contributed to reduced level of ash content in the fermented flour. It is however possible that some of the available minerals in the raw sweet potato as shown in Table 9 were utilized by the fermenting organisms in the sweet potato mash (Talaro, 2002), and this may result in the reduction in the ash content of the fermented sweet potato flour (Ihekeronye et al., 1985). The crude fibre content of the fermented sweet potato flour sample C and sample D was also higher than that in the raw tuber. The fat content in the fermented sweet potato flour (sample C and D) was lower than in the raw sweet potato and this may be due to the processing technique used in which after fermentation of the sweet potato, Some micro-organisms may also require some level of fat to thrive

(Talaro, 2002). Microorganisms require nutrients and minerals for growth and development (and this may be responsible for the reduction in some mineral after fermentation (Destrosier, 2004; Talaro, 2002). Heat produced during the drying operation (that is, drying the fermented mash) could also bring about reducing effect on some minerals such as calcium, phosphorus and iron, which are strongly adversely affected by heat.

Decrease in some minerals was observed in findings by Talaro et al. (2002) and reducing effect on some minerals such as phosphorus and iron, could be as a result of heat and the affinity for some of these elements by microorganisms.

Table 11 shows functional properties such as moisture content, water absorption capacity and swelling capacity of starter culture fermented sweet potato flour. The moisture content values of the fermented sweet potato flour sample C and D are 9.56 ± 0.21 and 10.42% respectively and this falls within acceptable limit for sweet potato flour (ISO 712). The moisture content in the starter culture fermented sweet potato flour and spontaneously fermented sweet potato flour was low enough for proper storage (Destrosier, 2004; Ihekeronye et al., 1985).

The low moisture content would enhance storability of the product, reduce post-harvest losses and subsequently tackle food insecurity. The water absorption capacity for the values of the fermented sweet potato flour samples C and D are 1.84 ± 0.01g/g and 1.77 ± 0.03 g/g respectively. The result reveals that there is no significanat difference in the values for both samples. Onimawo and Egbekun (1998) reported that the water absorption capacity gives an indication of the amount of water available for gelatinization during the heat processing. Shimles et al. (2006) also reported that water absorption of flours are influenced by a number of factors such as hydrophilic hydrophobic balance of amino acids, molecule size and shape.

The swelling capacity values 86.67 ± 3.33% and 96.67 ± 3.33% for samples C and D respectively. the swelling capacity of flour depends on particle size particles, types of variety and types of processing methods and unit operations (Adebowale et al., 2005).

Conclusion

Sweet potato has been reported to have a great potential in curbing malnutrition particularly in developing countries (Woolfe, 1992). Sweet potato can be consumed as fermented or unfermented. Moreover, these sweet potato products can be produced by small and medium scale entrepreneurs. Starter culture is one of the prerequisites for the establishment of small scale industrial production of fermented foods in Africa. Presently indigenous starter cultures are not available in Nigeria (Holzapfel, 1997). Nevertheless, this present study suggests that starter cultures can be used in the fermentation of sweet potato into flour and its use will help reduced fermentation period, ensure product consistency and improved hygiene. The use of the mixed cultures as starter cultures in the fermentation of sweet potato for 48 h had the best attributes as seen in terms of texture, flavour, rate of fermentation and consistency when prepared as stiff porridge. This product could serve as a form of meal. The study however recommends further research to be conducted by fermenting sweet potato with combination of various strains of lactic acid bacteria and yeast for different fermentation periods.

Conflict of Interests

The authors have not declared any conflict of interests.

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Microbiological evaluation of drinking water available in schools in Cruz das Almas, Brazil

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The current study assessed the drinkability of water in 25 schools in Cruz das Almas BA Brazil. Total coliforms, *Escherichia coli*, *Enterococcus*, mesophylls and psichrotrophic bacteria were evaluated, coupled with color, turbidity, free residual chlorine, dissolved oxygen and biochemical oxygen demands. Water samples were collected at four sites: the first tap within the supply network or within the alternative supply (first site), main reservoir (second site), kitchen water (third site) and drinking water troughs (fourth site). When the two assessment periods (during dry and wet seasons) were taken into account, contamination by total coliforms in approximately 65.3% of samples was detected, whilst *E. coli* and *Enterococcus* micro-organisms respectively occurred in 18.4 and 36.7% of samples. Counts of mesophyll microorganisms were greater during the dry period than during the rainy season. More than 34.7% of counts were above the legal limit. Although no apparent variation in color occurred, irregularity in turbidity and pH was detected in two schools. Inadequate rates for dissolved oxygen occurred in only two schools, during the dry period, whereas biochemical oxygen demand complied with legislation. Although, no coliform was extant in several samples, others had bacterial contamination requiring more efficacious practices for quality improvement. Results show that health risks in schools in Cruz das Almas may be solved by adequate water treatment, periodical cleaning of reservoirs and proper maintenance of taps and filters.

Key words: Water quality, hygiene, microbiology, schools, colleges.

INTRODUCTION

Water is important for all living organisms. Life, the functioning of ecosystems, communities and economies depend totally on water. However, water scarcity occurs in almost all developing countries, with immense expenses spent on its availability, quality, use and mortality by water-caused diseases (Brasil, 2014; Khan, 2012). Life quality is strictly linked to the quality of drinkable water since it is a relevant element employed in

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several daily tasks such as cooking, personal hygiene and cleaning. Personal well-bring requires access to drinkable water free from pathogen agents and/or toxic chemical products which transmit diseases to consumers (Brasil 2011; Xavier et al. 2011; WHO, 2011).

Several factors such as domestic drainage, industrial and agricultural effluents, deforestation, mining, solid wastes, effluents from swine breeding, diffuse pollution in urban areas, salinization, environmental accidents, dam building and aquiculture contribute towards water contamination (Brasil, 2006; Xavier et al., 2011; WHO, 2011). However, the best way for water preservation is avoiding contamination by animal and human wastes which contain a great number of bacteria, viruses, protozoa and helminths. In fact, failure in the effective treatment of water causes great risks to consumer´s health (Brasil, 2006; Azizullah et al., 2011; WHO, 2011; Rodrigues and Barros, 2012).

Several studies have shown that approximately two million tons of industrial drainage and agricultural wastes are deposited in water courses worldwide, resulting in illnesses due to contaminated water (UNEP, 2010). In fact, 2.2 million people, including 1.8 million children less than five years old, die every year from diarrhea and related diseases. United Nations Organization estimates show that almost 900 million people have some difficulty in accessing drinkable water and approximately 2.6 million do not have basic sanitation facilities. In Brazil, water availability reaches 91% of the population, with 98% in urban areas. Approximately 70% of rural populations have access to drinkable water, or rather, a rise in 85% within the last 25 years (UNEP, 2010; Brasil, 2012; Instituto Trata Brasil, 2015).

Brazilian legislation (Decree 2.914 of 2011, issued by the Ministry of Health, Brasil, 2011) forwards several parameters to characterize water as drinkable. Its parameters are based on universal rules derived from the Guidelines for Drinking-Water Quality of the World Health Organization (WHO, 2011). Current analysis evaluates drinking water available for consumption in several schools of Cruz das Almas BA Brazil.

METHODOLOGY

The current study was performed in 25 public school institutions (labeled A to Y) in the municipality of Cruz das Almas, Brazil. Analyses were undertaken during two seasonal periods. During the dry season (September to March), 25 water samples were collected between November and December, whereas samples were retrieved between June and August during the rainy season (April to August) (Bahia, 2013). Water samples were collected and transported to the Laboratory of Animal Parasitology and Microbiology of the Universidade Federal do Recôncavo da Bahia (UFRB) for analysis.

Water was collected at four sampling points in each school by retrieving 500 ml from each sampling point, amounting to 2 L of water from each school: water collected from the first tap of the water supply network or from the alternative supply (1st sampling point), main reservoir (2nd sampling point), kitchen water (3rd sampling point) and the main drinking water troughs (4th sampling point). Information on the water supply source was also retrieved. Collection points (tap or trough) were kept open with water flowing during two or three minutes; hygiene with alcohol 70% was undertaken for sample collection.

Total coliforms and *Escherichia coli* were analyzed by chromogenic substrates (*Colilert*®) based on two active substrates, σ-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferil-β-D-glucuronide (MUG), to detect total coliforms and *E. coli*, respectively. Coliforms produce the enzyme β-galactosidase which hydrolyzes ONPG and releases σ-nitrophenol, providing a yellowish color to the medium. *E. coli* produces enzyme β-glucuronidase which hydrolyzes MUG, forming the fluorescent compound 4-methylumbelliferone under ultraviolet light at 365 nm. Microorganisms were analyzed quantitatively, or rather for their presence or absence, by adding 100 mL of water samples in transparent plastic flasks (previously sterilized in autoclave). A powder substrate was added to each flask, homogenized and distributed on IDEXX™ Quanti Tray cards. The material was incubated in a buffer at 36°C for 24 h. After incubation, the yellowish color indicated total coliforms, whereas fluorescence blue under UV light (365 nm) in the dark indicated *E. coli*. Estimates of MPN.100 ml^{-1} at 95% confidence for each MPN rate quantified total coliforms and *E. coli*.

MPN of microorganisms *Enterococcus* was calculated by multiple tube technique, in a series of 5 or 10 tubes, in which 5/10 ml of water were inoculated in tubes with Glucose Azide Broth in double concentration and then incubated at 36±1°C for 48 h. Positivity would be demonstrated by the medium´s turbidity. Further, plates with medium Pfizer selective enterococcus (PSE) agar were striated in all tubes with turbidity to confirm the presence of typical colonies, featuring a dark brownish color with a brown halo (Apha, 1998).

Mesophyll microorganisms were counted by the Pour Plate technique in which 1 mL of each sample was deposited on the bottom of petri plates, adding (Plate Count Agar (PCA) culture medium previously fused and cooled at 40°C. After homogenized and solidified, the content was incubated at 35°C for 48 h. Counting was performed by colony counters on plates with 10 - 300 colony-forming units (CFU) (Apha, 1998).

Psichrotrophic microorganisms were calculated by the above-mentioned method described for mesophylls, with the exception of temperature and incubation period, respectively at 7°C for 10 days, in CFU.mL^{-1} (Apha, 1998).

Colorimeter and turbidity-meter were employed to determine the physical parameters color (UHazen) and turbidity (UNT), respectively. Further, pH rates of samples were obtained by pH-meter, while free residual chlorine was determined by NN diethyl paraphenylene diamine (DPD) with colorimeter HACH (Hanna, 1998).

Dissolved oxygen and biochemical oxygen demand of samples were measured by dissolved oxygen meters Hanna DO-5519 and Lutron DO-5519 (Apha, 1998).

RESULTS AND DISCUSSION

Microbiological assessment

Coliform microorganisms occurred only in samples collected during the rainy period, with approximately 66.6% contamination of the water analyzed at one out of the four collection sites. There was a 64% contamination of analyzed sites in samples during the dry period and

Table 1. Arithmetic means of most probable numbers (MPN) of total coliforms, *Escherichia coli*, *Enterococcus* and colony-forming units (CFUs) of mesophylls and psichrotrophic organisms in supply water of school units in Cruz das Almas BA Brazil, in two periods, between September and March 2015 (dry period) and between April and August 2015 (rainy period).

School/ college	Dry period					Rainy period				
	TC	EC	ENT	MSF	PSF	TC	EC	ENT	MSF	PSF
A	0.00	0.00	0.00	426.83	0.0	3.67	0.00	0.00	34.67	0.00
B	0.00	0.00	0.00	0.67	0.0	0.67	0.00	0.00	234.00	0.00
C	0.00	0.00	0.00	2.00	0.0	0.00	0.00	0.00	0.00	0.00
D	2419.60	18.80	4.07	1495.55	0.0	1535.48	0.00	13.23	317.50	6.75
E	0.00	0.00	0.00	442.42	0.0	634.53	0.00	4.03	322.25	1.75
F	2419.60	0.00	6.28	717.69	0.8	0.00	0.00	23.00	0.00	3.00
G	1.37	0.00	0.00	1026.92	46.0	0.00	0.00	19.55	0.00	46.00
H	12.30	0.00	0.00	167.75	0.0	0.00	0.00	20.70	0.00	0.00
I	1029.20	108.15	8.00	595.63	0.0	866.45	62.30	1.10	555.00	73.00
J	1769.75	0.00	8.00	1031.88	17.5	387.90	8.60	0.00	1275.00	0.00
K	2419.60	0.00	1.10	2250.63	0.5	686.70	0.00	0.00	1018.75	0.00
L	2.33	0.00	0.00	983.13	0.0	3.60	0.00	0.00	532.75	1.50
M	41.95	0.00	0.00	6500.00	0.0	0.00	0.00	0.00	1.25	0.00
N	0.00	0.00	0.00	439.08	0.0	0.00	0.00	0.00	1.00	0.33
O	0.78	0.00	0.00	1424.55	18.3	1.25	0.00	8.60	393.31	0.25
P	1853.80	11.20	3.30	1096.40	0.0	1313.25	0.80	2.95	153.33	0.00
Q	41.20	0.00	0.00	817.92	0.0	157.70	0.33	5.33	291.22	0.00
R	0.00	0.00	0.00	0.00	0.0	0.00	0.00	0.00	0.33	0.00
S	2.73	0.00	0.00	98.67	0.0	115.27	0.33	0.00	131.33	0.00
T	0.00	0.00	0.00	0.00	0.0	0.00	0.00	0.00	0.00	0.00
U	1.37	0.00	0.00	708.50	0.0	0.33	0.00	0.00	44.67	0.00
V	0.00	0.00	0.00	0.33	0.0	0.00	0.00	0.00	0.33	0.00
W	3.75	0.00	0.00	34.00	0.0	1.50	0.00	0.00	763.12	0.00
X	78.63	0.00	0.70	23.75	0.0	604.90	0.50	4.00	410.72	110.25
Y	-	-	-	-	-	11.10	0.00	6.65	394.62	0.00

TC– Total coliforms; EC– *Escherichia coli*; ENT– *Enterococcus*; MSF– Mesophylls; PSF– Psichrotrophic organisms.

thus inadequate for human consumption. A higher contamination rate was detected during the dry period when compared with that during the rainy one as averages (Table 1). In fact, results ranged between 0.0 (absence) and 2419.6 MPN.100 mL^{-1} of water. The above demonstrates that, regardless of the seasonal period or the supply form, and due to total coliforms, intervention of the sites under analysis should be made so that water could be safe for consumption (Table 1). Samples from the alternative supply had even higher contamination rates (Table 1), probably due to lack of chlorine treatment, mandatory by current legislation for drinkable water.

Rates contradict several studies that underscore the importance of data collection at different season periods. Review by Kostyla et al. (2015) on 22 studies on water quality and seasons in developing countries revealed that contamination is highest during the rainy period when bacteria, indicating fecal contamination, measurement methods, population definition, source type and equatorial climate zone are taken into account. The above is due to the fact that research related to water quality gives priority to the dry period because of accessibility, time, roads and other factors. However, there has been a recent trend to underscore the fact that water microbiological contamination is greater during the rainy season (WHO, 2010; UNICEF, 2010), which did not occur in the current study. Since collection periods were coupled with hygiene at the collection site in each school analyzed, the final result must have been affected.

Collection sites of the samples may also affect the research´s conclusion data since each specific site may produce a different result (WHO, 2010; UNICEF, 2010). In fact, the collection of samples at different sites in schools is very relevant so that greater representativeness would be obtained and the precise focus would be detected. Samples collected from WSS + ACS (B and U) show mild contamination by coliforms,

or rather, 0.67 and 0.33 MPN.100 mL^{-1} for averages of the four collection sites for the rainy season, and 0.0 and 1.37 MPN.100mL^{-1} for the dry period. In spite of low contamination rate, the samples are not the best for consumption and cross contamination of treated water may have occurred (Conama, 2005; Brasil, 2011, WHO, 2011).

The microorganism, *Escherichia coli* indicates recent fecal contamination and the possible presence of pathogens, since these microorganisms are strictly fecal. The detection of their presence is relevant since they cause several diseases, such as *E. coli* O157: H7 which may be lethal due to hemolytic-uremic syndrome (HUS), a very serious disease in children, elderly and immune-compromised people. Similar to legislation on total coliforms, the microorganism *E. coli* should be absent in water for human consumption in WSS and ACS (Conama, 2005; Brasil, 2011; WHO, 2011; Eden, 2014)

Table 1 shows averages in the collection sites. Variation in mean rates between 0.0 (absence) and 108.15 MPN.100mL^{-1} of the sample could be detected in the two season periods when the research was conducted. In fact, the microorganism was present in six schools during the rainy season and in three schools during the dry period. All the schools where *E. coli* was detected urgently require intervention due to harm caused to people by drinking contaminated water.

In the case of the two evaluation periods, the highest contamination levels occurred at collection sites 1 and 2. Since all water for consumption should be free from *E. coli*, all drinkable water should be treated and exhibit free residual chlorine rate so that micro-biological contaminations would be avoided (Brasil, 2011). Greater control is required to verify possible flaws in the water distribution system or in the building structure, hygiene of reservoir and water troughs.

Several studies by Jasper et al. (2012) have shown that school children exposed to inadequate water conditions and deficient sanitary installations may develop infectious, gastrointestinal, neural-cognitive and psychological diseases. The hygiene-sanitary segment of the premises should receive greater investments. Education is a right and the availability of water and sanitation in schools is highly relevant and requires criteria, norms and control so that the educational process would not be jeopardized.

Water may be contaminated in diverse manners as Rocha et al. (2010) exemplify in a similar study on schools. Contamination may initially occur in the public supply system, even though it commonly occurs by faulty distribution or by lack of hygiene of the water reservoir which supplies the schools. Faults in the maintenance of pipes and principally of the reservoir develop favorable conditions for microbial growth. Hygiene and the maintenance of water integrity for consumption avoid contamination.

Moosa et al. (2015) evaluated water quality in drinking troughs in schools and at the university in Ajman, United Arab Emirates, and detected *Pseudomonas aureginosas*, total coliforms and *E. coli*: 32.65% of the 49 samples assessed were contaminated by total coliforms and unfeasible for consumption according to the legislation of the country mentioned above. The authors reported that the drinking water should not contain the bacterium. As underscored by Rocha et al. (2010), cleansing of the premises and its surroundings has a key role in the maintenance of water quality for drinking. Another important factor in the study are stricter attitudes by health authorities to guarantee drinking water quality. The control and monitoring of water quality by school administrators and educators should involve the student community and the general society to supply water within the mandatory parameters. These activities must be backed by governmental policies to inform the population on quality parameters and how to maintain the best water quality (Souza et al., 2015). In a similar study, the above authors assessed input supply water, water exit from the reservoir and water in the drinking troughs and verified that 60.6% of water in the 33 school institutions in Mossoró RN Brazil were inadequate for human consumption, featuring total coliforms, and/or thermotolerant coliforms, with a high contamination index by total coliforms and *E. coli* in the schools´ drinking troughs.

The presence of significant amounts of coliform bacteria (total coliforms and *Escherichia coli*) in underground water indicates irregularities deep in the earth or a break down in the sanitary integrity of water wells. Inadequate protection associated with sanitary flaws highlights the need to establish plans for the protection of water supply for rural communities where water treatment is still unavailable. Hedging the premises adequately, enhancing regular maintenance and disposing correctly human and animal wastes (which infiltrate the soil and contaminate the place) are mandatory. They are strategies that ensure the safety of the water sources (Tsega et al., 2013; Bain et al., 2014).

Although, no specific legislation exists to determine the most probable number (MPN) for enterococcus micro-organisms in water for human consumption, high counts indicate potential sources of contamination caused by deficiencies in water treatment or in the distribution system (Conama, 2005; Brasil, 2011; WHO, 2011). Table 1 registers MPN of *Enterococcus* microorganisms during the two periods analyzed in the current study.

Positive results occurred during the dry and rainy periods, respectively in 29.16 and 44% of samples. Although, they may be found in the feces of animals or even in the environment as free organisms, enterococci are closely associated with human wastes, featuring fecal pollution in the water and lack of intervention when high rates were detected (Brasil, 2006). Further, these

microorganisms may be of great help to detect fecal contamination, albeit with certain restrictions, since they are integrated to food microflora. However, they are more resistant to chlorination when compared with the coliform group (WHO, 2011, 2003).

There were greater contamination levels by enterococci during the rainy period, confirming position by WHO and UNICEF (2010) and Kostyla et al. (2015) and by Table 1. In fact, the microbiological contamination of water is greater during the rainy period, confirming seasonal influences that enhance false-positive results in research that evaluates consumption water quality.

Assessment of water samples in plastic tanks and in wells (89 and 177) in an area within the metropolitan region of São Paulo, Brazil, revealed that enterococcus microorganisms were extant in 21 samples (23.5%) for plastic tanks and in 142 samples (80.2%) for wells, enhancing possible interventions due to fecal contamination (Razzolini et al., 2011).

Several studies have focused on enterococci especially with regard to their resistance capacity. A study assessed enterococci in water in bottles, hospitals and wells in Kerala, India, and detected contamination in 74% of the 270 samples. Results revealed that water may contain bacteria resistant to anti-microbial agents with severe health risks to consumers. Resistance is acquired by the microorganisms' capacity to form protecting films or biofilms (Peter et al., 2012). Concern on these microorganisms is highly important since they may damage human health regardless of their requirement by Brazilian legislation. Mesophyll and psychrotrophic microorganisms were selected for heterotrophic bacteria. In the case of strict and facultative aerobic mesophyll microorganisms, contamination level ranged between 0.0 (absence) and 6.500 $CFU.mL^{-1}$, respectively, reached high rates (Table 1) considered irregular by Decree 2,914/2011 of the Ministry of Health, with a limit of 500 $CFU.mL^{-1}$ in water samples. During the dry period, greater population counts of mesophyll microorganisms were reported as compared to those during the rainy period, with 45.83 and 20.00% of samples above the rates allowed by law.

No psychrotrophic microorganism rates above those permitted by Brazilian legislation were detected in the two periods analyzed and only small counts were registered. Since these microorganisms have an wide temperature range (between -10 and 30°C) for their development, great care should be taken to quantify them (Tortora et al., 2012) since they may be found in places without any refrigeration, such as sites 1 (reservoir) and 4 (water troughs), at room temperature or even regardless of temperature.

When they are used to indicate water quality, heterotrophic bacteria counts (HBC) have a wide range of results, or rather, they reveal bacteria of fecal origin or bacteria naturally occurring in water, since rates above 500 $CFU.mL^{-1}$ may indicate the occurrence of coliforms. Although they may not be prejudicial to health, high counts of these microorganisms are a warning on flaws in the water treatment, either due to disinfection and to the formation of biofilms, or in storage or faults in the distribution systems or the presence of organic matter in water. They may also indicate the existence of pathogenic agents such as *Acinetobacter, Aeromonas, Flavobacterium, Klebsiella, Moraxella, Serratia, Pseudomonas* and *Xanthomonas* (Ana, 2005; Brasil, 2011; WHO, 2011; Chowdhury, 2012). Similar to requirements for enterococcus microorganisms, the above is also required to evaluate bottled mineral water (Falcone-Dias and Farache Filho, 2013).

The parameter also indicates the efficiency of the disinfection process, improvement in the installations for water distribution and evaluation of water storage in tanks or in reservoirs (Diduch et al., 2016).

Physical and chemical assessment

Physical assessment showed no variation when the parameter color of water was evaluated. Only two of all the sites analyzed failed to comply with legislation on turbidity (Table 2), with rates up to 5.0 UNT (BRASIL, 2011). Means from each environment assessed revealed a variation between 0.54 and 7.26, with higher rates during the rainy period than during the dry period. Soil erosion, mining activities, drainage and industrial effluents were the main causes. Turbidity above the best legal rates directly affects consumer´s water acceptability. The less the turbidity rates, the more acceptable will the water be. Further, suspended particles may protect pathogenic microorganisms (BRASIL, 2006; WHO, 2011). High turbidity levels may indicate high levels of microbial contamination and other physical and chemical parameters. Therefore, turbidity rates may be used to select water sources. In fact, turbidity analysis is a low-cost key parameter and indicates the efficient removal of pathogens resistant to chlorination (Mann et al., 2007, WHO, 2011; Castaño and Higuita, 2016).In the case of chemical assessment, pH of samples B, D and O (Table 2) during the two collection periods featured rates below the ideal range (between 6.0 and 9.5, according to Brasil, 2011). Although, pH does not have a direct impact on the water used by the final consumer, it is a very important parameter. When it exceeds the ideal range, it may influence disinfection and chlorine clarification in the water. Flaws in the maintenance of the range may cause several liabilities, such as corrosion and incrustations in the pipes, which may change the water's taste and aspect due to the aggregation of the material that the water tubes are made of (Brasil, 2006; WHO, 2011). Free Residual Chlorine count was the parameter with the greatest variation. Decree 2,914 of 2011 of the Brazilian

Table 2. Arithmetic means of physical and chemical parameters of supply water in teaching institutions in Cruz das Almas BA Brazil, during two periods, between September and March 2015 (dry period) and between April and August 2015 (rainy period).

School/	Dry period						Rainy period					
College	Color	Turbidity	pH	FRC	DO	BOD	Color	Turbidity	pH	FRC	DO	BOD
A	0.00	0.64	6.84	1.93	4.70	0.79	0.00	0.70	7.39	1.02	7.70	0.71
B	0.00	0.57	5.58	0.69	3.87	0.66	0.00	1.02	4.39	0.23	7.63	1.62
C	0.00	0.82	6.64	7.16	7.93	0.37	0.00	0.60	7.11	2.72	7.87	0.23
D	0.00	2.90	6.41	0.26	7.40	1.05	0.00	7.26	5.54	0.35	7.35	0.44
E	0.00	1.18	6.28	0.98	8.13	0.73	0.00	0.53	7.00	0.42	7.78	0.13
F	0.00	0.78	7.10	0.06	7.18	0.52	0.00	1.06	7.65	0.43	7.90	0.20
G	0.00	1.18	6.28	0.98	8.13	0.73	0.00	0.86	7.48	0.98	8.00	0.23
H	0.00	0.63	7.73	0.08	7.35	0.43	0.00	0.70	7.82	0.37	7.83	0.45
I	0.00	1.49	6.15	0.03	5.90	1.02	0.00	1.38	6.89	0.13	7.20	0.29
J	0.00	1.04	6.83	0.04	3.65	1.36	0.00	1.46	7.44	0.14	7.50	0.82
K	0.00	0.62	7.25	0.11	6.50	0.67	0.00	1.75	6.55	0.11	7.00	0.29
L	0.00	0.61	7.65	0.02	7.85	0.69	0.00	0.53	7.74	0.12	7.55	0.51
M	0.00	0.66	6.60	0.10	7.95	0.14	0.00	0.87	7.86	0.83	7.63	0.59
N	0.00	0.60	6.66	1.33	7.70	0.80	0.00	0.72	7.85	2.26	7.47	0.47
O	0.00	0.54	7.03	0.79	7.58	0.85	0.00	0.65	7.73	0.13	7.80	0.55
P	0.00	0.96	6.00	0.22	7.78	0.68	0.00	5.54	5.85	0.11	5.18	1.11
Q	0.00	0.54	6.58	9.45	7.00	0.90	0.00	0.75	6.59	1.18	5.37	0.47
R	0.00	0.51	6.65	3.02	6.70	0.39	0.00	0.62	6.38	6.18	5.70	0.65
S	0.00	1.01	6.35	0.08	7.90	0.23	0.00	0.89	6.73	0.05	5.07	0.59
T	0.00	0.50	7.05	6.52	7.50	0.48	0.00	1.47	7.03	4.89	7.45	0.89
U	0.00	0.85	6.53	0.06	7.77	0.15	0.00	0.96	6.98	0.87	6.98	0.74
V	0.00	0.60	6.23	3.23	8.00	0.17	0.00	0.99	7.33	1.23	8.10	1.42
W	0.00	0.79	6.29	2.71	7.47	0.88	0.00	0.68	7.60	2.18	8.00	1.40
X	0.00	0.80	7.01	1.56	7.35	0.53	0.00	0.74	7.97	2.24	8.15	1.09
Y	0.00	-	-	-	-	-	0.00	1.45	7.26	0.85	8.05	0.28

pH– Hydrogenionic potential; FRC– free residual chlorine; DO– dissolved oxygen; BOD– biochemical oxygen demand.

Ministry of Health specifies an acceptable standard for the parameter, with a minimum of 0.2mg.L^{-1} of Free Residual Chlorine in the water for consumption up to 2 mg.L^{-1} throughout the supply system. When means in Table 2 are analyzed, samples I, J, K and L show that during the two assessment periods, rates were below the best level. Within the set of samples evaluated, only 34% of the schools during the dry period had rates which complied with the range proposed by the Ministry of Health. On the other hand, FRC rates comply with legislation in 48% of the schools during the rainy period.

Since chlorine is the principal active compound for water disinfection, several analyses have focused on the manner the product works without causing economic liabilities or any harm to people´s health. There are several strategies for its maintenance at adequate levels to avoid re-contamination after treatment and harm to the consumer´s health. Decrease in FRC levels frequently occurs when the pipes of the distribution system are made of copper as compared to PVC and galvanized pipes.

However, several motives, such as frequent stagnations, temperature increase, reduced discharge, stocking for large periods and others, may alter FRC levels (Zheng et al., 2015).

Contrastingly, high levels may also cause serious health risks to people. Although, chlorine´s residual load combats the risk of contamination by the pathogenic microorganisms in the water, contrary effects may occur when rates are high. Associated with high levels of FRC, one should mention the formation of trihalomethanes, compounds formed by the reaction between chlorine and organic compounds, originating cancerigenous chloroforms. Besides allergic symptoms, such as skin eruptions and intestinal symptoms, when ingested, the bacteria of lactic acid that line the colon are destroyed and the intestine is exposed to foreign pathogenic agents (Brasil, 2006; Siddique et al., 2011; Zheng et al., 2015). When they quantified the minimum legal rate of 0.2 mg.L^{-1} for FRC, Sanches et al. (2014) verified that 40.62% of

samples from kitchen taps and 31.25% of samples from drinking troughs from eight schools in Uberaba MG Brazil failed to comply with the accepted minimum. Moreover, Cardoso et al. (2007) reported that FRC rate in 33% of samples from 83 schools in Salvador BA Brazil was below the legal parameter. The above proves a common denominator in many Brazilian municipalities.

Dissolved oxygen is one of the most relevant parameters to evaluate the quality of water environments. Although, DO averages of samples analyzed for the two periods were very similar, three school institution registered rates lower than 5 $mg.L^{-1}$ during the dry period. Consequently, they failed to comply with legislation which states that rates should not be lower than 5 mg/L (Conama, 2005). Alterations in DO levels are due to temperature variations, pressure and water salinity. In other words, physical, chemical and biological processes act directly on water bodies (Brasil, 2014).

Several studies report that the association between temperature, pH and DO in irregular rates may affect water quality, especially in supply systems with copper pipes. When the re-contamination of water is evaluated, one may observe a close relationship between temperature, DO and heterotrophic bacteria counts. Best temperature rates and available oxygen enhance re-contamination of the environment since they lack FRC to eliminate re-contamination (Vargas et al., 2010; WHO, 2011; Lu et al., 2014).

All BOD rates complied with current legislation up to 5 $mg.L^{-1}$ (Table 2) (Brasil, 2014). There was a variation in averages between 0.50 and 0.79 $mg.L^{-1}$, considered the best rates. Although, BOD is a parameter to assess domestic and industrial effluents in natural non-polluted environments, the Brazilian Health Foundation registers that the ideal concentration in such environments varies between 1 and 10 $mg.L^{-1}$. This is a low rate, with alterations only with possible contaminations affecting color, turbidity and DO consumption by decomposing organisms (Brasil, 2014; Lu et al., 2014). Wanda et al. (2015) studied the quality of water in certain African regions and reported that high BOD, availability of organic matter in the medium, greatly interferes with water classification. When associated with a low DO rate plus microorganisms, great care must be taken with the assessed water treatment system

Conclusions

Although, assessments during two periods have beenundertaken to obtain a greater representation of the hygiene-sanitary quality of available water in schools in Cruz das Almas, a constant follow-up of the water quality is needed since students, school officers and teachers spend a large part of their time on the premise and use the water available.

Immediate intervention is required in sites where FRC rates exceed the acceptable range, since otherwise microbial growth and re-contamination will occur during the dry and rainy periods. Immediate provisions should be taken when fecal contamination occurs in the water. Conditions should be considered irregular even though high rates occur during the dry period. Current data are a help to school and municipal authorities so that water quality problems would be solved. Adequate and regular hygiene of water reservoirs that provide water to the kitchen and water troughs, change of water filters and the establishment of standard procedures are alternatives for the elimination of microorganisms to comply with Brazilian legislation.

In the case of schools that use alternative water supply solutions, the issue on the implantation of the public supply system and other options should be raised for the solution of the problem. In fact, the Ministry of Health has already recommended alternative disinfection such as diffused chlorination, lozenge chlorination, liquid chlorination and the establishment of home-treatment units.

Conflict of Interests

The authors have not declared any conflict of interests.

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