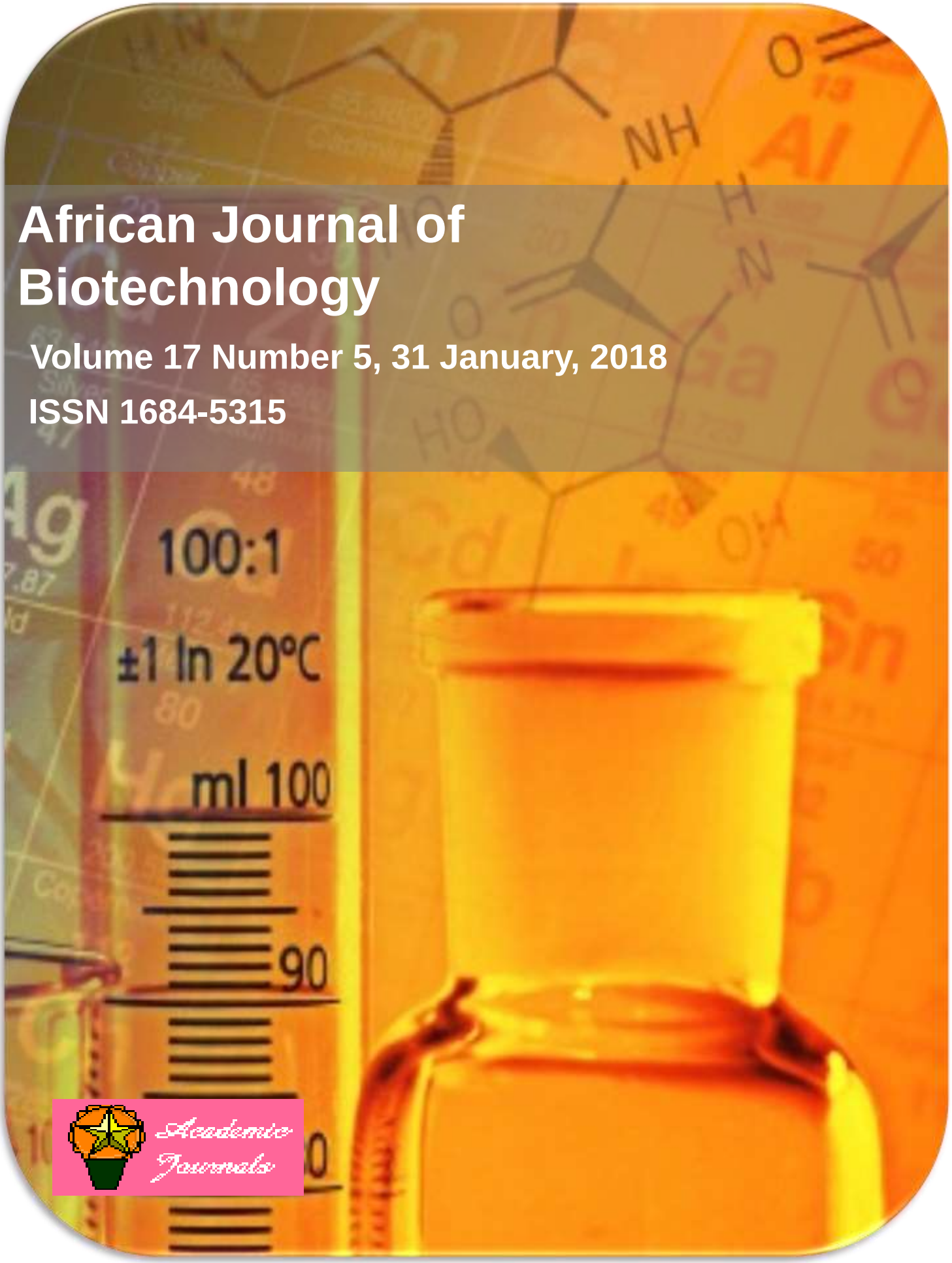

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Full Length Research Paper

Controlled fermentation of the *zoom-koom* dough using two isolates of lactic acid bacteria (LAB 1 and LAB 5) as starter cultures: Effect on hygienic, rheological, nutritional and sensorial characteristics of the final product

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Zoom-koom **is a popular non-alcoholic beverage in Burkina Faso, which is based on cereals and mainly produced by women with important socio-economic implications. This study aimed to evaluate the effect of controlled fermentation using two selected isolates of lactic acid bacteria (LAB) as starter cultures, on the rheological and hygienic quality of *zoom-koom.* The starter cultures were used singly in monoculture and both in mixed culture. Microorganisms dynamic during the controlled fermentation were followed and enumerated using pour plate methods. The titratable acidity, pH, viscosity, water, ash, crude protein (N×6.25), crude fat and total carbohydrates contents were determined on the final *zoom-koom* by using standards methods. Sensory analyses of *zoom-koom* samples were performed by a panel of 30 tasters. The enterobacteria counts of all the controlled fermented *zoom-koom* samples using starters cultures decreased totally and significantly ($p<0.05$) from 6.4 (LAB 1), 5.5 log CFU/g (LAB 5) and 3.8 (LAB 1 and 5 in mixed culture) to < 1 log CFU/g after 24 h of fermentation. However, those of natural fermentation without inoculum decreased significantly ($p<0.05$) but not totally (1.4 log CFU/g after 24 h of fermentation). The *zoom-koom* from LAB 5 presented the best production of exopolysaccharides and was more viscous and homogenous than the others. All the *zoom-koom* samples presented a low fat (4.74, 5.21, 5.36 and 5.55%/DM) and ash (0.32, 0.53, 0.49 and 0.69%/DM) contents with a high total carbohydrate (74.18, 76.24, 68.86 and 76.09%/DM) and protein (20.75, 18.02, 25.30 and 17.66%/DM) contents. The most appreciated *zoom-koom* by the tasters was the controlled fermented *zoom-koom* from mixed culture (LAB 1 and 5).**

Key words: *Zoom-koom*, starter cultures, fermentation, exopolysaccharides (EPS), hygienic quality.

INTRODUCTION

For Africans, the importance of traditional food fermentation lies in providing improved flavors to existing staples (for example cereals and root crops), and as a cheap way for food preservation and enhancement of the

nutritional quality and digestibility of the raw products (Olasupo et al., 2010). Frequently, fermented foods are considered to have health benefits, and in many regions, they are believed to aid in the control of some diseases, in particular intestinal disorders (Mathara et al., 2004). Traditional fermented foods still play a major role in the diet of numerous societies worldwide. The African dietary ethos includes both fermented and unfermented cereals and cassava products, wild legume seeds, but also meat, milk products and alcoholic beverages (Tamang and Samuel, 2010). *Zoom-koom* is one of common street-vended beverage and it is produced by crafts women. It is sold in all parts of Burkina Faso, mainly in cities such as Ouagadougou, Bobo-Dioulasso and Koudougou (Icard-Vernière et al., 2010). The grains of millet or sorghum are soaked overnight and then washed and mixed with spice (ginger and mint). The blend is ground into a dough, diluted with water, and then filtered using a clean muslin cloth to obtain *zoom-koom*, in which sugar and tamarind juice are added to give a sweet and sour taste. The production of *zoom-koom* is usually done in unhygienic environmental conditions (Besadjo-Tchamba et al., 2014; Soma, 2014; Tapsoba et al., 2017a).

Recently, study on the traditional process of *zoom-koom,* showed the positive impact of the fermentation on the hygienic quality of this drink (Tapsoba et al., 2017a). Some isolates of lactic acid bacteria (LAB) involved in the *zoom-koom* production process identified as *Weissella cibaria*/*confusa* had shown their ability to produce exopolysaccharides and antimicrobial compounds (Tapsoba et al., 2017b). These technological properties are very important for the improvement of the safety and the texture of the *zoom-koom* in controlled fermentation. For example, the use of exopolysaccharides (EPS)-producing LAB strains as ferment during the production of fermented milks improved the texture and decreased the syneresis (Zannini et al., 2016). The success of EPS application in the food industry is generally dictated by its ability to bind water, interact with proteins, and increase the viscosity of the milk serum phase. EPS may act as texturisers and stabilisers, and consequently, avoid the use of food additives (Duboc and Mollet, 2001; Zannini et al., 2016). The availability of LAB starter cultures to produce exopolysaccharides *in situ* during fermentation could be a suitable alternative for products whose polysaccharides addition requires the specification as food additives, which is a condition not much appreciated by consumer. *Zoom-koom* is a suspension of millet fermented dough in water, which settles quickly. The use of EPS-producing LAB isolates, for controlled fermentation could improve the physical stability of this beverage. Moreover, LAB are generally recognized as safe (GRAS) due to their long history of safe use in food production, and many of them have the qualified presumption of safety (QPS) status (Lahtinen et al., 2011; Caggianiello et al., 2016). Controlled fermentation using starter cultures allowed improvement of the hygienic and nutritional quality of traditional fermented products (Egounlety et al., 2007; Sawadogo-Lingani et al., 2008; Yao et al., 2009; Soma, 2014).

This study aimed to use two isolates of LAB producing EPSs and antimicrobial compounds (LAB 1 and 5) as starter cultures, to improve the rheological, nutritional, sensory and hygienic quality of *zoom-koom.*

MATERIALS AND METHODS

Origin of starters' cultures

The LAB isolates (LAB 1 and 5) used as starters cultures (pure cultures) were obtained from traditional fermentation process of *zoom-koom* (Tapsoba et al., 2017a). These isolates were previously characterized and identified as *W. confusa/cibaria* by using 16S rRNA gene sequencing and were able to produce EPSs and antimicrobial compounds against *Escherichia coli; Pseudomonas aeruginosa* and *Salmonella thyphimerium* (Tapsoba et al., 2017b).

Preparation of LAB inoculums

The two selected LAB isolates (previously stored in MRS-broth + glycerol at -20°C) were subcultured onto mMRS agar and incubated for 48 h at 37°C. The isolated colonies were then subcultured in 10 mL of MRS-broth and incubated for 24 h at 37°C. 0.1 mL of culture broth of each tube initially prepared was subcultured in MRS-broth (10 mL) and then incubated for 16 to 18 h at 37°C. For each isolate, the culture broth obtained after 16-18 h of incubation was distributed in sterile cryotubes (1 mL/tube) then centrifuged at 5000 *g* for 10 min. The supernatant of each tube was removed and the pellet (cells) of the tube was retained. To this pellet was added 1 mL of sterile diluent [0.1% (w/v) peptone (Difco), 0.85% (w/v) NaCl (Sigma), pH 7.2 ± 0.2] after vortexing, a further centrifugation was carried out at 5000 *g* for 10 min. The supernatant was again removed and the pellet was kept. One millimeter (1 mL) of sterile diluent was added to the pellet and, after stirring, the suspension of cells which constitutes the inoculum was stored in the refrigerator at 4°C. The concentration of viable cells of the inoculum was determined by enumeration on mMRS agar. The inoculum was used at a rate of 1% (v/v) (Sawadogo-Lingani et al., 2008; Soma, 2014) in the millet dough for controlled fermentation.

Controlled fermentation using the isolates

Controlled fermentation in monoculture was carried out at 30°C in an incubator (Binder 78532 Tuttlingen, GERMANY) using separately LAB 1 and 5. For each isolate, 20 mL of inoculums were prepared to inoculate 2 L of millet dough made with millet. For controlled fermentation in mixed culture (with both LAB 1 and 5), 20 mL of mixed inoculum (10 mL of LAB 1 inoculum + 10 mL of LAB 5 inoculum) was used to inoculate 2 L of millet dough. The controlled fermentation of the millet dough with the isolates were followed by

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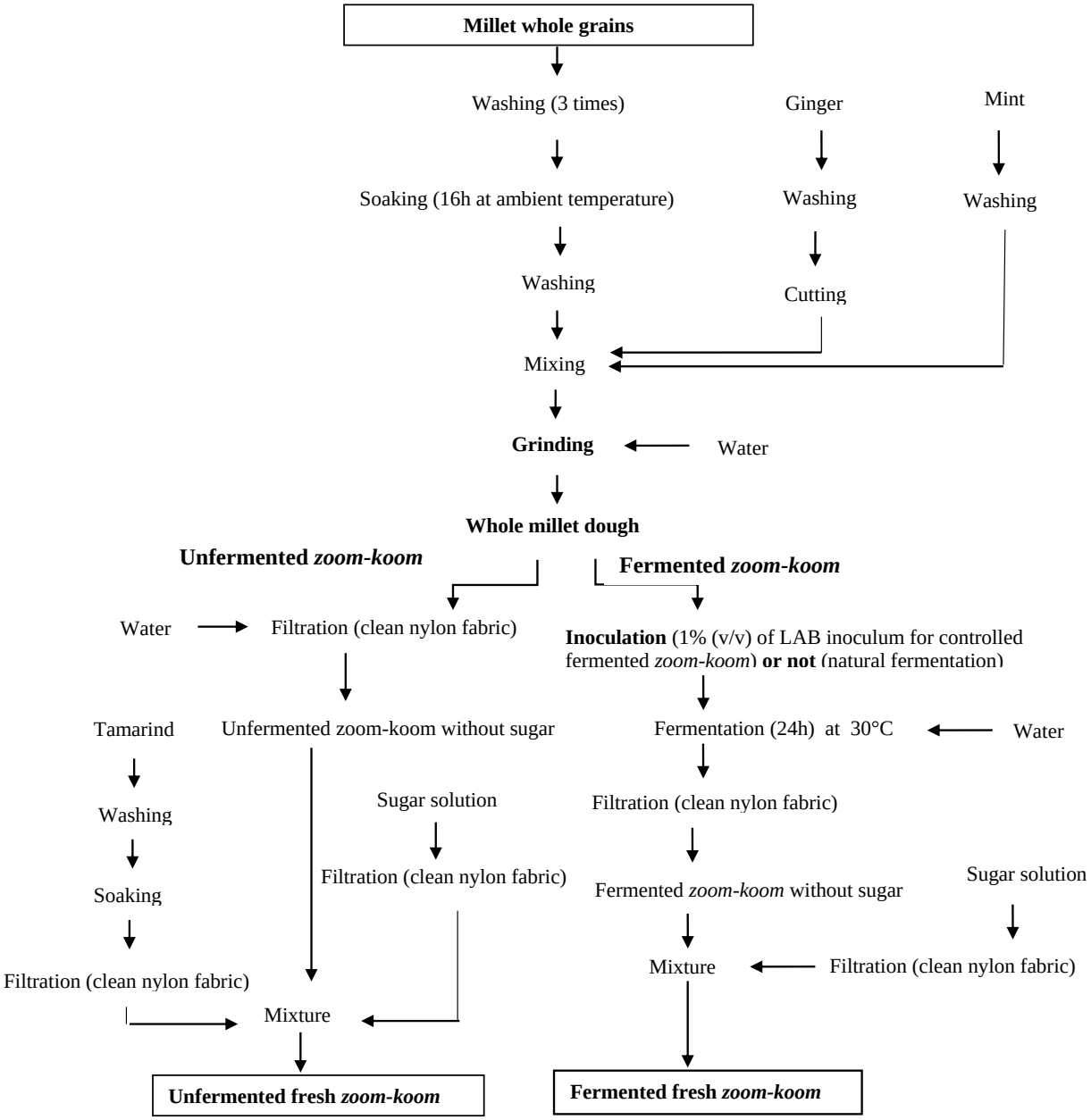

Figure 1. Fermented and unfermented millet *zoom-koom* production process diagram (Tapsoba et al., 2017a).

sampling at intervals of: 0, 4, 6, 8, 10 and 24 h for laboratory analyses. For each sample, pH, titratable acidity, mesophillic microorganisms, lactic acid bacteria, enterobacteria, yeasts and molds were measured or counted. A natural fermentation of the millet dough without inoculum was carried out simultaneously to serve as a control at each fermentation trials. For each isolate, the trial fermentation was done in duplicate. The flow diagram (Figure 1) of *zoom-koom* previously described (Tapsoba et al., 2017a) was adapted for the production with controlled fermentation.

Enumeration of microorganisms

For all the samples, 10 g of the product were soaked in 90 mL of sterile diluent (0.1% peptone, 0.8% NaCl, pH 7.0 ± 0.2) in a stomacher bag and homogenized in a stomacher (stomacher 400 lab blender, England) for 2 min at normal speed. From appropriate ten-fold dilutions, total mesophilic microorganisms were enumerated by pour plate on plate count agar (Liofilchem, Spain) incubated at 30°C for 72 h (ISO 4833, 2003). Yeasts were enumerated by pour plate on Dextrose Chloramphenicol Agar (Liofilchem, Spain), pH 6.6 ± 0.2, and incubated at 30°C for 3-5 days according to ISO 7954 (1988). Lactic acid bacteria (LAB) were enumerated on modified Man, Rogosa and Sharpe (mMRS: MRS-IM agar + maltose) agar (Liofilchem, Spain), incubated anaerobically in an anaerobic jar with anaerocult A at 37°C, for 72 to 96 h according to ISO 15214 (1998). Enterobacteria were enumerated on Violet Red Bile Glucose (VRBG) agar (Liofilchem,

Spain), and incubated at 37°C for 24 h according to ISO 7402 (1993). The results were given as CFU/g or mL of sample. The trial were done in duplicate.

Physico-chemical and nutritional analyses

The pH of the samples was measured with an electronic pH meter (Model HI 8520; Hanna Instrument, Singapore). For solid samples, 10 g of product were mixed with 20 mL of distilled water prior to pH measurement. For liquid samples, the pH was measured directly (Sawadogo-Lingani et al., 2007). For titratable acidity determination, 5 g or 5 mL of sample suspended in 30 mL of ethanol (90°) was mixed 1 h, using an automatic agitator, and centrifuged for 5 min at 3500 *g*. From the supernatant, 20 ml was transferred to a 50 ml measuring flask and was titrated with NaOH 0.1 N using 1% phenolphthalein as indicator (Soma, 2014). The titratable acidity (as g lactic acid per 100 ml or g of sample) was calculated according to Amoa-Awua et al. (1996). Water content was determined by oven drying the sample at 105 ± 2°C for 12 h (NF V03-707, July 2000); ash content was determined by incineration at 650°C overnight according to the French standard V03-760 (1981); crude protein content (N×6.25) was determined by the Kjeldahl method after acid digestion (NF V03 50, 1970); crude fat content was determined by soxhlet extraction using n-hexane (ISO 659, 1998). Total carbohydrates content were determined by spectrophotometric method at 510 nm using orcinol as reagent (Montreuil and Spik, 1963). The values were expressed in g/100 g of dry matter. The trial were done in duplicate

Determination of viscosity

The viscosity measurement of the *zoom-koom* samples resulting from the controlled fermentations by the LAB 1 and LAB 5 isolates, was carried out by using a viscometer (CSC scientific 1-800-458-2558). This measure consisted sinking 10 mL of the *zoom-koom* samples on a viscometer and measuring the flow rate. The result was expressed in cm/s. The types of zoom-koom were left for settling to observe their homogeneity at different times (25 min and 24 h).

Sensory analysis of *zoom-koom* samples

The sensory analysis consisted of evaluating the sensory profile of *zoom-koom* samples : A test of differentiation of the controlled fermented *zoom-koom* samples compared to the unfermented *zoom-koom;* used as control sample; a test of the classification of the *zoom-koom* samples according to the tasters were also performed. Thirty (30) members tasting panel were composed of men and women aged between 15 and more, who had already consumed the *zoom-koom*. The sensory profile was related to the color (nice, acceptable and mediocre), mouthfeel (very pleasant, pleasant and unpleasant), sweetened taste (very sweet, sweet and little sweet), aroma (very good, good and fair) and acidity (very acidic, acidic and fair acidic).

Statistical analysis

All the data (except sensorial analyses data) were subjected to Analysis of Variance (ANOVA) with the statistical software XLSTAT-Pro 7.5.2 and the means were compared using the test of Student Newman-keuls to the probability level $p<0.05$. The curves were obtained using Microsoft Excel 2013. The data of sensorial analyses were performed using the Chi^2 test with the statistical software SPSS.

RESULTS

Microbial growth during fermentation

The inoculum counts were 10^6 CFU/mL. All the control samples showed the same trend with their corresponding controlled fermentation trials. In this study, one control was presented to illustrate the other controls.

From the results, it is shown that during all the fermentation trials, the enterobacteria counts decreased significantly ($P<0.05$) after 24 h of incubation (Figures 2, 3, 4 and 5). Thus, from the fermentation using LAB 1 and 5 isolates (singly) as starters cultures in monoculture, the enterobacteria counts decreased from 6.4 (0 h) to < 1 log CFU/g (24 h) (LAB 1 in monoculture) and from 5.5 (0 h) to < 1 log CFU/g (24 h) (LAB 5 in monoculture) as shown in Figures 2 and 3. From the fermentation using both isolates LAB 1 and 5 in mixed culture, the enterobacteria counts decreased from 3.8 (0 h) to < 1 log CFU/g (24 h) as shown in Figure 4. The natural fermentation of millet dough (control) also showed a significant decrease ($P<0.05$) in enterobacteria counts (Figure 4). However, the enterobacteria counts at 24 h of fermentation were not < 1 log CFU/g. These counts were 1.3 log CFU/g for the natural fermented millet dough samples at 24 h of fermentation (Figure 4). All the final products (*zoom-koom*) did not contain enterobacteria except the natural fermented *zoom-koom* samples (Table 1). The yeasts, LAB and mesophillic microorganisms counts increased significantly ($P<0.05$) after 24 h of fermentation (Figures 2, 3, 4 and 5). Thus, the yeasts counts increased from 4.2 (0 h) to 7.1 log CFU/g (LAB 1 in monoculture) as shown in Figure 2, from 5.3 (0h) to 7.2 log CFU/g (LAB 5 in monoculture) as shown in Figure 3 and from 5.0 (0 h) to 6.8 log CFU/g (both LAB 1 and 5 in mixed culture) as shown in Figure 4. The natural fermentation showed the same trend. The LAB counts increased from 8.3 (0 h) to 8.7 log CFU/g (LAB 1 in monoculture), from 7.9 (0 h) to 8.6 log CFU/g (LAB 5 in monocuture) and from 6.7 to 8.7 log CFU/g (both in mixed culture). The natural fermentation showed the same trend. No moulds were observed after 24 h of fermentation for all the fermentation trials. For all the fermentation trials (natural and controlled fermentation) the LAB, mesophillic microorganisms and yeasts counts decreased a little in the final product (*zoom-koom*) after diluting and filtering of the dough (at 24 h of fermentation) as shown in Table 1.

From the means comparison of all the controlled fermentation trials, the LAB, mesophillic microorganisms and yeasts counts at 24 h of fermentation were significantly different ($P<0.05$) from those of 0 h (Figures 2, 3 and 4). The enterobacteria, mesophillic microorganisms, LAB and yeasts counts of natural fermentation at 24 h of fermentation of the dough, were significantly different ($P<0.05$) from those of 0 h as shown in Figure 5.

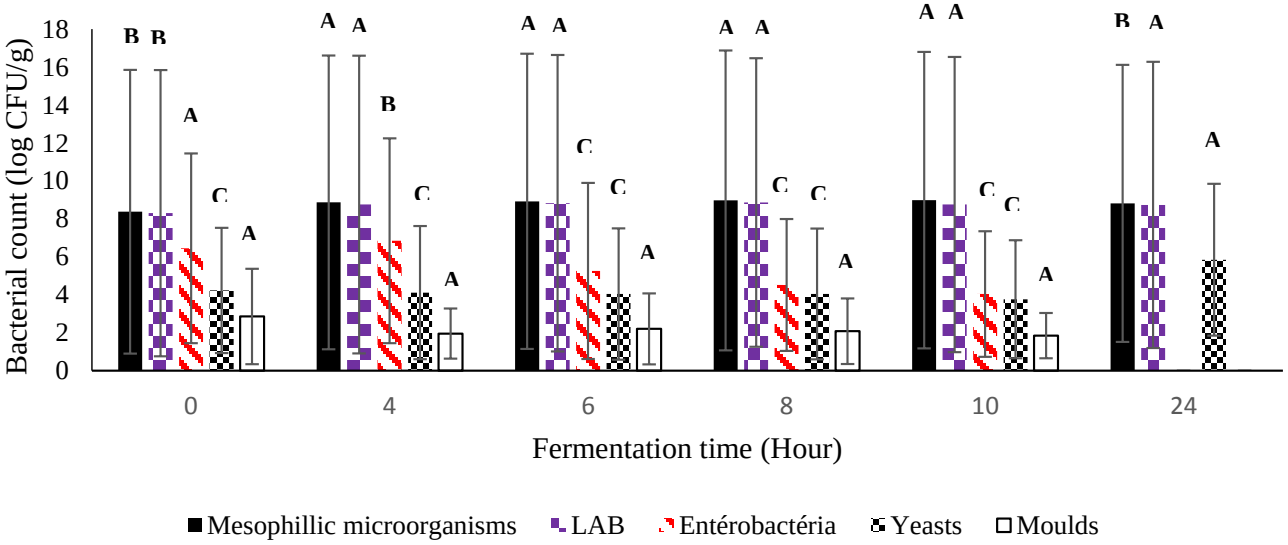

Figure 2. Evolution of the microbial population during the controlled fermentation of the millet dough using the isolate LAB1 as inoculum (each parameter having a common letter during the fermentation time, are not significantly different according to the Student Newman Keuls test threshold of 5%).

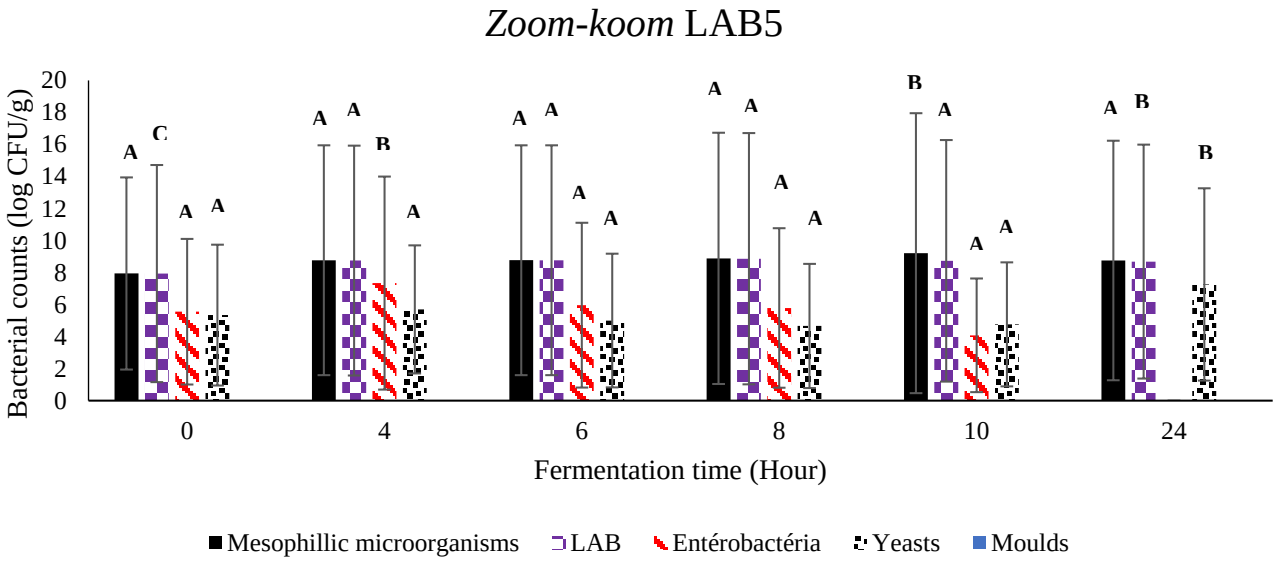

Figure 3. Evolution of the microbial population during the controlled fermentation of millet dough using the isolate LAB 5 as inoculum (each parameter having a common letter during the fermentation time, are not significantly different according to the Student Newman Keuls test threshold of 5%).

Physicochemical parameters during fermentation

pH

The pH of controlled fermented samples evolved similarly during all the trials fermentation processes. The pH values decreased significantly after 4 h of fermentation ($p<0.001$). The pH obtained with the fermentation in mixed culture (LAB 1 and LAB 5) showed the lowest decrease after 4 h of fermentation (from 6.2 to 5.4). The pH decreased slowly (from 6 to 10 h) before stabilizing at pH 4.0 (10 to 24 h) for monoculture fermentation (Figure 6). It should also be noted that the pH measured during the natural fermentation of the *millet* dough without inoculum (control) showed a similar evolutionary trend as that performed with the LAB 1 and LAB 5 isolates (Figure 6).

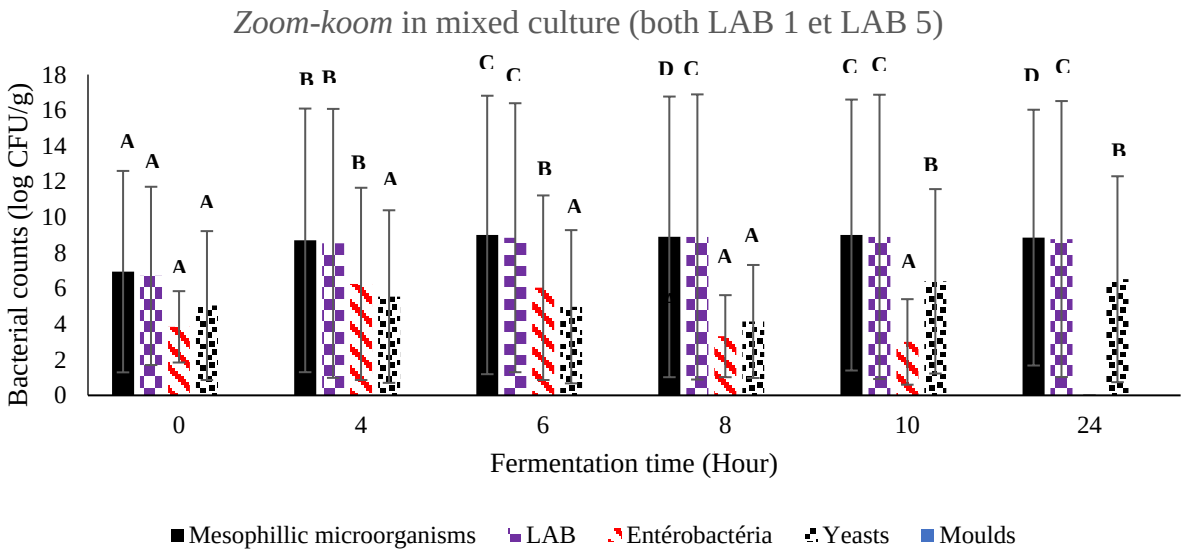

Figure 4. Evolution of the microbial population during the controlled fermentation of millet dough using both isolate LAB 1 and LAB 5 as inoculum (mixed culture): each parameter having a common letter during the fermentation time, are not significantly different according to the Student Newman Keuls test threshold of 5%).

Table 1. Microbiological and physicochemical analyses of *zoom-koom* samples after diluting and filtration of the millet dough from 24 H of fermentation.

	Microorganisms counts (log CFU/ml) ; Titratable acidity (g/100g of lactic acid)			
Samples / Parameters	***Zoom-koom*** **LAB 1**	***Zoom-koom*** **LAB 5**	***Zoom-koom*** **LAB 1 + LAB 5**	***Zoom-koom*** **control**
Enterobacteria	< 1	< 1	< 1	1.1 ± 0.6
Yeasts	5.1 ± 4.4	6.9 ± 5.7	5.2 ± 4.3	6.3 ± 5.8
Moulds	< 1	< 1	< 1	< 1
LAB	8.1 ± 7.4	8.2 ± 7.3	8.1 ± 7.2	8.0 ± 7.3
Mesophillic microorganisms	8.3 ± 7.8	8.6 ± 7.2	8.2 ± 7.2	8.1 ± 7.5
pH	4.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1	4.0 ± 0.1
Titratable acidity	0.29 ± 0.03	0.33 ± 0.02	0.33 ± 0.03	0.3 ± 0.01

Titratable acidity

The titratable acidity of all the samples showed the same evolutionary trend during the trials fermentations. The results show that titratable acidity evolved significantly from 0 to 24 h for all trials fermentations. The highest acidity value was recorded with the monoculture fermentation using the LAB 1 isolate at 24 h (1.24 g of lactic acid/100 g). After dilution and filtration of the 24 h fermented dough, the titratable acidity values of all the fermentations decreased significantly ($p<0.001$) as shown in Figure 10. It should also be noted that the titratable acidity measured during natural fermentation of the millet dough without inoculum (control) showed a similar evolutionary trend as that performed with the LAB 1 and LAB 5 isolates (Figure 7).

Viscosity

From the results of viscosity, the flow tests showed that the *zoom-koom* fermented by the isolate LAB 5 was the most viscous and homogeneous with a flow of 0.22 cm/s as compared to the natural fermented *zoom-koom* (control for LAB 5) without inoculum (0.14 cm/s). The flow of the other types of *zoom-koom* fermented in monoculture with the isolate LAB 1 and in mixed culture with the isolates both LAB 1 and 5 were different from that of the control and less homogeneous than the *zoom-koom* with the isolate LAB 5. The unfermented *zoom-koom* was the least viscous and decanted faster than other fermented types. After 25 min and 24 h of settling, the control (natural fermented) *zoom-koom* settled faster than the controlled fermented *zoom-koom* using LAB 5.

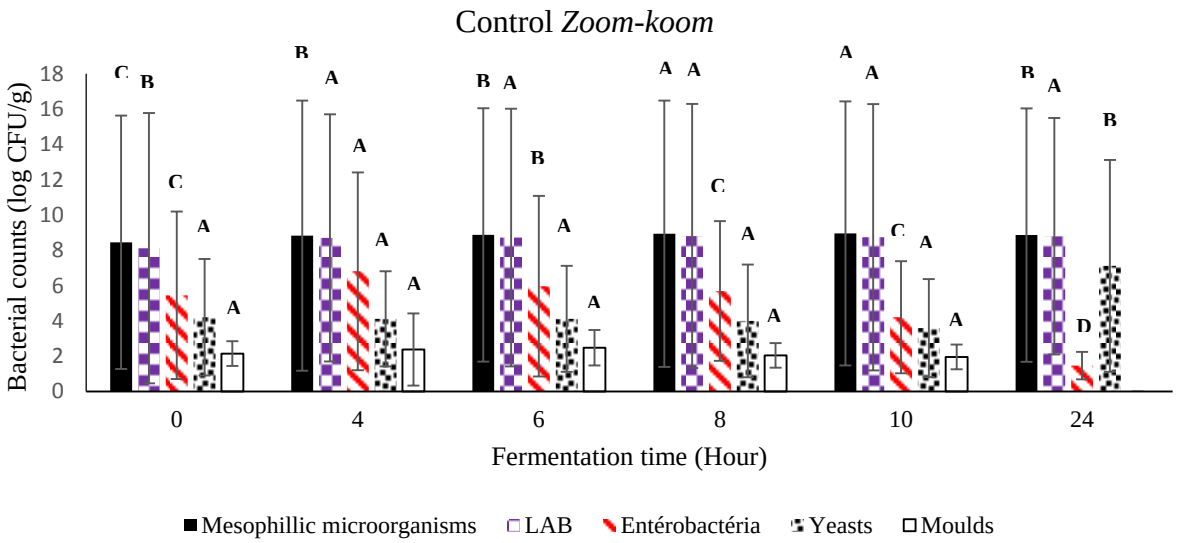

Figure 5. Evolution of the microbial population during the natural fermentation process of the millet dough without inoculum (control): each parameter having a common letter during the fermentation time are not significantly different according to the Student Newman Keuls test threshold of 5%.

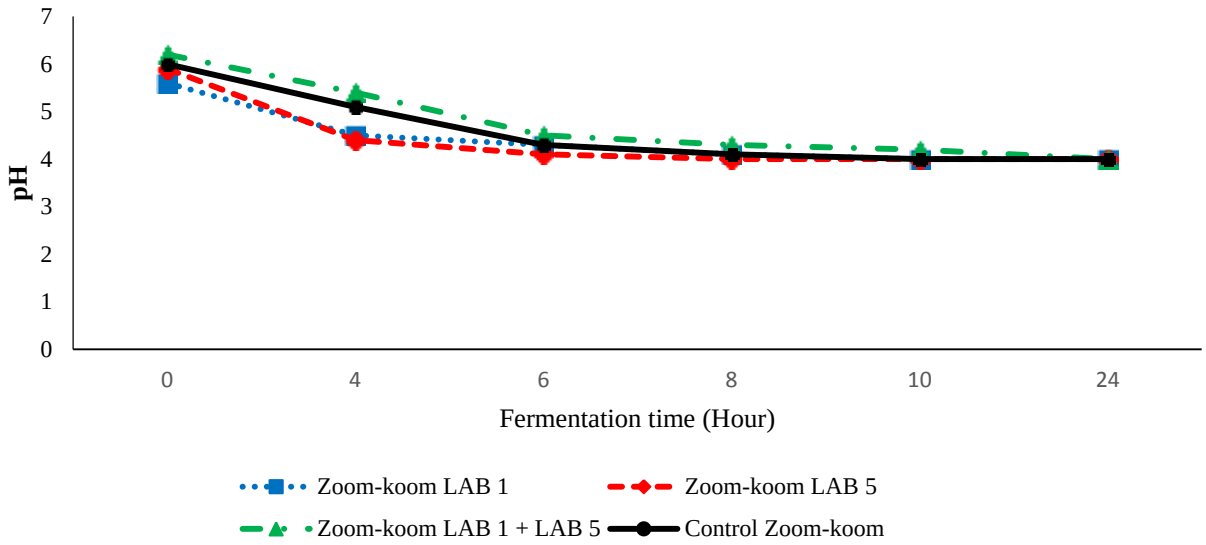

Figure 6. Evolution of the pH during the natural fermentation of the millet dough without inoculum (control) and the controlled fermentation of millet dough using LAB 1 and LAB 5 isolates in monoculture and mixed culture.

The last one was more viscous and cloudy.

Nutritional characteristics of fermented *zoom-koom* samples

The fermented *zoom-koom* sample from monoculture with the isolate LAB 5 contained less water and more dry matter content than the others, but not significantly different on statistical plan ($p<0.05$) (Table 2). This sample contained more fat, total carbohydrates and ash than the *zoom-koom* sample with isolate LAB 1. However, the *zoom-koom* sample with isolate LAB 1 contained more proteins as compared to the *zoom-koom* with isolate LAB 5. Both samples contained more sugars than the mixed culture fermentation sample. The control sample (natural fermentation without inoculum) contained more fat and ash than the others. The highest ash contents were obtained with the *zoom-koom* LAB 5 samples and the natural fermented *zoom-koom* sample

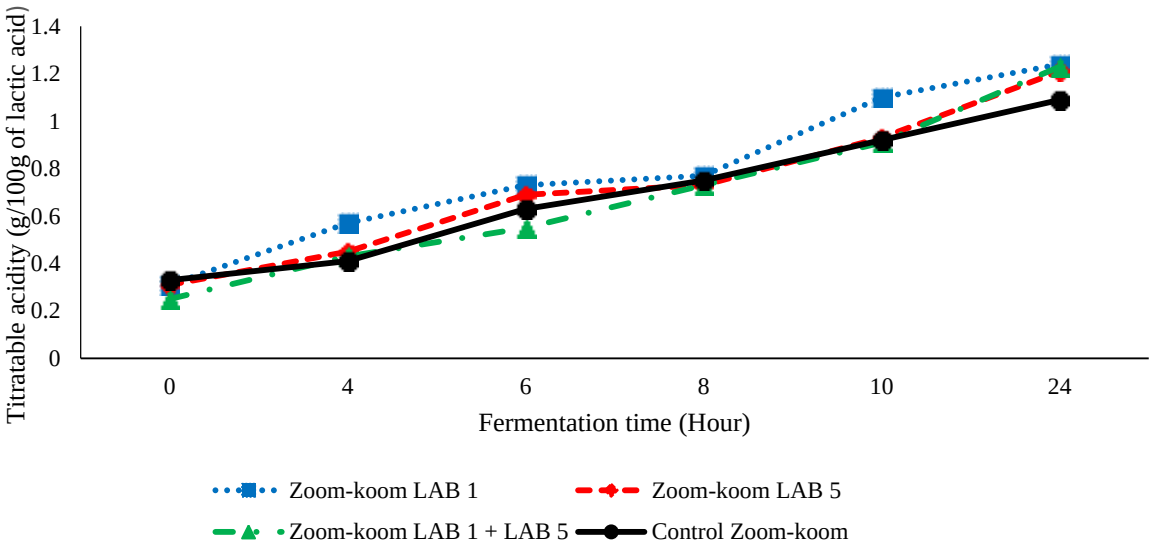

Figure 7. Evolution of titratable acidity during the natural fermentation of the millet dough without inoculum (control) and the controlled fermentation of millet dough using LAB 1 and LAB 5 isolates in monoculture and mixed culture.

Table 2. Nutritional characteristics of fermented *zoom-koom* samples.

Samples	Water content (%)	Dry matter (%)	Crude fat (%/DM)	Total carbohydrates (%/DM)	Crude proteins (%/DM)	Ash (%/DM)
Zoom-koom LAB 1	81.51 ± 0.00^{a}	18.49 ± 0.00^{a}	4.74 ± 0.31^{a}	74.18 ± 0.02^{a}	20.75 ± 0.01^{a}	0.32 ± 0.00^{a}
Zoom-koom LAB 5	81.04 ± 1.10^{a}	18.96 ± 1.10^{a}	5.21 ± 0.05^{b}	76.24 ± 0.12^{a}	18.02 ± 0.02^{b}	0.53 ± 0.00^{b}
Zoom-koom LAB 1 and LAB 5	81.49 ± 0.01^{a}	18.51 ± 0.01^{a}	5.36 ± 0.28^{b}	68.86 ± 0.01^{b}	25.30 ± 0.05^{c}	0.49 ± 0.01^{c}
Zoom-koom control (without inoculum)	81.25 ± 0.02^{a}	18.75 ± 0.02^{a}	5.55 ± 0.34^{b}	76.09 ± 0.04^{a}	17.66 ± 0.05^{b}	0.69 ± 0.01^{d}

For each column, the values with a common letter are not significantly different according to the Student Newman Keuls test at the 5% threshold. DM, Dry matter.

without inoculum (control). Natural fermented *zoom-koom* (without inoculum) showed the best fat levels and the lowest value of proteins. All the samples showed a low level of fat. The highest protein content was obtained with the *zoom-koom* sample from fermentation in mixed-culture (Table 2), probably due to the high contribution of isolate LAB 1. No significant difference ($p<0.05$) was observed for water content. The protein content of the *zoom-koom* sample from the fermentation in mixed-culture was significantly different from that of the other samples ($p<0.05$). The ash contents of the different samples were significantly different from each other ($p<0.05$). The total carbohydrates in the *zoom-koom* from mixed culture fermentation were significantly different from the others ($p<0.05$).

Sensorial characteristics of fermented *zoom-koom* samples

From the sensory analysis results, it appeared that 70% of the tasters found that the *zoom-koom* resulting from the monoculture fermentation with the isolate LAB 1 and the *zoom-koom* resulting from the fermentation in mixed culture with both isolates LAB 1 and LAB 5 had a nice color. However, 50 and 13.3% of the tasters found that the *zoom-koom* from the monoculture fermentation with the isolate LAB 5 and the control *zoom-koom* showed a nice color (Figure 8). The *zoom-koom* with the isolate LAB 1 and the *zoom-koom* from the fermentation in mixed-culture showed a better aroma (46.7 and 46.7% of the tasters, respectively) than the *zoom-koom* with the isolate LAB 5 and the control *zoom-koom* (40 and 43.3%, respectively) according to the tasters (Figure 8). The mouth feel after tasting the *zoom-koom* in mixed culture (both LAB 1 and Lab 5) and the control *zoom-koom* appeared pleasant (63.3 and 63.3%, respectively). Approximately 60% of the tasters appreciated pleasant mouth feel after tasting the *zoom-koom* resulting from monoculture fermentation with isolate LAB 1 on one hand and isolate LAB 5 on the other hand. The tasters (73.3%) also found that the *zoom-koom* with isolate LAB 1 and

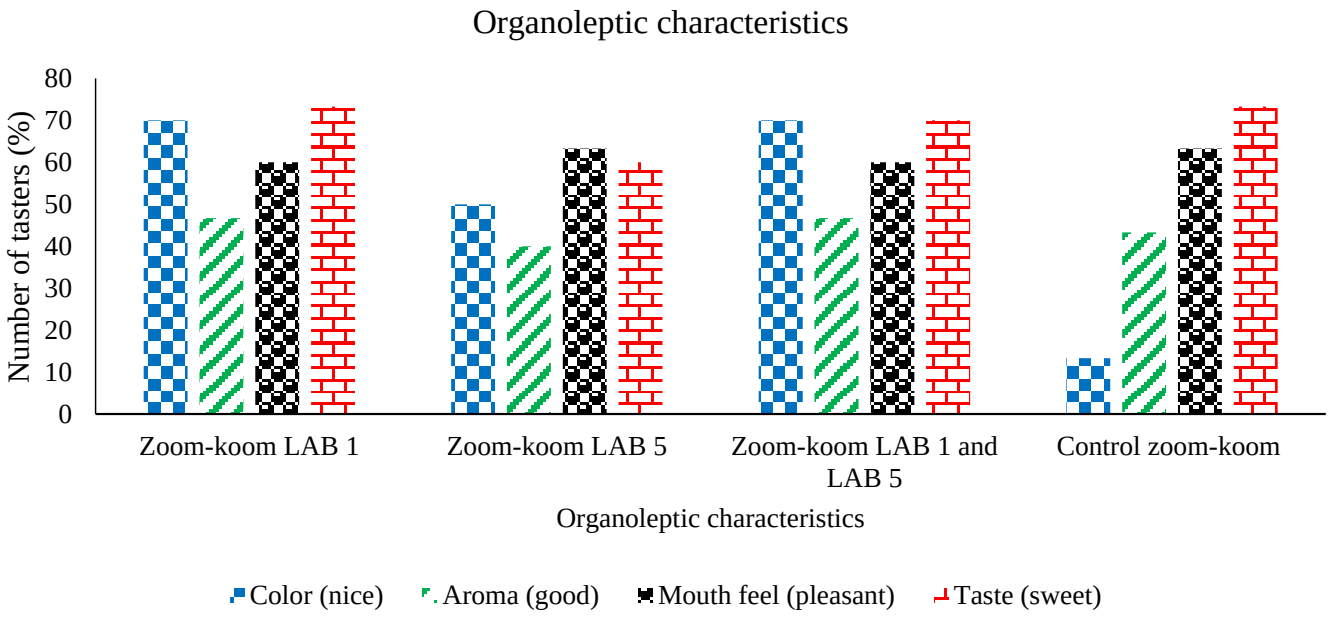

Figure 8. Organoleptic characteristics of *zoom-koom* samples according to the tasters.

the control *zoom-koom* were sweet. However, 60% of the tasters found that the *zoom-koom* resulting from the monoculture fermentation with the isolate LAB 1 was sweet, while 70% of the tasters found that the *zoom-koom* in mixed culture (both LAB 1 and LAB 5) was sweet (Figure 8). The control *zoom-koom* for sensory analysis is an unfermented *zoom-koom*.

As for the acidity, 70, 66.7 and 60% of the tasters found that *zoom-koom* with LAB 1, LAB 5 isolate in monoculture and mixed culture, respectively had normal acidity against 13,3% of tasters who thought that the control *zoom-koom* was fairly acid (Figure 9). A differentiation test based on the homogeneity and texture of the different types of *zoom-koom* was performed as compared to the control *zoom-koom*. From this, it appeared that all the fermented *zoom-koom* were different from the control *zoom-koom* according to the tasters.

Also, 83.34% of the tasters found that the control *zoom-koom* sample had more liquid (less viscous) than the controlled fermented *zoom-koom* samples. Among the three controlled fermented *zoom-koom* samples, the *zoom-koom* obtained with isolate LAB 5 was the most viscous and cloudy according to the tasters. All tasters did not notice any pungent taste of ginger in all *zoom-koom* samples after tasting. The overall ranking of tasters were the *zoom-koom* resulting from mixed-culture fermentation at the first place (33.3%), followed by *zoom-koom* with isolate LAB 1 in second place (36.7%), *zoom-koom* with isolate LAB 5 was in third place (36.7%) and the *zoom-koom* control was fourth (63.3%) according to the proportion of tasters for each rank (Figure 10).

DISCUSSION

The enterobacteria, mesophillic microorganisms and LAB counts increased after 4 h of fermentation, while the yeast counts remained almost unchanged. This increase could be due to the fact that at the beginning of the fermentation, the medium was rich in nutrients with a favorable temperature which allowed the growth of microorganisms. Indeed, water activity and the presence of nutrients could promote the activation of spores, the growth of bacteria, yeasts and molds (Tawaba et al., 2013). LAB are generally described as mesophilic microorganisms with an optimal growth temperature of 30°C (van de Guchte et al., 2002). Gymnase (2011) also indicated that cereals contain prebiotics which stimulate the growth of bacteria like enterobacteria and LAB for the present study and *zoom-koom* is a cereal based beverage. The decrease in enterobacteria counts during the fermentation (8, 10 and 24 h) is probably due to the growth of LAB which are well known to produce antimicrobial substances such as organic acids (lactic, acetic, formic and caproic phenolic), carbon dioxide, hydrogen peroxide, ethanol and bacteriocins during fermentation (Messens and De Vuyst, 2002).

The pH of the fermented dough remained stable at pH 4.0 from 10 to 24 h and this induced an effective action of the acidity on enterobacteria. The results corroborate those of Soma (2014) who observed a decrease in enterobacteria counts in the fresh *zoom-koom* after 24 h of fermentation, using a strain of *Lactobacillus fermentum* as starter. This result also confirmed previous study of Tapsoba et al. (2017a) where a decrease in

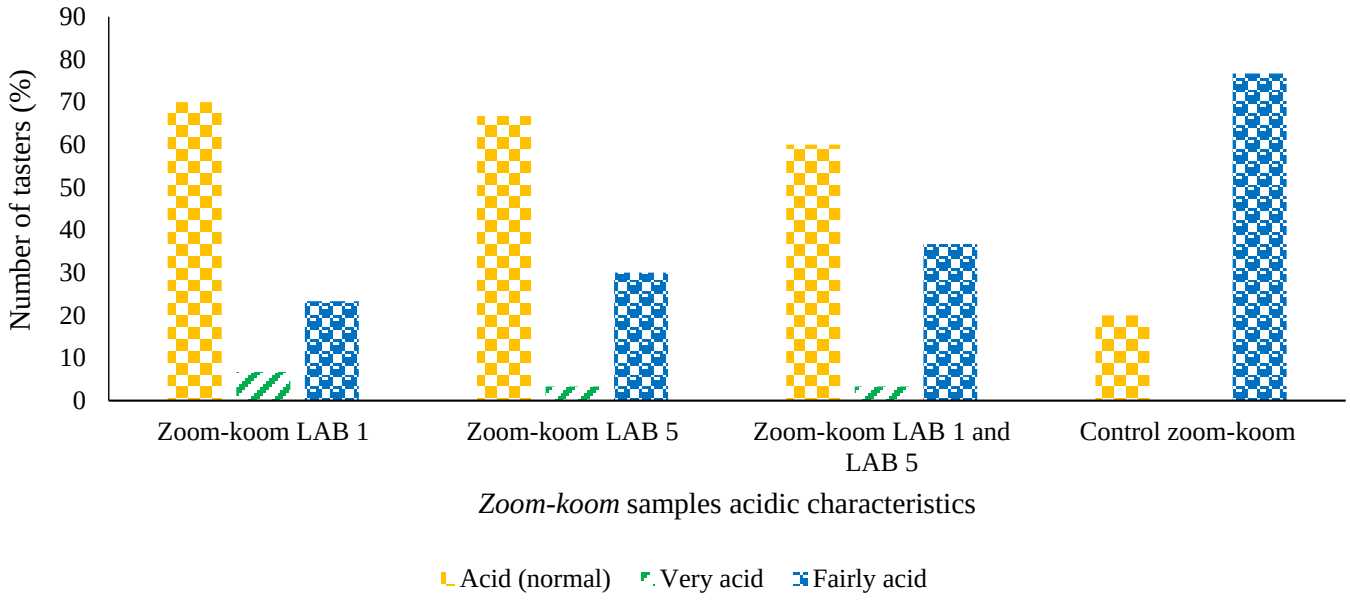

Figure 9. Acidic characteristics of the *zoom-koom* samples according to the tasters.

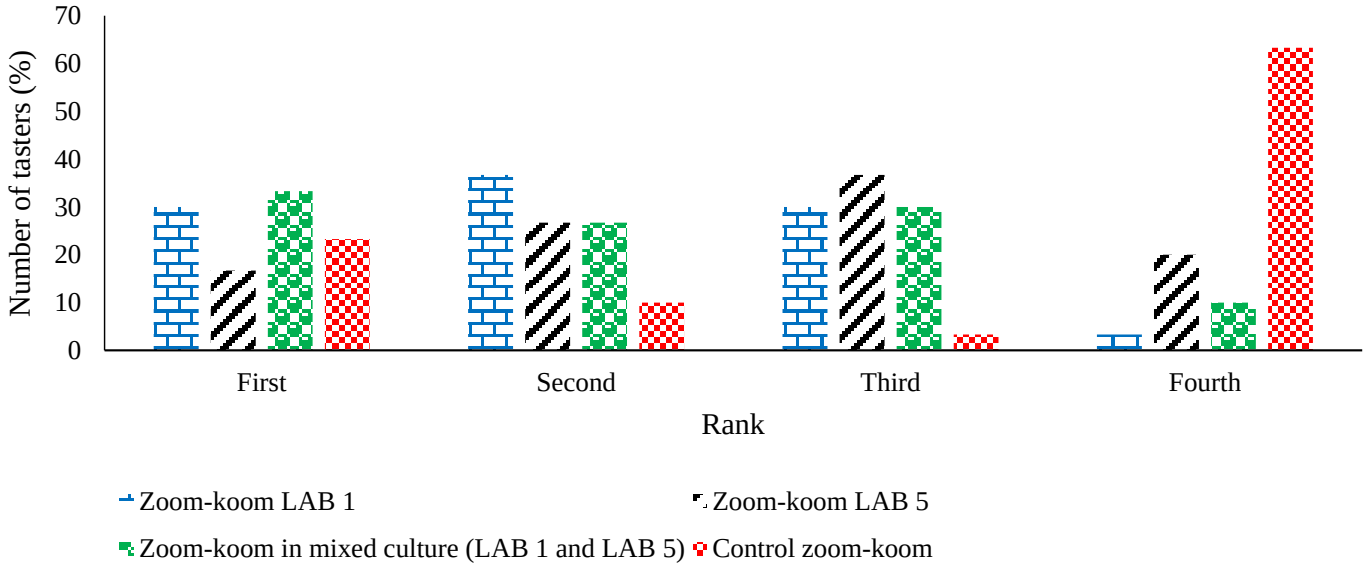

Figure 10. Ranking of *zoom-koom* after tasting according to the tasters.

enterobacteria and yeasts counts was found after 10 h of natural fermentation of the *zoom-koom* based on millet and red sorghum dough. In addition, the LAB isolates used as starter in this study have been selected on the bases of their antibacterial and antifungal activities (Tapsoba et al., 2017b).

The presence of enterobacteria in the control dough at 24 h and their absence in the dough with the inocula, means that the selected isolates have maintained and expressed their antibacterial properties. In fact, these selected isolates were able to produce bacteriocins like compounds or similar metabolites according to the antimicrobial activities performed (Tapsoba et al., 2017b). It was also observed in previous study that the bacteriocin produced by *W. confusa* had a broad spectrum of antimicrobial activity inhibiting both Gram-positive and negative bacteria (Hweh and Koshy, 2015). The results also highlighted an increase of yeasts population after 24 h of fermentation, while those of LAB decreased; this could be due to the fact that LAB by their

carbohydrates metabolism acidify the medium which becomes favorable to the growth of yeasts and molds (Tchekessi et al., 2014). According to Yao et al. (2009), the acid environment created by LAB promoted yeasts growth. From a hygienic point of view, this acidification is a major asset because it prevents the growth of most pathogenic bacteria (Tchekessi et al., 2013). After 24 h of fermentation, the population of enterobacteria decreased totally during the controlled fermentation with the selected isolates. In general, the manufacturing of foods and beverages sold on the streets involves manual processes without any good hygiene practices and are subject to numerous contaminations (Sunday et al., 2011; Bsadjo-Tchamba et al., 2014). This contamination was not observed in our *zoom-koom* produced from controlled fermentation with starter cultures where the conditions were better controlled than previous productions.

The low water content of *zoom-koom* samples could be due to EPS produced during the dough fermentation with the isolates. Indeed, exopolysaccharides have been shown to increase the viscosity and softness of the milk product and have the ability to retain water molecules, thereby reducing the separation of whey and milk coagulated caseins (Zannini et al., 2016). The water contents of the samples were lower than those of Soma (2014) on unfermented *zoom-koom* and fermented *zoom-koom* using a strain of *Lactobacillus fermentum* as starter. The fermented *zoom-koom* samples also contained more total carbohydrates but less protein and ash than the *zoom-koom* samples produced by Soma (2014). The crude fat content of this fermented *zoom-koom* samples had approximately the same levels than the *zoom-koom* samples produced by Soma (2014). This difference could be explained in part by the dilution rate and the fact that the *zoom-koom* samples were produced from whole grains of millet, while those of Soma (2014) were produced from dehulled millet grains.

All the controlled fermented *zoom-koom* samples were a good source of energy and nutrients. Concerning the sensory characteristics, overall, the fermented *zoom-koom* samples were found to be more acidic than the unfermented *zoom-koom* which was slightly acidic, due to the effect of fermentation. All the *zoom-koom* samples showed a nice color, pleasant taste and good aroma. The best flavor was obtained with the unfermented *zoom-koom* sample, followed by the *zoom-koom* from the mixed-culture fermentation. This difference in flavor is due to the fact that with the addition of the tamarind, the *zoom-koom* presents a better aroma. Nevertheless, LAB allows the development of aroma in the fermented products. These bacteria in mixed cultured during fermentation could diversify aroma production better than when they are in monocultures. Indeed, LAB are well known to produce a variety of compounds that contribute to the taste, flavor, color, texture, consistency, nutritional quality and safety of fermented products (Davidson, 1997; Ayad et al., 2004; Sawadogo-Lingani et al., 2008). Because they are different from the unfermented *zoom-koom*, the *zoom-koom* from the mixed-culture fermentation was preferred by the tasters, followed by the *zoom-koom* in monoculture with the isolate LAB 1 and then the isolate LAB 5 and finally the unfermmented *zoom-koom*, on the basis of their organoleptic qualities. Since the strains of *Weissella* spp. occupy an important place in certain African fermented foods, or in European fermented dough, the use of these specific strains as starter cultures can be envisaged (Fusco et al., 2015).

Conclusion

This study showed the efficacy of selected LAB isolates (EPS producer and antimicrobial properties) for controlled fermentation of *zoom-koom* dough. During this fermentation, the enterobacteria counts reduced to maximum after 24 h of fermentation. Overall, the LAB counts in controlled fermented *zoom-koom* with inocula were higher than that of natural fermented *zoom-koom* without inoculum. The *zoom-koom* obtained with isolate LAB 5 was the most homogeneous and viscous as compared to the other types of *zoom-koom*. All the types of *zoom-koom* contained more carbohydrates and protein than fat and ash. The fermented *zoom-koom* in mixed culture and the *zoom-koom* control (unfermented) presented the best aromas. From the acid taste point of view, the unfermented *zoom-koom* was not very acidic as compared to fermented *zoom-koom*. All *zoom-koom* types have a good taste after tasting and a nice color. The preferred *zoom-koom* of the tasters was that resulting from the fermentation in mixed culture. The controlled fermentation using selected LAB isolates allows (i) scaling up of the production of *zoom-koom* by moving from household to semi-industrial level, (ii) standardizing of the flow diagram of *zoom-koom* and (iii) improvement of the hygienic, nutritional and organoleptics characteristics of *zoom-koom*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Identification and major technological characteristics of *Lactococcus* and *Lactobacillus* strains isolated from "hamoum", an Algerian fermented wheat

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Twenty-eight strains of lactic bacteria were isolated from fermented wheat "hamoum" and phenotypically attributed to the following species: seven strains of *Lactococcus lactis* subsp. *lactis*, six strains of *Lactobacillus brevis* and 15 strains of *Lactobacillus plantarum*. The acidifying behavior of the strains is considerably variably demanding on considered strain. The amounts of lactic acid produced reached 9.7 g for lactococci. Strains (27) showed proteolytic activity in the presence of 1% skimmed milk. The lipolysis activity of *L. lactis* strains was greater than that expressed by lactobacilli. The search for aromatic activity showed that four out of ten citratase producing strains can produce acetoin. The results indicate that *L. plantarum* is the most dominant strain in the "hamoum" with the most important technological characteristics.

Key words: "Hamoum", *Lactococcus lactis* subsp. *lactis*, *Lactobacillus brevis*, *Lactobacillus plantarum*, identification, proteolysis, lipolysis, exopolysaccharides (EPS), aromatic activity.

INTRODUCTION

Cereals are by far the most important food resource in the world for both human and animal. Wheat (*Triticum* species), by its important nutritional power, remains one of the main human food resource (Cassman, 1999).

In Algeria, wheat was historically conserved in underground silos called "Matmor" or "Matmora". Due to the accidental infiltration of precipitation water into the "matmor", the humidified or flooded wheat grains undergo a spontaneous fermentation at the periphery and depth of the silo, which depends also on the nature of the soil. Humidity, uncontrolled temperature and the absence of air in the matmor cause microbial fermentation phenomena that can last several years (≤ nine years). Fermented wheat taste is then discovered and entered into the eating habits for the manufacture of fermented wheat, bread or couscous, "lemzeiet", "elmechroub" or "hamoum". This fermented wheat has a variety of flavors, textures and aromas that are highly coveted by consumers in specific regions (Bekhouche et al., 2013).

Balance of total microbial population present in wheat grains can be affected by many factors (Wang et al., 2015). Elements of this imbalance include climatic conditions, mainly temperature and humidity, and biotic conditions associated with insect and mold attack and

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pesticide application. Among the microorganisms associated to wheat grains, lactic acid bacteria play a very important role in preserving the balance of the microbial flora and stabilizing the final fermentation products (Corsetti et al., 2017).

Lactic acid bacteria are a heterogeneous group of microorganisms producing lactic acid as the main product of metabolism. They colonize many food products such as dairy products, meat and vegetables. They are involved in a large number of spontaneous fermentations of food products and intervene in the dairy industry and fermentation of many other food products. They contribute to both texture and flavor of food and the production of aromatic compounds. They constitute a group of bacteria united by a multitude of morphological, metabolic and physiological characteristics. In general, they are described as Gram-positive bacteria, immobile, rods and cocci, non-sporulating, free of cytochromes and catalase, anaerobic micro-aerophiles, strictly fermentative, with complex nutritional requirements (amino acids, peptides, vitamins, salts, fatty acids, fermentable carbohydrates) and produce lactic acid as the main end product during carbohydrates fermentation (Axelsson, 2004).

Currently, lactic acid bacteria encompass 13 different bacterial genera: *Lactobacillus*, *Bifidobacterium*, *Leuconostoc*, *Lactococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Oenococcus*, *Weissella*, *Aerococcus*, *Tetragenococcus* and *Vagococcus*. Classification can be done according to phylogenetic criteria by the use of molecular methods. However, the classical phenotypic/biochemical characterization remains practical in the preliminary identification of micro-organisms. Some phenotypic characteristics are used to identify species within genus such as ability to ferment carbohydrates, different bile concentrations toleration, extracellular polysaccharides production, growth factors requirement, acetoin production, and some enzymes synthesis. The G+C composition of the DNA, the fatty acid composition, the electrophoretic mobility of lactate dehydrogenase are criteria that can also be studied for the identification of lactic species (Vandamme, 1996; Stiles and Holzapfel, 1997; Ho et al., 2007).

Lactobacillus is one of the most important genus involved in food microbiology, due to its role in food production and preservation. Lactobacilli contribute to the flavor of fermented foods by diacetyl production. The genus *Lactobacillus* was proposed by Beijerinck in 1901. They are long and fine (sometimes curved) rods often grouped in chains, immobile, non-sporulated, with negative catalase, and developed at 30 to 40°C. Lactobacilli have very complex nutritional requirements lactic acid bacteria. It is therefore quite heterogeneous, contains species with a wide phenotypic/biochemical variety, and physiological properties. Heterogeneity is reflected by the type of molecular percentage G+C of the DNA in the species of this genus (Schleifer and Ludwig, 1995; Axelsson, 2004; Hammes and Hertel, 2006).

The lactococci have been used primarily as starter cultures for various dairy products (yogurt, Cheddar, and hard cheeses). For most parts, they have been limited to N and D Streptococci and *Leuconostoc cremoris* and *Leuconostoc dextranicum*. The lactococci are Gram-positive cocci, nonmotile, grow at 10 and 40°C, but not 45°C, grow in 4% NaCl (except for *L. cremoris*); some species grow in 0.1% methylene blue milk medium. The lactococci ferment glucose by the hexose diphosphate pathway with the formation of L(+) lactic acid. In general, *Lactococcus* species produce smooth colonies with an entire edge on agar media (Carr et al., 2002).

Lactococcus lactis is predominantly found on plant material and in the dairy environment. It is extensively used in dairy fermentations, which is mainly due to its role in the development of texture and flavor through, for example proteolysis and the production of volatile flavor compounds. It also contributes to food preservation through the production of organic acids and bacteriocins such as nisin. Four *L. lactis* subspecies have been defined: subsp. *lactis*, subsp. *cremoris*, subsp. *hordniae*, and subsp. *tructae* (Backus et al., 2017).

To our knowledge, very few studies have been carried out on the fermented wheat “hamoum”, but without a special interest regarding the technological interest in lactobacilli and lactococci. The metabolic activities of bacterial species sought by food industry, like production of lactic acid, aroma or thickening saccharides. Then, the objectives of this study were to isolate these lactic acid bacteria from “hamoum” in order to identify and highlight their technological characteristics.

MATERIALS AND METHODS

Isolation and storage of lactic acid bacteria

To carry out this study, 28 strains of lactic acid bacteria were isolated from fermented wheat "hamoum": three samples of hammoum of three different matmors (underground silos) were taken in sterile bottles; an aliquot of 5 g was homogenized with Stomacher, then was added to 10 ml of sterile skimmed milk and placed to coagulate at 30°C for 24 h in order to promote the development of the endogenous lactic flora. After coagulation of milk, the first dilutions were prepared by mixing 1 ml of each milk sample with 9 ml of physiological water (0.90% w/v NaCl solution). Decimal dilutions were then made in the same solution. Purification of bacterial strains was performed by the method of the streaks on solid MRS medium (De Man et al., 1960). Incubation was carried out at 30°C for 48 h. Obtained colonies were examined macroscopically and bacteria were characterized microscopically after Gram staining. The search for catalase activity was performed for all strains. Gram positive and catalase negative bacteria were then stored at -20°C in MRS medium supplemented (v/v) with 40% glycerol.

Strains identification

Physiological and biochemical study of strains

Bacterial growth was followed by spectrophotometric measures

(A_{600nm}) at different temperatures: 10, 15, 37, and 45°C, different pH: 4; 4.5 and 8 and different concentrations of NaCl: 2, 4 and 6.5% in liquid MRS medium. Each strain was seeded into two tubes containing the Falkow medium (Falkow et al., 1958): a control tube (without arginine) and a test tube (with arginine). The presence of arginine dihydrolase results in turning the pH indicator towards violet, whereas glucose fermentation in the control tube leads to the turning of the colored indicator towards the yellow.

Fermentation type was searched out for all the strains. The strains were seeded in tubes containing 10 ml of MRS medium and a Durham tube, and then incubated at 30°C for 48 h. The accumulation of gas in the Durham tube shows that the path of degradation of the sugar is heterofermentary, otherwise it is homofermentary.

Identification with API50 CHL galleries

Fermentation profiles of the strains were established using the API50 CHL biochemical galleries according to manufacturer instructions. The identification of strains was performed using Apiweb™ software of Biomerieux.

Study of technological characteristics of strains

Measurement of acidity produced by bacteria: The acidity produced by the bacteria in MRS medium was estimated by pH-meter using Dornic soda (N/9) (Karam and Karam, 1994) after an incubation period of 24 h at 30°C. Results were expressed in Dornic degrees according to the formula: Acidity (°D) = n × 10 (n = average volume of soda to titrate 10 ml of milk; 1 °D = 0.1 g/L of produced lactic acid) (Accolas et al., 1971).

Bacterial proteolysis activity: Cells capacity for proteolysis was sought in MRS medium Na/Na_2-phosphate buffered to pH 7 supplemented with 2% of reconstituted sterile skimmed milk at 10%, according to the method described by Van Den Berg et al. (1993) and adapted by Roudj et al. (2009).

Lipolytic activity: Lipolytic activity was sought on solid MRS medium Na/Na_2-phosphate buffered to pH 7 and supplemented with 1% of milk fat as sole lipid source.

Aromatic activity: (1) Search for acetoin (Hydroxy-3-butanone-2 or acetylmethylcarbinol): Bacteria were inoculated into Clark and Lubs medium. After incubation at 30°C for 48 h, the production of acetoin was demonstrated by means of Voges-Proskauer colored reaction (Eddy, 1961). (2) Search for citratase: The production of citratase was demonstrated by bulk culture in semi-solid agar with citrated milk; prepared by adding 0.5 ml of 10% sodium citrate solution to 10 ml of milk with 1% (0.1 ml) of the preculture and then adding 4 ml of molten agar at 48°C (Harrigan, 1998).

Exopolysaccharides (EPS) production: The production of EPS was sought on Mayeux medium (*Leuconostoc* specific medium) and on hypersaccharosed solid MRS medium containing 50 g of sucrose per liter (Messens et al., 2002).

RESULTS AND DISCUSSION

Strains identification

After purification series on MRS medium (pH 5.4), bacterial colonies presented the following characteristics: small, whitish, smooth, curved and with regular outline. Results of morphologic tests have also shown that all strains were Gram positive and catalase negative. Microscopic observation has revealed two cell forms: 7 strains have presented the shape of small cocci rallied in chains, recalling the form of *Lactococcus* strains, and 21 strains whose cells have presented the shape of isolated rods or short chains belong to *Lactobacillus* genus.

Among the lactobacilli, 14 isolates were homofermentatives and did not possess arginine dihydrolase. All these strains did not grow at 10°C but grew at 15 and 37°C. Out of them, only nine were able to grow at 45°C. These isolates belong to Group II of lactobacilli (Streptobacteria) according to Axelsson (2004) and Hammes and Hertel (2006) recommendations. The six other strains of bacilli belong to Group III of the lactobacilli (Betabacteria) because they are heterofermentatives with positive ADH, and grew at 15°C but not at 45°C. Eight strains of *Lactococcus* were homofermentary, possessed ADH and grew at 15, 37 and 45°C. Table 1 shows the physiological and biochemical characteristics of the strains.

Results of fermentation of the carbohydrates on the API 50CHL gallery allowed the identification of the strains. The results (Table 1) show that the 7 strains of *Lactococcus* belong to *L. lactis* subsp*. lactis*, the 6 heterofermentative strains of *Lactobacillus* are part of *Lactobacillus brevis.* The 15 homofermentative lactobacilli belong to *Lactobacillus plantarum*; which was confirmed by the ATCC 14917 *L. plantarum* carbohydrate fermentation profile obtained from Biomerieux database.

Technological characteristics of strains

Acidity produced by bacteria

The results (Figure 1) lead us to note that the acidifying behavior of these bacteria is variable from one strain to another in the same species. In this study, comparison between means of acidity production in all readings has not revealed any significant difference in all strains, which reflects stability of this characteristic (Table 2).

The strains of *L. plantarum* has produced acidity varying from 10 to 90°D, which is greater than that produced by strains of *L. brevis.* These results are in agreement with those of Zhang and Vadlani (2014). *L. plantarum* is known to be homofermentative to hexoses, producing 2 moles of lactic acid per hexoses mole (Passos et al., 1994). However, the higher acidifying behavior was that of the strains of *L. lactis*. They produced lactic acid amounts of up to 9.7 g/L, which is in agreement with the work of Åkerberg et al. (1998). In general, lactobacilli ferment lactose by producing lesser amounts than *Lactococcus* and this was also suggested by Herreros et al. (2003). In fact,comparison between means of acidity production of the different strains has revealed a significant difference ($P<0.05$) (Table 3).

Table 1. Phenotypic characteristics of strains isolated from "hamoum".

Species	Code of the strain	Arginine dihydrolase	Gas production	Growth at different									
				Temperature (°C)				% NaCl			pH		
				10	15	37	45	2	4	6.5	4	4.5	8
Lactobacillus brevis	HMTK10	+	+	-	+	+	-	-	-	-	+	+	-
	HMTK24	+	+	-	+	+	-	+	-	-	+	+	-
	HMTK29	+	+	-	+	+	-	+	+	-	-	-	-
	HMTK52	+	+	-	+	+	-	+	-	-	-	-	-
	HMTK56	+	+	-	+	+	-	-	-	-	-	-	-
	HMTK57	+	+	-	+	-	-	-	-	-	-	-	-
Lactobacillus plantarum	HMTK2	-	-	-	+	+	+	+	+	+	+	+	+
	HMTK6	-	-	-	+	+	+	+	+	-	-	-	-
	HMTK8	-	-	-	+	+	+	+	+	-	-	+	-
	HMTK9	-	-	-	+	+	+	+	+	+	-	-	+
	HMTK21	-	-	-	+	+	-	+	-	-	-	+	-
	HMTK23	-	-	-	+	+	+	+	+	-	-	-	+
	HMTK25	-	-	-	+	+	-	+	-	-	-	-	-
	HMTK26	-	-	-	+	+	-	+	-	-	-	-	-
	HMTK28	-	-	-	+	+	-	+	-	-	-	-	-
	HMTK50	-	-	-	+	+	-	+	-	-	+	+	-
	HMTK51	-	-	-	+	+	-	+	+	-	+	+	-
	HMTK53	-	-	-	+	+	+	+	-	-	-	-	-
	HMTK58	-	-	-	+	+	+	+	+	-	-	+	-
	HMTK59	-	-	-	+	+	+	+	+	-	-	-	+
Lactococcus lactis ssp *lactis*	HMTK1	+	-	-	+	+	+	+	+	-	-	-	+
	HMTK3	+	-	-	+	+	+	+	+	+	-	-	-
	HMTK4	+	-	-	+	+	+	+	-	-	+	+	-
	HMTK7	+	-	-	+	+	+	+	-	-	-	-	+
	HMTK20	+	-	-	+	+	+	+	+	+	-	+	+
	HMTK22	+	-	-	+	+	+	+	+	-	-	-	+
	HMTK54	+	-	-	+	+	+	+	+	-	-	-	+
	HMTK55	+	-	-	+	+	+	+	+	-	-	-	+

+, Positive reaction; -, negative reaction.

According to these results, it may be suggested that strains with good acidifying activity can be proposed for application in the dairy industry, in which they lead to pH decrease, which plays an important and essential part in the coagulation of milk by destabilizing the casein micelles on one hand and giving the product its distinct and characteristic taste, thus contributing to flavor and aroma production. They may also act as inhibitors of undesirable micro-organisms.

Proteolysis activity

All the tested strains except the HMTK24 strain showed a growth with proteolysis activity confirmed by the appearance of a clear halo around the colonies seeded in a touch on the surface of the MRS medium supplemented with 1% of skimmed milk reconstituted at 10% (Figure 2).

According to Vuillemard (1986), the strain is consider as a proteolytic one if it presents a lysis zone with a diameter of 5 to 15 mm. In comparison with this data, our strains are revealed to be proteolytic, with proteolysis zone diameters between 6 and 14 mm. *L. lactis* subsp. *lactis* strains are more proteolytic than *Lactobacillus* strains, of which 50% have a lysis zone greater than or equal to 10 mm. The statistical study showed significant differences ($p < 0.05$) between the results obtained for the three species (Table 4). These results are consistent with those of Hassaïne et al. (2007). *L. lactis* possesses a complex proteolysis system comprised of multiple intracellular peptidases and a single protease anchored

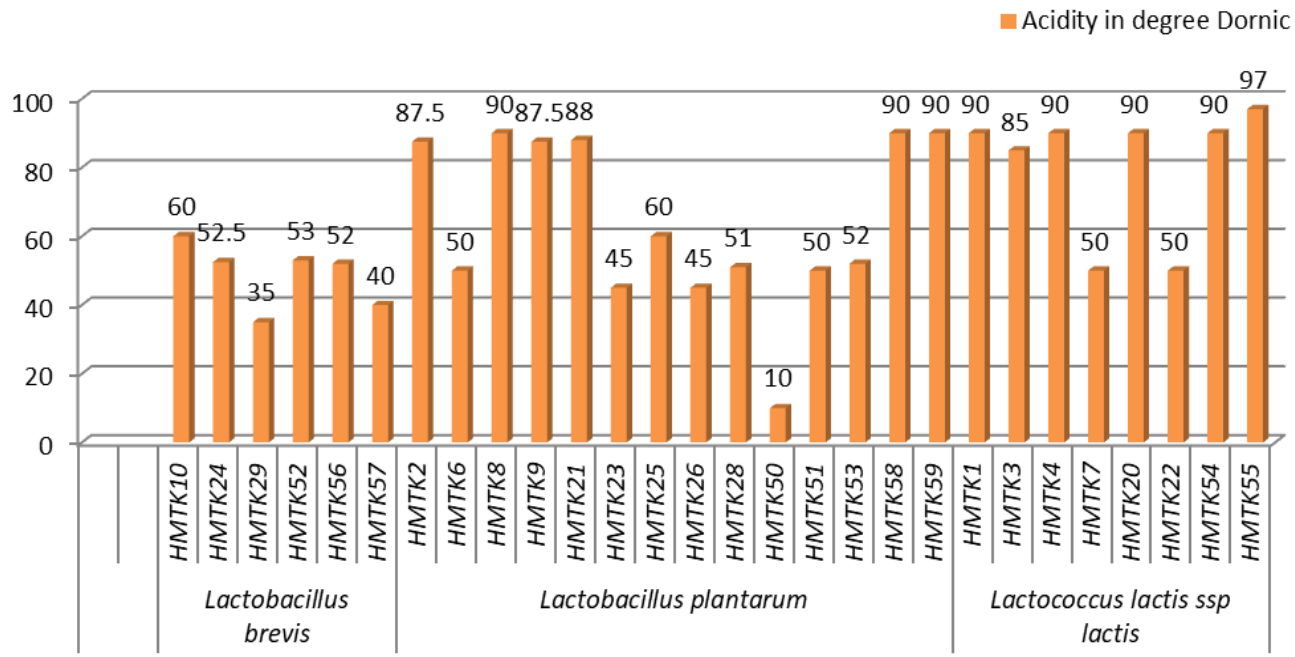

Figure 1. Acidity produced by the strains.

Table 2. Mean ± standard deviation of acidity produced by strains during experiment assays.

Parameter	*Lactobacillus brevis*	*Lactobacillus plantarum*	*Lactococcus lactis*
1st assay	46.50±8.41	64.00±24.91	80.25±18.95
2nd assay	49.25±8.75	64.04±24.80	79.75±18.85
3rd assay	48.25±10.01	64.68±24.77	80.88±18.87

to the cell surface, PrtP, whose gene is plasmidic, a serine protease that allows growth in milk by hydrolyzing caseins (Kunji et al., 1996). Some strains of *L. lactis* possess a surface protease specific to the maturation of the precursor of nisin. A functional protease of the HtrA family was demonstrated in *L. lactis* (Poquet et al., 2001).

The strains of *Lactobacillus* exhibit proteolytic activity with lysis zones with a diameter ranging from 6 to 12 mm, which is in line with the results of Roudj et al. (2009). *L. brevis* have shown a moderate level of proteolysis compared to *L. plantarum* with lysis diameters not exceeding 8 mm, these results being in agreement with the work of Belkheir et al. (2017). Strains exhibiting high proteolytic activity could be used with other ferments as complement or secondary culture. These strains can contribute to the development of the flavors during the maturation stage of cheese or in the manufacture of the fermented beverages.

L. lactis and *Lactobacillus* are largely deficient in the capacity of amino acid biosynthesis, which is compensated for by the ability to synthesize a large number of peptidases, amino acid permeases and multiple oligopeptide transport systems (Opp) (Klaenhammer et al., 2005). A large number of *Lactococcus* and *Lactobacillus* peptidases have been purified and biochemically characterized; in most cases, the corresponding gene has been cloned and sequenced (Kirsi et al., 2006). The first step in the use of casein by lactic acid bacteria is performed by CEP. Five different types of these enzymes (PrtP endoprotease, 2 general PepN and PepC aminopeptidases, PepO1 endopeptidase and Opp oligopeptide transport system) were cloned and characterized, including PrtP from *L. lactis,* whose gene (*prt*P) can be found either on plasmidic or chromosomic DNA, while the CEPs of lactobacilli are coded by genes on chromosomic DNA (Holck and Naes, 1992; Guédon et al., 2001; Kelleher et al., 2017).

Lipolytic activity

The lipolytic activity of the strains of the same species is highly variable. An example of the result is as shown in

Table 3. Acidity production expressed by different strains.

Strain	Mean ± standard deviation
Lactobacillus brevis	48.75±9.33 [(a)]
Lactobacillus plantarum	64.00±24.91 [(a)]
Lactococcus lactis subsp *lactis*	80.25±18.95 [(a)]

Values are mean ± standard deviation. [(a)]No significant difference was obtained by Ducan's test between three assay.

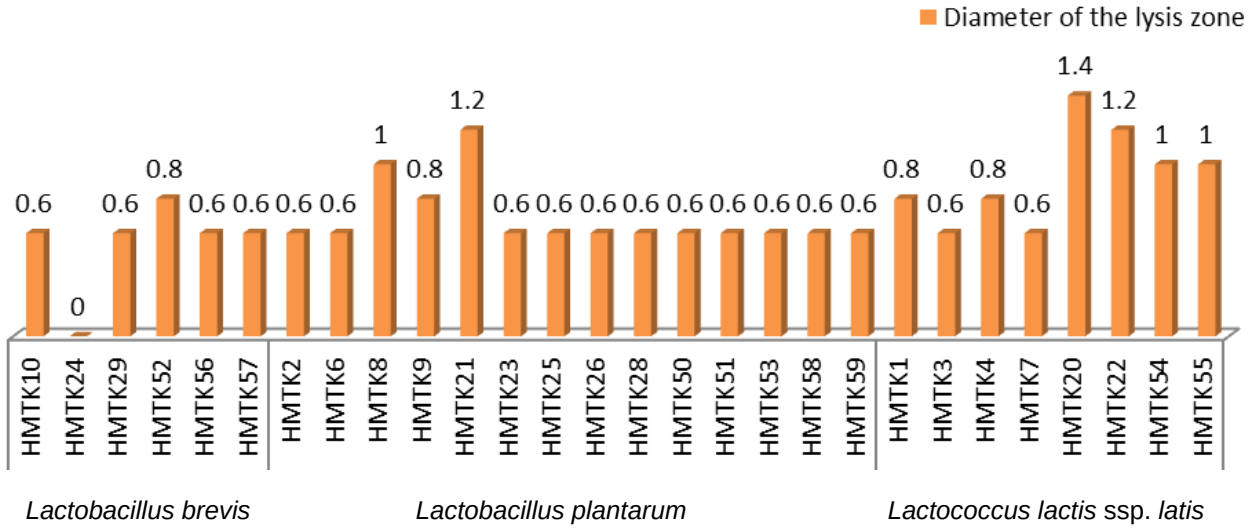

Figure 2. Proteolytic activity of the bacterial strains.

Table 4. Proteolysis expressed by different strains.

Strain	Mean ± standard deviation
Lactobacillus brevis	0.533±0.273 [(a)]
Lactobacillus plantarum	0.6857±0.1875 [(a)]
Lactococcus lactis subsp *lactis*	0.9250±0.2816 [(a)]

Values are mean ± standard deviation. [(a)]No significant difference was obtained by Ducan's test between three assay.

Figure 3. The ratio of the clear halo to the colony diameter (h/c) expressing the lipolytic activity was calculated for each strain (Figure 4). The statistical study of the means obtained for the lipolytic activity shows significant differences ($p < 0.05$) by Duncan's post hoc test (Table 5). The strains of *Lactococcus lactis* ssp *lactis* express a higher activity with ratios ranging from 2 to 5 by comparing it with that expressed by *L. plantarum*. The two species preferentially hydrolyze short chain fatty acids knowing that the milk fat is rich of these fatty acids. *L. lactis* exhibits a stronger esterase activity than that of *L. plantarum* according to Macedo et al. (2003) and Karam et al. (2012), which confirms the results of the present study. Several studies have characterized the esterases of *L. plantarum* (Gobbetti et al., 1997; Brod et al., 2009; Esteban-Torres et al., 2013, 2014, 2015; Kim et al., 2017). The lipolytic activity of *L. brevis* remains the lowest with ratios (h/c) not exceeding 3.

The study of Herreros et al. (2004) suggests that *L. plantarum* strains hydrolyze C8 and C14 fatty acids while those of *L. brevis* have shown greater esterase activity on C4 and C8 fatty acids. The study of the genome of *L. plantarum* WCFS1 reveals the presence of a rich repertoire of esterases and lipases suggesting their important role in cellular metabolism, among them LpEst

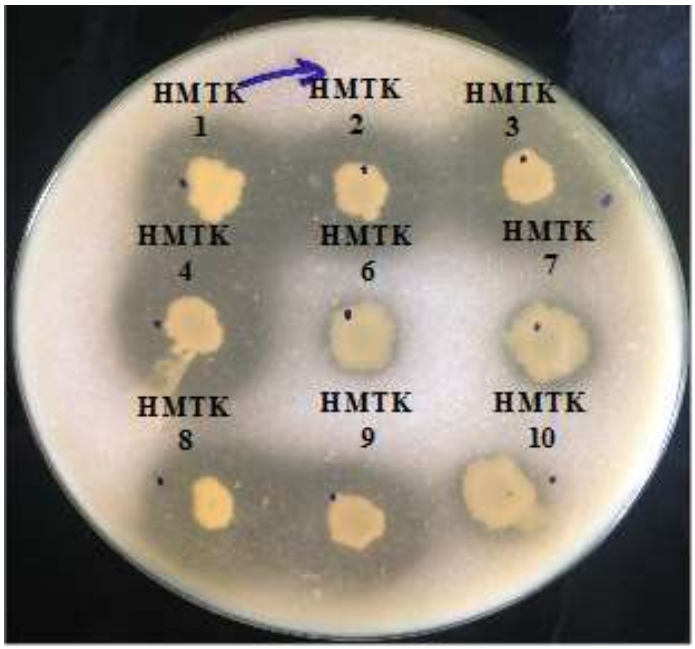

Figure 3. Lipolytic activity on MRS medium deprived of tween 80 and supplemented with 1% milk fat.

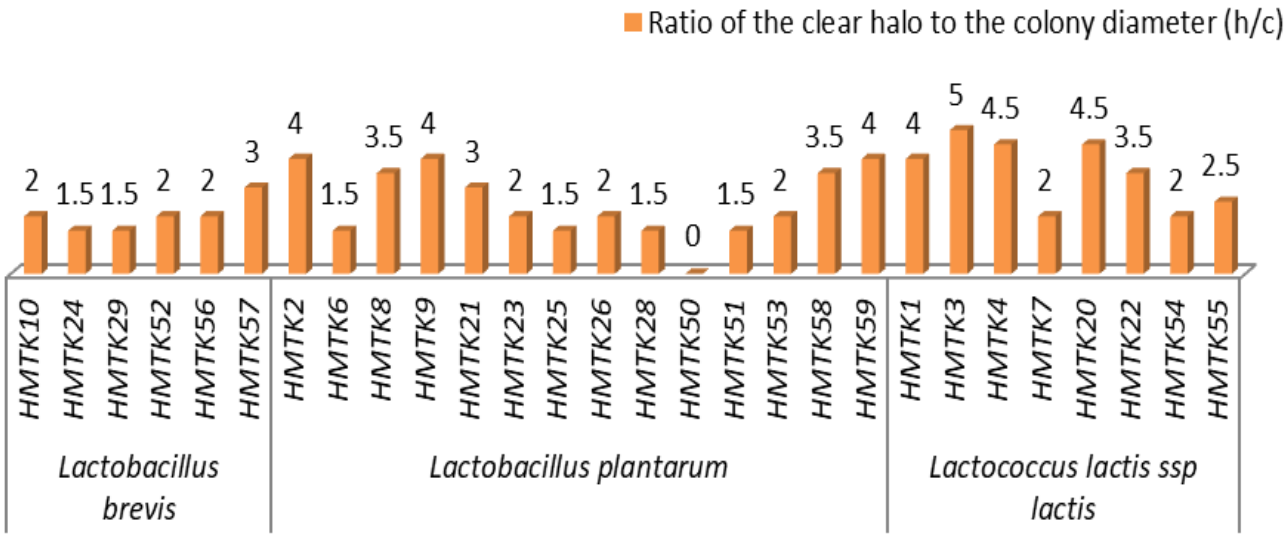

Figure 4. Lipolytic activity expressed by the strains on the milk fat.

carboxylesterase (Alvarez et al., 2014).

Lipases find promising applications in various fields: hydrolysis and synthesis of long-chain acylglycerols, manufacture of detergents, manufacture of food ingredients, application in the paper industry and biocatalysis of stereoselective transformations. They are widely used in the treatment of products of organic chemistry, the manufacture of cosmetic and pharmaceutical products as well as increased stability or enantioselectivity (Kapoor and Gupta, 2012).

Production of exopolysaccharides

The production of EPS by lactic bacteria is a favorable trait to many industrial food processes. The main advantage of the use of exopolysaccharide-producing lactic bacteria in lactic ferments during the production of

Table 5. Lipolysis expressed by different strains.

Strain	Mean ± standard deviation
Lactobacillus brevis	2.000±0.548 [(a)]
Lactobacillus plantarum	2.429±1.238 [(a)]
Lactococcus lactis subsp *lactis*	3.500±1.195 [(a)]

Values are mean ± standard deviation. [(a)]No significant difference was obtained by Ducan's test between three assay.

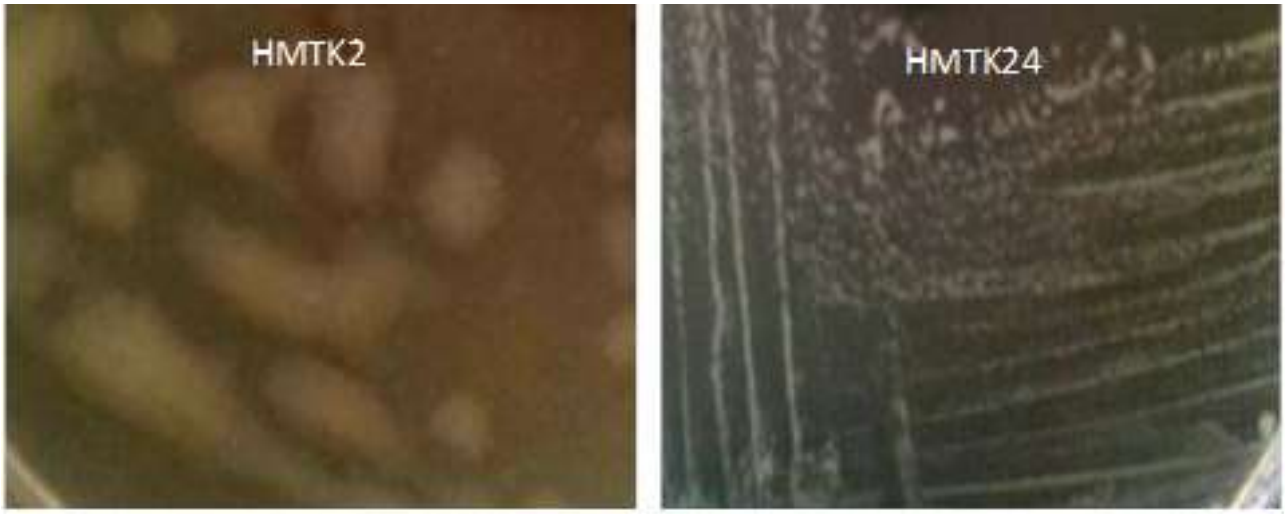

Figure 5. Production of exopolysaccharides on hypersaccharosed MRS medium.

fermented milks is the improvement of the texture and the reduction of the syneresis (expulsion of liquid from a gel). According to this test, negative results were obtained on the Mayeux medium for all the lactic strains, which were unable to develop by forming colonies with a more or less glutinous aspect, testifying to the production of a thickening agent, exopolysaccharides. Nevertheless, four strains, HMTK2, HMTK4, HMTK10 and HMTK24, produce EPS on hypersaccharosed MRS medium (Figure 5).

Several works focus on the EPS production by *Lactobacillus* (Ismail and Nampoothiri, 2010; Dilna et al., 2015; Fontana et al., 2015; Salazar et al., 2015; Oleksy and Klewicka, 2016). To date, about 30 species of *Lactobacillus* producing EPS have been identified, the most well-known being *Lactobacillus casei, Lactobacillus acidophilus, L. brevis, Lactobacillus curvatus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus helveticus, Lactobacillus rhamnosus, L. plantarum* and *Lactobacillus johnsonii*. The genetic determinants of EPS are carried either by a plasmidic or a chromosomal DNA. The genes encoding for proteins responsible for EPS synthesis by mesophilic lactic bacteria are generally located on a plasmid. In *Lactococcus*, the production of EPS is less stable, the main reasons being the plasmidic location of the production genes and the presence of a mobile insertion sequence (IS, e.g. ISS1, IS981) (Sanlibaba and Çakmak, 2016).

Aromatic activity

Co-metabolism of citrate, fermentable sugar is very important in lactic bacteria since it is closely related to the aromatic activity. The strains HMTK2, HMTK4, HMTK8, HMTK10, HMTK20, HMTK21, HMTK24, HMTK50, HMTK51 and HMTK58 were found to produce citratase (Figure 6).

Lactic bacteria using citrate play an important role in many dairy processes. They are responsible for the production of aromatic compounds (diacetyl and acetoin). Diacetyl is essential for establishing the flavor of dairy products such as butter and buttermilk and sometimes, young cheeses. Because of these properties, these lactic bacteria are often called aroma bacteria. During the citrate metabolism, CO_2 is also produced, which leads to eye formation in certain types of cheese.

The strains HMTK 8, HMTK20, HMTK21 and HMTK58 show a positive result for the production of acetoin (Figure7).

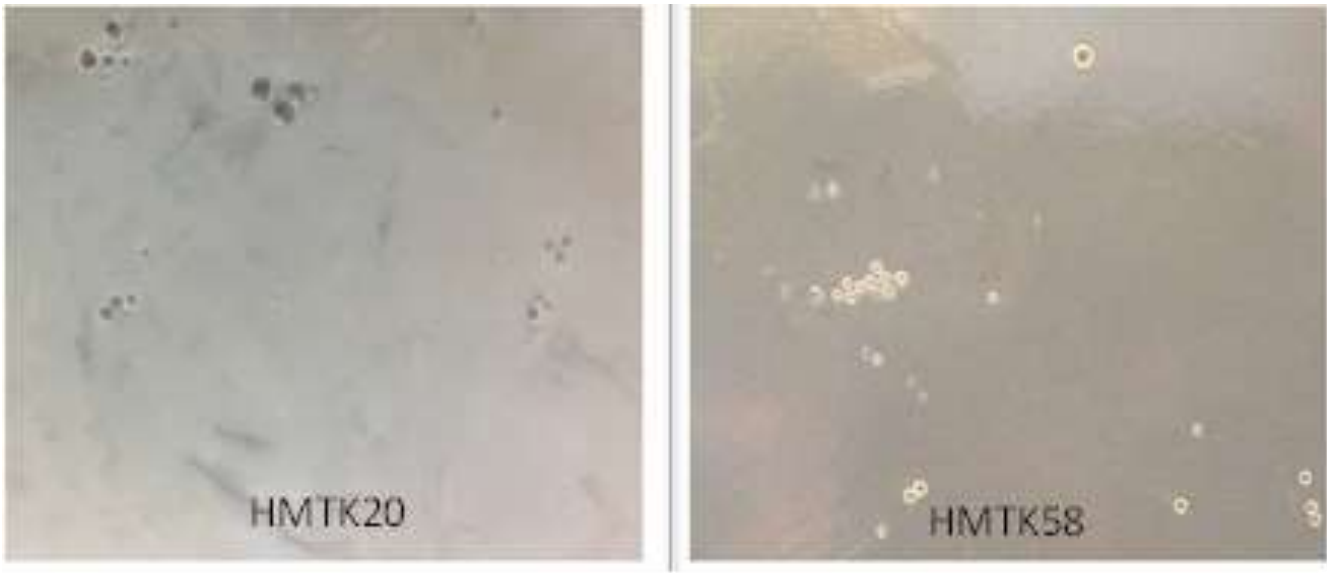

Figure 6. Citratase production in semi-solid agar with citrated milk.

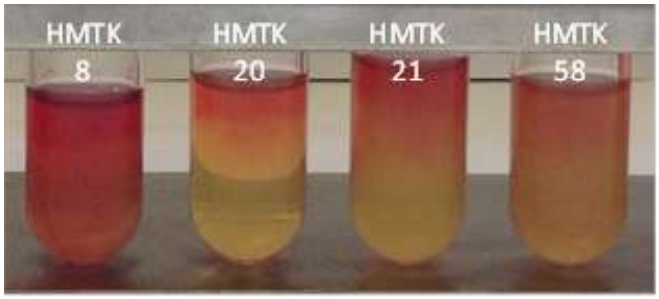

Figure 7. Production of acetoin revealed by Voges-Proskauer colored reaction.

Conclusion

This study shows the interesting technological properties presented by strains of *L. lactis* ssp *lactis, L. plantarum* and *L. brevis* isolated from hamoum. Some of them produce amounts of lactic acid of up to 9.7 g/L. They express proteolytic activity of milk proteins and lipolytic of milk fat. Ten strains possess citratase and four strains produce acetoin. These strains are good candidates for use in the food industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Bio-preservation of Nigerian soft-white cheese in submerged consortium of bacteriocinogenic lactic acid bacteria culture

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Bio-preservation of Nigerian soft-white cheese (wara) in submerged consortium of bacteriocinogenic lactic acid bacteria (LAB) culture was investigated. *Lactobacillus acidophilus* PIT17 and *Lactococcus lactis* PIT30 were isolated from pito using the pour plate technique on MRS medium. The selection of *L. acidophilus* PIT17 and *L. lactis* PIT30 for the bio-preservation studies were based on their ability to produce acidophilin and nisin to inhibit the growth of the test isolates. The 'wara' were submerged in consortia of the *L. acidophilus* PIT17 and *L. lactis* PIT30 culture and were kept/stored at 2, 4, 6, 8, and 10°C and at room temperature. The shelf-life of the wara alongside control was determined. Physico-chemical and proximate analysis, microbial counts and organoleptic characteristics of the wara were also carried out before and after the bio-preservation. The shelf life of the wara were observed with a significant difference ($p < 0.05$) at storage temperatures of 2°C (5 days), 4°C (6 days), 6°C (4 days), 8°C (3 days), 10°C (6 days) and 24±1°C (3 days). The proximate analysis of wara showed significant difference ($p < 0.05$) at different storage temperatures employed. The total viable bacterial count (TVBC) of the cheese (wara) decreased significantly ($p < 0.05$) after 24 h of submerged bio-preservation of wara from 9.8×10^5 cfu/ml, 1.3×10^6 cfu/g to between 6.2×10^5 and 7.5×10^5 cfu/g. The organoleptic characteristics of wara revealed that there were significant difference ($p < 0.05$) in colour, texture, aroma, taste, and general acceptability.

Key words: Organoleptic characteristics, proximate, physico-chemical, cheese, consortium.

INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of microorganisms with different metabolic activities. This diversity makes them very adaptable to a range of conditions and is largely responsible for their success in acid food fermentation (Beuchat, 1995). LAB have no strict taxonomic significance although they had been

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shown by serological technique and 16S ribosomal RNA cataloguing to be phylogenetically related. They share a number of common features (Adams and Moss, 2008). Historically, bacteria from the genera *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc* and *Pediococcus* are the main species involved. Several more have been identified but with minor significance or role in lactic acid fermentations (Food and Agriculture Organization, FAO, 2013). LAB produce various compounds such as organic acids and bacteriocin during lactic acid fermentation (Lindgren and Dobrogosz, 1990).

Bacteriocins are naturally occurring antibiotic peptides produced by Gram positive bacteria and may contain as much as 24 amino acids. Some bacteriocins are lantibiotics, which means that they are post translationally modified so as to encompass the amino acid lanthionine or "Lan" (Chatterjee et al., 2005). In recent years, the interest increased in bacteriocin-like inhibitory substances (BLIS) producing LAB because of their potential use as natural antimicrobial agents to enhance the safety of food products. Bacteriocins from LAB are described as "natural" inhibitors and as a result LAB had acquired generally recognized as safe (GRAS) status. The BLIS from LAB are antimicrobial compounds that possess bacteriocin requisites but that have not yet been characterized for their amino acid sequence (Jack et al., 1995). Bacteriocins from the GRAS-LAB have received significant attention as a novel approach to the control of pathogens in foods (Settani et al., 2005).

Pito is one of the indigenous alcoholic beverages. Mainly, pito is produced from the grains of guinea corn (*Sorghum vulgare* and *Sorghum bicolor*). Sorghum is one of the cereals cultivated in the tropical regions of Africa and is about the largest cultivated crop in the Northern Guinea Savanna areas of Nigeria (Okoro et al., 2011). The process of pito production is similar to burukutu production which involves malting, mashing, fermentation, and maturation as described by Okoro et al. (2011). *Geotrichum candidum* and *Lacobacillus* species have been described to be responsible for souring pito (Okoro et al., 2011). From plant extracts of tea leaf (*Camelia* species), cashew tree bark (*Anacardium occidenttale*) and the bark of mango tree (*Magnifera indica*), pito can be produced. Steeping and boiling are the process involved in production of unfermented pit (Kolawole et al., 2007). Most of the bacterial cultures found during the production of pito include LAB, which include those bacteria capable of metabolising fructose, galactose, lactose lactic acid that lowers pH of product. The LAB have optimum pH range between 3 and 6.8. Many bacteria species had been found in fermented pito. They included *Leuconostoc mesenteriodes, Bacillus subtitis, Staphylococcus* species*, G. candidum* and *Lactobacillus* spp. The species are responsible for the souring of pito. Due to consumers demand for the locally fermented beverages such as pito, the bacteriocin producing organisms are considered a potential source of biological preservatives for such local drinks (Okoro et al., 2011).

Soft white-cheese (wara) can be defined as consolidated curd milk solid in which fat is entrapped by coagulated casein. The physical characteristics of cheese are far removed from milk, this is because protein coagulation proceeds to a greater extent as a result of the use of proteolytic enzymes and much of the water content of the milk separates and it is removed in the form of whey (Taylor et al., 1997). Some examples of cheese include soft ripened cheeses which include camemberti and blue cheeses. The cheese starter culture is the combination of *Streptococcus cremoris* and *Lactobacillus lactis* (O' Sullivan et al., 2002).

Bio-preservation has gained increasing attention as natural means for controlling the shelf-life and safety of food products. The application of bio protective cultures to ensure the hygienic quality is a promising tool although, it should be considered only as an additional measure to good manufacturing, processing, storage and distribution practices (Amani, 2012). The application of bacteriocins as natural antimicrobial substances in biopreservation (the use of living cells and/or their products for preservation purposes) has focused mainly on foods and foodstuffs from animal origin (Cleveland et al., 2001; Devlieghere et al., 2004; Stiles, 2004). Consequently, bio-preservation systems such as bacteriocinogenic LAB cultures and/or their bacteriocins have received increasing attention and new approaches to control pathogenic and spoilage microorganisms have been developed (Ross et al., 1999). This study is aimed at bio-preservation, physicochemical, proximate, microbial analysis and evaluation of organoleptic characteristics of Nigerian soft-cheese in submerged consortium of bacteriocinogenic LAB culture.

MATERIALS AND METHODS

Collection of study samples

Pito samples were purchased from Unguwan Kaje, Minna in sterile bottles and were taken to the laboratory for LAB isolation. Samples of soft-white cheese (wara) were purchased from Bosso Market and deposited in sterile conical flask for the bio-preservation and organoleptic characteristic studies.

Culture media

The standard laboratory methods as prescribed by Cheesebrough (2003) were used to prepare the culture media. The media used in this study include nutrient agar (NA) (Oxoid), urea agar base (Analar), mannitol salt agar (MSA) (Oxoid), Simon's citrate agar (Oxoid), De Man Rogosa Sharpe (MRS) broth (Oxoid) and De Man Rogosa sharpe (MRS) medium (Oxoid). The MRS is a selective medium for the growth of LAB.

Isolation of LAB

One milliliter of pito was aseptically transferred into 9 ml buffered

peptone water, Bpw (Oxoid) to obtain 1:10 dilution. In 0.1% peptone water, serial dilution of the pito was carried out. The serially diluted samples of pito were plated on MRS medium and were incubated at 37°C for 24 h. Colonies/Growth that appeared on the culture plates were counted using the colony counter (Stuart, 6339, Co. Ltd. Great Britain). The result of the count was recorded as colony forming units per milliliter (cfu/ml). Repeated sub-culturing of the isolates on fresh media was used to obtain pure cultures. The pure culture was maintained on agar slant for further characterization and identification (Bromberg, 2004; Oyeleke and Manga, 2008).

Characterization and identification of microbial isolates

The microbial isolates were identified based on colony morphology, cell morphology and biochemical tests (Fawole and Oso, 1998; Cheesbrough, 2003; Manga, 2008). The biochemical tests include Gram's reaction, motility, oxidase, ammonia from arginine, coagulase, catalase, citrate utilization, indole test, gelatine liquefaction, carbohydrate utilization profiles, and mannitol activity. The LAB were characterized and identified as *Lactobacillus acidopholus* PIT 17 and *Lactococcus lactis* PIT 30 using standard scheme.

Selection of LAB for bio-preservation studies

The *L. acidopholus* PIT 17 and *L. lactis* PIT 30 were selected from other LAB after vigorous screening with reference amount of bacteriocin produced using the methods described by Kacem et al. (2005) and Mohammed et al. (2013).

Inoculum preparation of LAB

The bacteriocinogenic LAB (*L. acidophilus* PIT17 and *L. lactis* PIT30) were inoculated into nutrient broth medium and then incubated at 37°C overnight, serial dilutions was carried out thereafter. The total count of microorganisms per milliliter (ml) of the stock suspension was determined by means of the surface viable count (SVC) technique. The McFarland standard was prepared by mixing 0.85% of 1% sodium chloride and 9.95 ml of 1% sulphuric acid in a separate test tube. While Microbial cell dilutions of the *L. acidophilus* PIT17 and *L. lactis* PIT30 in normal saline initially prepared were compared with the turbidity that matches that of the 0.5 (10^8 cells/ml) McFarland standard prepared. Thus, standard inoculums for the culture consortia of *L. acidophilus* PIT17 and *L. lactis* PIT30 were prepared. The 0.5 McFarland standards is comparable to a bacterial suspension of 10^8 cells/ml. From the inoculums, wara were preserved/submerged in 10^8 cells/ml (w/v) of the consortia of *L. acidophilus* PIT17 and *L. lactis* PIT30 cultures employed in this study (McFarland, 1907; Sanaa et al., 2008).

Bio-preservation studies of soft-white cheese using consortium of bacteriocinogenic LAB

From the inoculum preparations, the cheese (wara) were submerged and preserved in 10^8 cells/ml (w/v) of the consortia of *L. acidophilus* PIT17 and *L. lactis* PIT30 cultures. The preserved wara were kept/stored at refrigeration temperature (2, 4, 6, 8 and 10°C) and room temperature (24±1°C) to determine the shelf life of the wara under study. The experimental control (wara without consortia bacteriocinogenic LAB) were set aside (Mcfarland, 1907; Techno serve, 1994; Food Storage Time Guide Line, FSTGL, 2003; Food Safety Authority of Ireland, FSAI, 2005; Sanaa et al., 2008).

Proximate analysis of soft-white cheese (wara)

Percentage moisture content

In an oven at 80°C, the metallic dishes were dried for 20 min and were allowed to cool in desiccators and weighed. About 5 g of wara were placed in the dishes and were weighed. The dishes with the wara samples were then dried at 80°C in an oven for 24 h to achieve a constant weight. These were quickly transferred to desiccators to cool. It was then weighed immediately with minimum exposure to the atmosphere. The loss in weight of the wara sample during drying is the moisture content (AOAC, 2005).

$$\%\ \textbf{Moisture} = \frac{W_2 - W_3}{W_2 - W_1} \text{x } 100$$

Where, W_1 = Initial weight of empty crucible, W_2 = weight of crucible + food before drying, W_3 = final weight of crucible + food after drying.

% Total solid (dry matter) = 100 - % Moisture

Percentage protein content

The concentrated H_2SO_4, concentrated NaOH (40%), K_2SO_4 and $CUSO_4$ were used to digest the wara sample. About 5 ml of the digested wara samples each were placed into a micro-Kjeldahl distillation apparatus with excess concentrated NaOH to make the solution strongly alkaline. Ammonia were distilled into 5ml of boric acid indicator in a titrating flask separately. About 45 ml of the distillates were collected. Titrations were done with 0.01M HCL. The end points of titration were light green (AOAC, 2005).

% Protein = %N × F

Where, F = Conversion factor = 100 / (%N in food protein)

and

% Nitrogen (N) = ($V_S V_B$ ×Nacid × 0.01401 / W) × 100

Where, V_S = vol. (ml) of acid required to titrate sample, V_B =vol. (ml) of acid required to titrate blank, N acid = Normality of acid (0.1N), and W = weight of sample in grams.

The common factor used for most food and food mixture is 6.25

Percentage total ash content

Ten grams of wara samples were weighed into a small dry crucible of known weight separately. The wara samples in the separate crucible were charred on a low furnace. At 550°C, the charred samples of wara were ashed in a muffle furnace for 2 h. The ashed materials were removed from the furnace and cooled. The materials were placed in the desiccators and were weighed (AOAC, 2005).

$$\%\ \textbf{Ash content}(\textbf{dry basis}) = \frac{W_3 - W_1}{W_2 - W_1} \text{x } 100$$

Where, W_1=Weight of empty crucible, W_2=weight of crucible + food before drying and/or ashing, and W_3=weight of crucible + ash.

Percentage total fat contents

The percentage fat content of wara was determined by direct

Soxhlet extraction using petroleum either (bp = 40 to 60°C) as solvent. The 0.5 g of wara samples were measured into separate filter papers and were placed in the extractor. The set-up was placed on a heating mantle separately. The heat source was adjusted such that the solvent was boiled gently and refluxed several times for 6 h until the ether had siphoned over and the barrel of the extractor was empty. On removal, the filter paper was placed in an oven at 50°C and dried to constant weight. The percentage of fat was then calculated (AOAC, 2005).

$$\% \textbf{ Fat} = \frac{\text{Weight of Fat}}{\text{Weight of Sample}} \text{x } 100$$

Total carbohydrate content (%)

The total percentage of carbohydrate content of wara was calculated by adding percentage moisture, ash, protein, fat, and fibre contents of the wara and subtracting it from 100% (AOAC, 2005).

Microbial counts

The pour plate method was used. Serially, diluted sample of the bio-peserved wara was inoculated into nutrient agar and incubated at 37°C for 24 h for the presence of aerobic viable bacteria. Colonies that appeared on the plates were counted using colony counting chamber and were recorded as colony forming unit per millilitre (cfu/ml) or (cfu/g) of samples (Cheesbrough, 2003; Oyeleke and Manga, 2008). Microbial counts were taken before biopreservation of products, every 24 h after first preservation of dairy products in consortia of *L. acidophilus* PIT17 and *L. lactis* PIT30 cultures and at the expiration of shelf life of the wara.

Organoleptic characteristics of soft-white cheese (wara)

The method of Ranganna (2008) was employed. Sensory quality attributes such as colour, aroma, texture, taste, and general acceptability of the biopreserved wara were evaluated using six-point Hedonic scale. For this purpose, the wara samples were served to ten panellists for rating on Six-point scale as score 1 (dislike very much/most undesirable), 2 (dislike much), 3 (dislike), 4 (like), 5 (like much), and 6 (liked very much/most desirable) compared with the control samples of wara. The organoleptic scores generated were analyzed statistically.

Statistical analysis of data

Data generated in this study were subjected to statistical analysis using analysis of variance (ANOVA), that is, one way analysis of variance (ANOVA), SPSS 19.0 version package and Pearson's correlation with MINITAB 14 package to determine the level of significance between variables.

RESULTS

Isolation, characterization, identification of bacteriocinogenic LAB and it selection for bio-preservation studies

The pito analyzed had varying species of LAB in them. *L. acidophilus* PIT 17 and *L. lactis* PIT 30 were isolated, characterized and identified (Table 1). The *L. acidophilus* PIT 17 and *L. lactis* PIT 30 were selected after vigorous screening based on its ability to grow in MRS broth to produce acidophilin and nisin. Also, through spectrophotometric analysis at the 580 nm wavelength, bacteriocin activity (AU/mL), pH and potentials for use as food preservative. It was observed that *L. acidophilus* PIT 17 and *L. lactis* PIT 30 had growth ability of 0.89, at pH of 5.00 and 0.91 at pH of 5.80 and bacteriocin activity of 8200 and 9400 AU/mL, respectively with significant differences ($p < 0.05$) (Table 2).

Proximate analysis of bio-preserved soft-white cheese (wara)

The proximate analysis of the wara such as %moisture, protein, total ash, fat and carbohydrate showed significant difference ($p < 0.05$) at different storage temperatures employed. The variation in the proximate compositions could be attributed to effects of the LAB consortium used and/or differences in storage temperatures employed in this study (Table 3).

Microbial count of bio-preserved soft-white cheese (wara)

The total viable bacterial count (TVBC) of the cheese (wara) decreased significantly ($p < 0.05$) after 24 h of submerged technique of bio-preservation of wara from 9.8×10^5 cfu/ml, 1.3×10^6 cfu/g to b 6.2×10^5 and 7.5×10^5 cfu/g. Shelf life extension days of wara were observed with a significant difference ($p < 0.05$) at storage temperatures of 2°C (5 days), 4°C (6 days), 6°C (4 days), 8°C (3 days), 10°C (6 days) and 24±1°C (3 days) (Table 3).

Organoleptic characteristics of bio-preserved soft-white cheese (wara)

The organoleptic characteristics of the wara revealed that there were no significant difference ($p > 0.05$) in colour, texture, aroma, taste and general acceptability compared with the wara not submerged in the consortia culture of the bacteriocinogenic LAB. This could be as a result of the effects of the bacteriocinogenic LAB and/or storage temperatures employed when compared with the wara not submerged in the consortia culture of the bacteriocinogenic LAB (control) (Table 4).

DISCUSSION

The pito analysed showed the presence of LAB. Similarly, the occurrence of LAB in locally fermented

Table 1. Morphological and biochemical characteristics of bacteriocinogenic LAB isolated from fermented food product.

Isolate code	Colony morphology	Cell morphology	Gram staining	Oxidation test	Mannitol activity	Catalase	NH_3 activity from arginine	Gelatine liquefaction	Sugar fermentation				Probable organisms
									Glucose	Sucrose	Fructose	Lactose	
PIT17	Circular, convex	Rods	G+	-	-	-	+	-	G	A	AG	A	*Lactobacillus acidophilus*PIT1 7
PIT30	Convex	Cocci in chains	G+	-	-	-	+	-	AG	A	A	AG	*Lactococcus lactis* PIT30

PIT, Pito; G+, Gram positive; +, positive result; -, negative result; G, gas production; A, acid production; AG, acid and gas production.

Table 2. Production of bacteriocin by *Lactobacillus acidophilus* and *Lactococcus lactis*.

Coded organisms	Growth of LAB (580 nm)	pH of bacteriocins	Bacteriocin activity (AU/mL)
Lactobacillus acidophilus PIT1 7	0.89	5.00	8200 ± 0.00[a]*
Lactococcus lactis PIT30	0.91	5.80	9400 ± 0.00[a]*

PIT, Pito; Au/ml, Activity unit per milliliter; nm, nanometer. Results with the same superscript are significantly different ($p > 0.05$).

foods were also reported by Oyeleke et al. (2006) who reported frequent isolation of *L. bulgaricus* and *L. acidophilus* with 29% each of occurrence, followed by *Streptococcus thermophilus* (25%), *S. cremoris* (10.6%) and *L. lactis* (6.4%) products. This is in conformity with the report of Mohammed and Ijah (2013) who isolated and characterized LAB from fermented milk (nono), cheese (wara) and yoghurt and revealed that 13 (86.6%) out of 15 samples analysed, harboured LAB. Nono had the highest LAB counts (9.8×10^6 cfu/ml), while yoghurt had the lowest LAB counts (3.1×10^6 cfu/ml). The LAB were identified as *Lactobacillus bulgaricus* (31.6%), *L. lactis* (15.8%), *L. acidophilus* (10.5%), *S. thermophilus* (15.8%), *S. cremoris* (10.5%), *Pediococcus halophilus* (5.3%) and *Saccharomyces cerevisiae* (5.3%). The bio-preservation of wara (w/v) in consortia of *L. acidophilus* PIT17 and *L. lactis* PIT30 culture revealed that pH, storage temperature and microbial load played significant roles in shelf life determination. Similarly, FSAI (2005) reported that the shelf life of many food products is dependent on storage temperature and microbial load. At refrigeration, storage temperatures of 4 and 10°C, fermented milk products in this study were also better preserved than other storage temperatures (2, 6, 8 and 24±1°C) employed in this study. This could be due to the inability of some the spoilage pathogenic organisms to grow at those temperatures and/or the presence of consortia culture of LAB employed. This is not the same but similar with the report of Mohammed et al. (2013) worked on bio-shelf life extension of fresh beef in *Lactobacillus plantarum* FALB 33 culture at different storage temperatures and revealed that at refrigeration storage temperatures of 4 and 10°C, fresh beef were best preserved than other storage temperatures (2, 6, 8 and 24±1°C) employed in their study. This finding is similar to the report of Techno serve (1994) that most commercial products, like milk products are refrigerated at 10°C which also encourages the growth of many psychrophiles like *Pseudomonas*, *Alkaligenes, Flavobacterium* and *Micrococcus* species at room storage temperature (24±1°C). This is similar to the result of the present study where the preserved wara with the consortium proved effective and extended the shelf life by 2 to6 days at different storage temperatures. This agrees with the report of O'Sullivan et al. (2002) that as an alternative to using bacteriocin itself for bio-preservation of foods, direct introduction of live bacteriocin-producing culture of LAB as a protection starter has been investigated extensively and has achieved favourable results in some food systems. For example, the nisin-

Table 3. Physicochemical, proximate and microbial qualities of soft-white cheese bio-preserved in consortium of *L. acidophilus* PIT 17 and *L. lactis* PIT30 culture.

Milk product	Moisture content (%)	Protein content (%)	Total ash content (%)	Fat content (%)	Total carbohydrate (%)	pH of fermented milk products
White cheese (wara)	67.20±.13^{b,c}	12.16±.03^{a}	2.80±.23^{a}	13.11±.06^{b,c,d}	3.95±.02^{f}	4.0
	67.22±.06^{b,c}	14.63±.02^{e}	2.63±.02^{a}	13.13±.08^{c,d}	2.12±.01^{b}	4.0
	67.31±.01^{b,c}	13.77±.03^{c}	2.63±.01^{a}	13.10±.06^{b,c,d}	3.04±.02^{d}	4.0
	67.31±.02^{b,c}	14.14±.08^{d}	2.61±.02^{a}	13.22±.13^{d}	2.01±.01^{a}	4.0
	68.26±.14^{d,e}	14.24±.06^{d}	2.71±.01^{a}	13.25±.03^{d}	3.62±.01^{e}	4.0
	67.90±.16^{c,d}	13.20±.12^{b}	2.67±.02^{a}	12.91±.19^{a}	3.10±.06^{d}	4.0
Control	68.45±.58^{e}	13.71±.06^{c}	2.65±.03^{a}	12.82±.12^{a,b}	1.94±.02^{a}	4.68

	Storage temperature (°C)	Microbial counts (cfu/ml or cfu/g) before inoculation with/in LAB (10^6)	Microbial counts (cfu/ml or cfu/g) after 24 h of inoculation of milk products with/in LAB (10^5)	Microbial counts (cfu/ml or cfu/g) after shelf life of milk products (10^6)	*Days of original shelf life of milk products	Days of Improved shelf life (elongation) of milk products
White cheese (wara)	2	1.2 **	7.0**	1.1	14	19**
	4	1.2	7.2	1.2	14	20**
	6	1.2	7.5	1.4	10	14
	8	1.2	7.8	1.3	7	10**
	10	1.2	8.0	1.0**	7	13**
	24±1	1.2	8.4**	1.6**	1	4
Control	-	1.4	9.5	1.9	-	-

*Recommended period (days) of shelf life (elongation) of milk products (Techno serve, 1994; FSTGL, 2003; DSGL, 2004; DHEC, 2013). FCF, Fermented corn flour. Proximate analyses results represent mean ± standard error mean of triplicate determinations. Results with the same superscript on the same column are not significantly different at ($p > 0.05$), **($p < 0.05$).

Table 4. Organoleptic characteristics of white cheese (Wara) bio-preserved in consortium of *L. acidophilus* PIT 17 and *L. lactis* PIT30 culture.

Parameter	Colour	Aroma	Texture	Taste	General acceptability
White cheese	5.50±0.00^{c}	5.40±0.00^{f}	5.60±0.00^{p}	5.60±0.00^{o}	5.80±0.00^{o}
Control	6.00	6.00	6.00	6.00	6.00

Results with the same superscript on the same row are not significantly different ($p > 0.05$).

producing starter has been shown to have the potential to inhibit *L. monocytogenes* in Camembert cheese manufacture. Furthermore, it was reported that *Lactobacillus* or *Pediococcus* strains producing an antilisterial class IIa bacteriocin could inhibit *L. monocytogenes* growth in meats and meat products. The lacticin 481-producing and lacticin 3147-producing cultures have been used successfully to improve the

quality of Cheddar cheese through the inhibition of NSLAB (Ryan et al., 1996; O`Sullivan et al., 2003). O`Sullivan et al. (2003) reported a reduction of 4 log units in the number of NSLAB after 4 months of ripening in experimental Cheddar cheese with lacticin 481-producing strain *L. lactis* CNRZ 481 used as an adjunct to the lactococcal starter culture *L. lactis* HP, compared with the same number of bacteria in the control cheese (obtained with the standard starter culture only). The recorded decrease in the number of NSLAB was 2 log units achieved at the end of the ripening period (after 6 months). Nisin Z-producing strain *L. lactis* IPLA 729 has been successfully applied on the inhibition of the spoilage strain of *Clostridium tyrobutiricum* CECT 4011, a late blowing agent, in semi-hard Vidiago cheese making as reported by Rilla et al. (2003).

The results of the proximate analysis of the bio-preserved wara showed major differences at different storage temperatures employed. The variation in the proximate compositions could be attributed to presence of the biopreservation culture or differences in storage temperatures employed in this study. This is similar to the report of Elewa (2009) who revealed that the proximate analysis of cheese samples produced using combined culture of *Lactobacillus bulgaricus* and *L. plantarum* had the highest protein content (29.5%), while the sample produced by natural fermentation had the lowest protein content (26.66%). The moisture content of the samples ranged between 26.64 and 32.09%. This is also similar to the report of Ramzi and Ahmed (2013) who worked on soft-white cheese and revealed that the total solids, fat, crude protein, titratable acidity, pH, and volatile fatty acids were affected by the storage period, while there was no significant difference ($p>0.05$) in ash contents.

The organoleptic characteristics observed in this present study on the wara revealed that there were significant difference ($p < 0.05$) in colour, texture, aroma, taste and general acceptability compared with the control. This is similar to the findings of Papetti and Carelli (2013) who worked on composition and sensory analysis for quality evaluation of a typical Italian cheese and revealed that cheese were evaluated for various sensory attributes (taste, flavour, texture, and overall acceptability) during storage. In the affective tests, the panellists evaluated the samples for overall quality. The results proved that months of production had significant effects on the sensory quality of the cheese. This is also similar to the report of Katikou et al. (2005) who worked on sensory changes in colour and odour of sliced-bio preserved-refrigerated beef with bacteriocin and revealed that instrumental colour measurements changed with storage time, but no treatment effects were observed during the whole 28-day storage period. This is not the same but similar to the report of Mohammed et al. (2014) who worked on sensory evaluation of African Catfish (*Clarias gariepinus*) bio-preserved in culture of *Lactobacillus sake* FMB 9 and revealed that the shelf life of the African catfish was extended significantly ($p < 0.05$) between 2 and 5 days at the different storage temperatures employed. The implication of these research findings is that bio-preservation of wara using consortia culture can extend the shelf life of the products particularly at refrigeration temperatures of 4 and 10°C, respectively. In conclusion, the findings of this study demonstrate that the use of consortium of bactriocinogenic LAB improved the nutritional quality, shelf life and acceptability of the wara.

RECOMMENDATION

It is therefore recommended that this method of biopreservation be used in food and dairy industries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of genetic diversity of okra accessions [*Abelmoschus esculentus* (L. Moench)] cultivated in Burkina Faso using microsatellite markers

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Okra is a traditional vegetable grown throughout Burkina Faso. Despite a food and non-food valorization of all parts of the plant, its genetic diversity is still little known. Thus, 50 accessions of okra from Burkina Faso were characterized using 19 microsatellite markers in order to determine the level and structure of genetic diversity. The results reveal a total of 34 alleles including 3 rare alleles and a number of 2.58 effective alleles. A polymorphic information content (PIC) value between 0.11 and 0.86 and markers polymorphism rate of 42.10% were also obtained. Mean expected heterozygosity and Shannon diversity index were 0.46 and 0.77, respectively. In addition, a structuring of the 50 accessions in three genetic groups with indices of very similar accessions of 88 to 95% between climatic zones and 83 to 95% between ethnic groups were observed. The diversity obtained could be exploited in the program of selection and varietal improvement of okra.

Key words: Genetic variability, simple sequence repeats (SSR) markers, varietal selection, valorization, genetic differentiation.

INTRODUCTION

Okra [*Abelmoschus esculentus* (L. Moench)] is a fruit vegetable of the Malvaceae family. It is cultivated all over the world but especially in Africa and Asia (Koechlin, 1989). In Burkina Faso, okra is one of the main vegetables used in the preparation of sauces. It is especially popular for its fruits rich in trace elements, vitamins, fiber and mucilage (Hamon et al., 1997 ; Marius et al., 1997). Despite its potential, okra has long been neglected by government policies and research. There are practically no improved local varieties of okra in

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Table 1. Characteristics of 19 markers (Schafleitner et al., 2013).

Marker	Sequence 5'- 3'	Sequence 3'- 5'	Repeated pattern
SSRs AVRDC-okra **1**	ATGGAGTGATTTTTGTGGAG	GACCCGAACTCACGTTACTA	(AAG)13
SSRs AVRDC-okra 8	TGCTGTGGAAGGTTTTTACT	ATGACGAAAGTGGTGAAAAG	(AAG)8
SSRs AVRDC-okra 9	ACCTTGAACACCAGGTACAG	TTGCTCTTATGAAGCAGTGA	(AAT)12
SSRs AVRDC-okra 17	ACGAGAGTGAAGTGGAACTG	CTCCTCTTTCCTTTTTCCAT	(AGA)7
SSRs AVRDC-okra 21	TCATGTCTTTCCACTCAACA	CCAAACAAAATATGCCTCTC	(AGA)9
SSRs AVRDC-okra 28	CCTCTTCATCCATCTTTTCA	GGAAGATGCTGTGAAGGTAG	(ATT)8
SSRs AVRDC-okra 39	TGAGGTGATGATGTGAGAGA	TTGTAGATGAGGTTTGAACG	(AG)16
SSRs AVRDC-okra 52	AACACATCCTCATCCTCATC	ACCGGAAGCTATTTACATGA	(CAT)8-(TCA)9
SSRs AVRDC-okra 54	CGAAAAGGAAACTCAACAAC	TGAACCTTATTTTCCTCGTG	(GAA)10
SSRs AVRDC-okra 56	GGCAACTTCGTAATTTCCTA	TGAGTAAAAGTGGGGTCTGT	(GAA)44
SSRs AVRDC-okra 57	CGAGGAGACCATGGAAGAAG	ATGAGGAGGACGAGCAAGAA	(GAA)9-GAG)7
SSRs AVRDC-okra 63	GTGTTTGAAAGGGACTGTGT	CTTCATCAAAACCATGCAG	(TCT)12
SSRs AVRDC-okra 64	AAGGAGGAGAAAGAGAAGGA	ATTTACTTGAGCAGCAGCAG	(TCT)22
SSRs AVRDC-okra 66	CACCAGAATTTCCCTTTTG	ACTGTTGTTTGGCTTATGCT	(TTC)12-(TTC)13
SSRs AVRDC-okra 70	GTAGCTGAACCCTTTGCTTA	CTATCATGGCGGATTCTTTA	(TC)11
SSRs AVRDC-okra 77	CTGTTTGTTCGTCGTAATCA	AAAGTTTCTTCCTTTCCACC	(GAAATA)4-(GAAACA)7
SSRs AVRDC-okra 78	CTCCGACAATTCAAGAAAAG	CACCCAATCAAGCTATGTTA	(TAT)11-(TATTGT)4-(TATCGT)4
SSRs AVRDC-okra 86	ATGCAAACAAGCTAGTGGAT	ATTCTCTTCAGGGTTTCCTC	(AGC)8
SSRs AVRDC-okra 89	TTTGAGTTCTTTCGTCCACT	GTATTTGGACATGGCGTTAT	(AGC)8

Burkina Faso (Balma et al., 2003 ; Sawadogo et al., 2009; Jiro et al., 2011). The genetic diversity of okra remains poorly known (Hamon, 1988). Evaluations of okra diversity performed are essentially based on phenotypic traits (Ariyo, 1993; Martinello et al., 2001; Akotkar et al., 2010; Bello et al., 2017). However, prior knowledge of the genetic diversity of a crop is essential for a better valuation of the species.

The present study on the genetic diversity of okra using specific microsatellite markers was conducted to better knowledge of the genetic diversity of okra cultivated in Burkina Faso. The objective was to determine the level and structuring of genetic diversity in order to contribute to a better management of the okra genetic resources and to establish an improvement program.

MATERIALS AND METHODS

Plant

Fifty okra accessions were characterized. These accessions were collected in the three climatic zones (Sahelian, Soudanese and Sudano-Sahelian) of Burkina Faso within four ethnic groups (Bissa, Bobo, Bwaba, and Mossi Gourounsi).

Molecular markers

Nineteen specific microsatellite markers (Table 1) of *A. esculentus*, developed by Asian Vegetable Research and Development Center (AVRDC) nowadays called Word Vegetable Center were used. They are polymorphic, codominant, and neutral markers with a high polymorphic information content (PIC) (Schafleitner et al., 2013).

Extraction of DNA with the FTA card method

Extraction of the total DNA of the 50 accessions was performed using FTA technology on young leaves of about 10 days. These are the first three leaves of the same plant that were picked, then crushed on an FTA map using a mortar and a parafilm. These cards were dried at room temperature and stored in a desiccator in the laboratory. For the recovery of the DNA, a disk 1 mm in diameter of the card prints was taken using a punch (Haris). Each disc was washed with Ethanol (70°) and incubated with TE 1X (Tris EDTA) according to the following steps: (i) two successive washes with 200 µl of ethanol 70° per disc, for 5 min each time to rid the samples of chlorophyll, leaf and cell debris and other impurities; (ii) two successive incubations with 200 µl of TE (Tris EDTA) per disc for 5 min each time during which the DNA molecule is solubilized.

The disk is then dried at ambient temperature and then directly transferred to the polymerase chain reaction (PCR) tube for amplification.

PCR amplification and revelation

The PCR amplification was carried out with an Eppendorf brand thermocycler. During the different reactions, each tube contained 1 µl of each microsatellite primer, 5 µl of premix PCR (1 µl of Taq polymerase, 250 µM of the different dNTPs, 10 mM of KCl, and 1.5 mM of $MgCl_2$), 18 µl of ultra-pure water and finally the disk from the FTA card and carrying the DNA to amplify.

The PCR program used consisted of an initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation at 94°C for 30 s, hybridization at 55°C in 45 s and a step of final extension at 72°C for 5 min. The PCR products were stored at 4°C after each amplification. The amplified products were revealed by 3% agarose gel electrophoresis in the presence of 5% Ethydium Bromide used as a fluorescent developer under ultraviolet light at a voltage of 100 V (1h). The deposits were made in the presence of a molecular weight marker consisting of two microsatellites of different sizes

Table 2. Genetic diversity parameters.

Primer	A	Ae	I	He	PIC	A^r
AVRDC-Okra 1	1	1.22	0.33	0.18	0.19	0
AVRDC-Okra 8	1	1.32	0.44	0.25	0.26	0
AVRDC-Okra 56	3	5.19	1.81	0.88	0.70	0
AVRDC-Okra 28	2	2.97	0.83	0.56	0.46	0
AVRDC-Okra 52	3	5.05	1.66	0.97	0.53	0
AVRDC-Okra 63	3	3.97	1.13	0.69	0.54	0
AVRDC-Okra 64	5	6.44	1.66	0.99	0.86	2
AVRDC-Okra 70	2	3.03	0.97	0.63	0.54	0
AVRDC-Okra 77	2	2.80	0.78	0.49	0.75	1
AVRDC-Okra 9	3	3.94	0.91	0.56	0.85	0
AVRDC-Okra 17	1	1.42	0.47	0.30	0.32	0
AVRDC-Okra 21	1	1.17	0.28	0.15	0.15	0
AVRDC-Okra 54	1	1.68	0.59	0.40	0.48	0
AVRDC-Okra 57	1	1.13	0.23	0.11	0.11	0
AVRDC-Okra 66	1	1.99	0.69	0.50	0.78	0
AVRDC-Okra 78	1	1.22	0.33	0.18	0.19	0
AVRDC-Okra 86	1	1.77	0.63	0.44	0.53	0
AVRDC-Okra 89	1	1.17	0.28	0.15	0.15	0
AVRDC-Okra 39	1	1.63	0.57	0.39	0.45	0
Mean	1.79	2.58	0.77	0.46	0.47	-
Standard deviation	1.13	1.62	0.49	0.28	0.25	-

A, Number of alleles/marker; Ae, number of effective alleles; I, Shannon diversity index; He, average expected heterozygosity; PIC, polymorphism information content; A^r, number of rare alleles.

ranging from 25 to 100 bp. A Canon PowerShot A620, 7.1 megapixel camera was used to photograph the migration gel.

Statistical analysis of molecular data

From the bands revealed by the markers, a binary coding 1 or 0 was made, respectively in case of presence and absence of bands. The GenALEx version 6.501 software (Nistelberg et al., 2013) was used to estimate genetic parameters such as total number of alleles (A^t), allelic richness or number of alleles per marker (A), effective number of alleles (A_e) [(A_e = 1/(1-h) = 1/Σpi2, where pi is the frequency of the allele, i is the locus under consideration and h = heterozygosity], number of rare alleles (A^r), polymorphic information content (PIC) (Smith et al., 2000), and polymorphism of markers (P). The Shannon genetic diversity index (I) (I = -1 [(p × ln (p) + q × ln (q))]) and the expected mean heterozygosity (He) or Nei gene diversity index (D) (He = 1/N [n/n-1(1-Σpi2)], where N is the number of loci, n is the number of accessions, pi is the frequency of the allele i, at the relevant locus were also performed with the same software. The genetic diversity structure was carried out using DARwin V5.0 software (Perrier et al., 2006) from the dissimilarity matrix of accessions according to the "simple matching" procedure according to the Neighbor-Joining method. The genetic differentiation between genetic groups based on Fst (Weir and Cockerham, 1984) and minimum distance of Nei between pairs of genetic groups were estimated using too FSTAT software V2.9.3.2.

RESULTS

Level of diversity of markers

All 19 markers allowed amplification of the individuals tested, but only eight revealed more than one allele (Table 2). A total of 34 alleles including three rare alleles with a size between 25 and 500 bp (Figure 1) were observed. The mean number of effective alleles and the average expected heterozygosity were 2.58 and 0.46, respectively.

The AVRDC-Okra 64 marker (Figure 2) showed the highest number of alleles (5 alleles). The Shannon genetic diversity index (I) ranged from 0.23 to 0.97 with an average of 0.77. The Polymorphic Information Content ranged from 0.11 for the AVRDC-Okra 57 primer to 0.86 for the AVRDC-Okra 64 primer with an average of 0.47.

Organization of the genetic diversity of okra accessions

Structure of genetic diversity

The analysis of genetic diversity using the "Neighbor-Joining" algorithm divided the 50 accessions of okra into three genetic groups (Figure 3). Indeed, genetic groups I and III consisted respectively of 62 and 30% of the accessions of the collection coming from the three climatic zones while the genetic group II contained 8% of the accessions coming from the Sudano-Sahelian.

Genetic group I contains all alleles (34) observed including the three rare alleles (Table 3). Genetic group II has the lowest genetic indices while genetic group III has

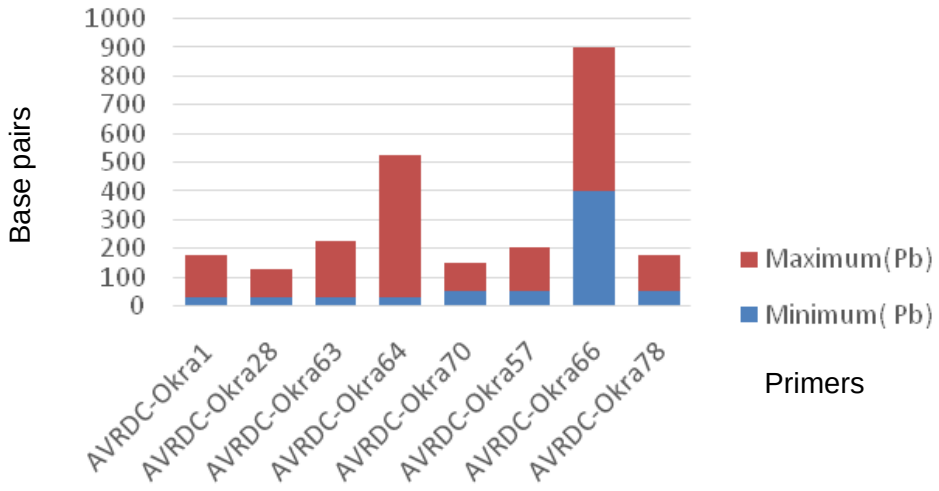

Figure 1. Microsatellite markers bands size.

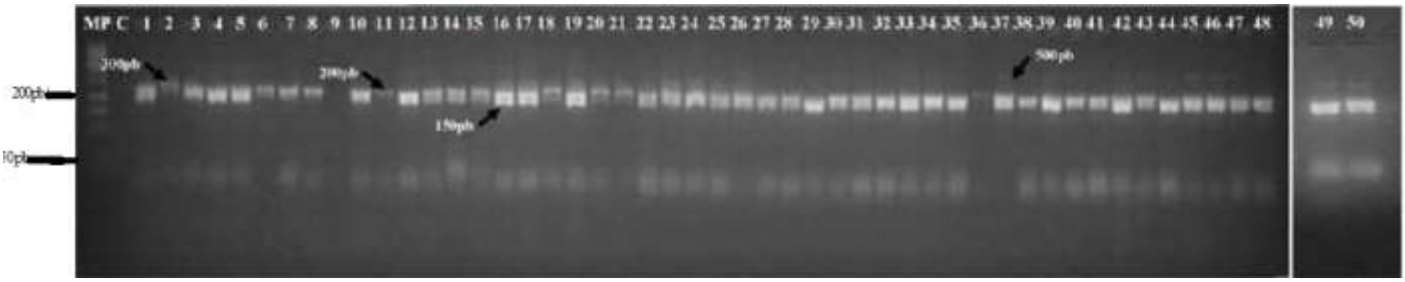

Figure 2. Migration profile of AVRDC-Okra marker 64.

the highest values of effective alleles number (2.94), Shannon genetic diversity index (0.93) and expected heterozygosity (0.66).

Differentiation between genetic groups

Genetic differentiation (Fst) showed a significant difference only between groups I and III (Table 3). The highest Nei minimal genetic distance (0.087) was observed between genetic groups I and II (Table 4).

The structure of genetic diversity is very weakly influenced by climatic zone and ethnic group factors. Indeed, low genetic differentiation (Fst) and strong indices of similarity of accessions were observed between ethnic groups (Table 5) and between climatic zones (Table 6)

DISCUSSION

The 19 primers used showed a genetic diversity of okra accessions. The average PIC of 0.47 shows that these markers are all informative because according to Smith et al. (2000), the PIC of simple sequence repeats (SSR) marker is an important estimate of the discriminating power of this marker. The value of PIC ranging from 0.11 to 0.86 confirms the reliability of these SSR markers. Indeed, Schafleitner et al. (2013) found a PIC value between 0.43 and 0.84 on okra with the same SSRs. Kumar et al. (2017) also reported a PIC ranging from 0.11 to 0.80 for 30 polymorphic SSRs used on okra genotypes. The AVRDC-Okra marker 64 and AVRDC-Okra marker 9 with respective PIC values of 0.86 and 0.85 are the most polymorphic.

The values of the effective alleles number (Ae = 2.58) and average number of alleles per marker (A = 1.78) indicate that this genetic diversity would be relatively small. These results confirm those of Hamon (1988) who showed a low genetic diversity of cultivated species of okra from West African origins. In fact, okra producers in Burkina preferentially select short-cycle and green-fruit okra cultivars (Ouédraogo et al., 2016), which could explain the low genetic diversity of the 50 accessions. However, greater diversity has been reported by Sawadogo et al. (2009) who achieved a higher mean

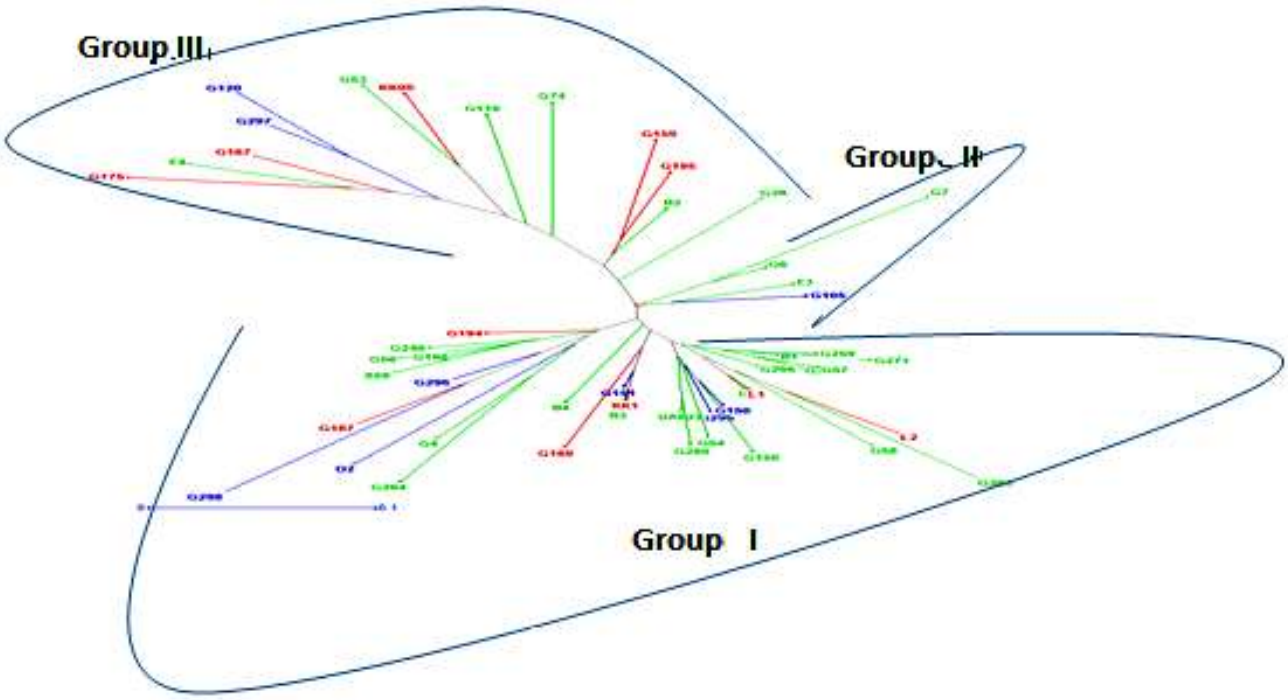

Figure 3. Radial representation of the dendrogram of the 50 accessions of okra cultivated in Burkina Faso constructed from the dissimilarity matrix according to the Neighbor-Joining method.

Table 3. Characteristics of genetic group diversity.

Genetic group	Number of accessions	A^t	A	A_e	I	He	P(%)	A^r
I	31	34	1.78	2.83	0.89	0.61	100	3
II	4	27	1.42	2.39	0.47	0.36	44.12	0
III	15	33	1.73	2.94	0.93	0.66	94.12	0

A^t , Total number of alleles; A, number of alleles per marker, Ae, number of effective alleles; I, Shannon genetic diversity index; He, average expected heterozygosity; P, percentage of polymorphism; A^r, number of rare alleles; AP, number of distinct or private alleles.

Table 4. Genetic differentiation (FST) and Nei genetic distance.

Genetic group	I	II	III
I	0	0.087	0.027
II	0.1737^{ns}	0	0.087
III	0.236*	0.0197^{ns}	0

FST (below the diagonal), genetic distances of Nei (above the diagonal).

Table 5. Matrix Fst (below the diagonal) and index of average genetic similarities of accessions between ethnic groups (above the diagonal).

Ethnic group ethnique	Bissa	Bobo	Bwaba	Mossi	Gourounsi
Bissa	0	0.95**	0.86**	0.94**	0.88**
Bobo	$0 .082^{ns}$	0	0.90	0.94**	0.89**
Bwaba	-0.063^{ns}	0.006^{ns}	0	0.92**	0.83**
Mossi	0.027^{ns}	-0.027^{ns}	-0.026^{ns}	0	0.94*
Gourounsi	0.036^{ns}	0.013^{ns}	0.045^{ns}	0.066^{ns}	0

Table 6. Genetic differentiation indices (FST) (Below Diagonal) and indices of mean genetic similarity of accessions between climatic zones (Above the diagonal).

Climatic zone	Sahelian	Soudanese	Sudano-Sahelian
Sahelian	0	0.89**	0.93**
Soudanese	-0.0099^{ns}	0	0.96**
Sudano-Sahelian	0.0105^{ns}	0.0277^{ns}	0

**Very significant.

number of allele per locus (A = 4.8) over 20 traditional okra varieties from Burkina Faso. This important diversity revealed by this author can be explained by the origin of these accessions which come from a selection of phenotypic diversity. Many previous studies (Schafleitner et al., 2013; Nasser, 2014; Kumar et al., 2017) report an influence of the origin of cultivars on the genetic diversity of okra. The high similarity indices of okra accessions show that climatic zone and ethnic group factors do not significantly influence their genetic variability. The genetic differentiation of okra subpopulations therefore depends on the genotype of accessions. These results are similar to those of Kiébré (2016) who found that the "genetic group" is the only factor that significantly influences the genetic differentiation of *Cleome gynandra* subpopulations.

The low genetic differentiation of accessions observed between climatic zones and between ethnic groups could be explained by the fact that producers use the same seeds. Nana (2010) reported also a low ecotypic differentiation of okra cultivated in Burkina Faso. In fact, the farmers exchange seeds with each other and also take with them the seeds during the exodus.

Genetic distance is a function of the level of genetic diversity in the population. The three genetic groups and the low genetic distance of Nei observed between them suggest a low genetic diversity of the okra collection. However, Sawadogo et al. (2009) obtained five genetic groups of okra ecotypes with a higher Nei genetic distance (0.51 to 0.77). The high values of the polymorphism rate of markers (91%), the Shannon genetic diversity index (0.77) and the presence of the three rare alleles explain the relatively higher level of diversity in the Sudano-Sahelian zone of Burkina. This zone, which is the largest of the three climatic zones, is characterized by ecological diversity that favors the differentiation of okra ecotypes. On the other hand, on *Cleome gynandra*, Kiébré (2016) observed a higher diversity in the Sudanian zone of Burkina as compared to the other two zones.The presence of rare alleles only in the Sudano-Sahelian zone could reflect an interaction between genotypes of cultivated accessions and the environment. According to Akinyele et al. (2011), the environment alone can affect the genotype of okra by stimulating permanent changes in the genome of the varieties. The rare and private alleles could be a potential genotype for the production and resistance to diseases of okra. Sawadogo (2015) reported that rare alleles would be of great interest if they are only related to some particular genotypes.

Conclusion

Molecular markers revealed a genetic diversity of okra accessions cultivated in Burkina Faso. The 19 SSRs markers developed by AVRDC specifically for the gombo were all discriminating and informative. This study showed also a moderate value for expected heterozygotie, a high value of Shanon' indice and an organization of the 50 okra' accessions in three groups. The three genetic groups suggest the existence of several genotypes of cultivated okra accessions. Moreover, the genetic diversity of okra is more influenced by genotype than climatic zone or ethnic group factors. The results of this study provide a basis for implementing an *in situ* and *ex situ* conservation program and improving the genetic resources of okra cultivars. In the perspective of a better valorization of okra in Burkina Faso, a biochemical characterization and organoleptic tests of accessions is necessary. Also, the characterization of these accessions with SNP markers could allow to better appreciate the level of genetic diversity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibacterial effect of *Thymus* sp. and *Boswellia* sp. extracts on *Streptococcus pneumoniae* and *Klebsiella pneumoniae* isolates

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The antimicrobial activity of essential oils of *Boswellia* and *thyme* (*Boswellia* sp., and *Thyme* sp.) was evaluated against 20 clinical isolates of *Streptococcus pneumoniae* and 5 isolates of *Klebsiella pneumoniae*. Essential oils were prepared using methanol and water (1:1) with HPLC technique. Antimicrobial activity and minimum inhibitory concentration (MIC) were measured using disk diffusion method against 20 isolates of *S. pneumoniae* and 5 isolates of *K. pneumoniae* isolated from different patients. Flavonoids and phenolic compounds are the main constituents of *Boswellia* and thyme which may have the antimicrobial activity. *Boswellia* extract was more efficient than thyme extracts; 60% of *S. pneumoniae* isolates and one *K. pneumoniae* isolate were sensitive to *Boswellia* extract, 30% of *S. pneumoniae* isolates were sensitive to thyme extract, and no effect on *K. pneumoniae* clinical isolates was observed. Inhibition zones ranged from 1-12 mm with thyme extract, while *Boswellia* extracts showed 2 to 30 mm diameters of inhibition zone. This study is significant due to the widespread problem of microbial drug resistance and the need for natural antibiotic to fight diseases.

Key words: *Thymus* sp., *Boswellia* sp., antibacterial effect, *Pneumonia.*

INTRODUCTION

Based on the World Health Organization (WHO), pneumonia disease is considered as a common serious illness that threatens health, especially in developing countries. According to WHO, 15% of the total death of children is due to pneumonia (WHO, 2015). Pneumonia disease caused by *Klebsiella pneumoniae* is the major cause of infants and patients injury in hospitals intensive care units (WHO, 2014).

Medicinal plants and their essential oils always had choice of use for different purposes such as treatment of some diseases all over the world; therefore it has high economic value (Joshi et al., 2011). *Boswellia* is one of the most effective medicinal plants. Genus *Boswellia,* belonging to Burseraceae family includes both trees and shrubs; a family that contains 20 species widespread in the dry regions (Hussain et al., 2013). Frankincense is the oligomer resin that is extracted from tapping in the inner bark of the trees. *Boswellia* essential oils and

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oligomer have medicinal properties such as immune enhancing, antibacterial, anti-inflammatory, wound healing and anticancer properties (Shareef, 2011). Gum is used traditional as a diuretic and for the treatment of diarrhea, dysentery, cardiac disease, cough, hemorrhage, dyspnea, polyuria, urinary troubles, piles, ulcers and burns (Ismail et al., 2014). Most of the research on *Boswellia* focused on the effectiveness of its extracts on memory regions of the brain (Farshchi et al., 2010).

Thyme sp. is also one of the medicinal plants. Genus *Thymus* belonging to the family Lamiaceae consists of 928 species, native to Europe, and grows in the Mediterranean basin and northern Europe, as well as other parts of the world such as Asia, South America, and Australia. Thyme is also used in traditional medicine for the treatment of coughs, upper respiratory infections, acute and chronic bronchitis, whooping cough; also in cases of respiratory system disorders due to its antitussive, antioxidant, anti-inflammatory, and antimicrobial properties. Thyme is employed for external use as a mouth wash in gargles to treat laryngitis. Recent data suggest that the essential oil of thyme is one of the best choices to fight inflammations, with its main components being carvacrol and thymol (Nabavi et al., 2015). Thymol and carvacrol are phenolic compounds with strong antifungal and therapeutic properties (Lakis et al., 2012). Thymol compound reduces bacterial resistance to some antibiotics such as penicillin (Nabavi et al., 2015); however, many studies indicated the antimicrobial activity of *Boswellia* and thyme extracts, especially against *K. pneumonia* (Javed et al., 2015). Ismail et al. (2014) found that *K. pneumoniae* was sensitive against high concentrations of frankincense extracts of *Boswellia serrata* whereas investigations on *Cinnamon* and frankincense essential oil for their antibacterial activity against pathogenic bacterial isolates, showed that *K. pneumoniae* was less sensitive to cinnamon giving negative results with frankincense; however, frankincense essential oil showed good antimicrobial activity against the other tested bacteria (Shareef, 2011). Fournomiti et al. (2015) investigated the antimicrobial activity of some medicinal plant extracts: oregano (*Origanum vulgare*), sage (*Salvia officinalis*), and thyme (*Thymus vulgaris*) against three clinical bacteria isolates (*Escherichia coli, Klebsiella oxytoca, K. pneumoniae*), and the results showed that *K. pneumoniae* was the second most sensitive strain to thyme and oregano essential oils were the most efficient. Sienkiewicz et al. (2012) screened the antimicrobial activity of thyme essential oil against clinical multidrug resistant strains of *Staphylococcus, Enterococcus, Escherichia* and *Pseudomonas*, with the results indicating that thyme essential oil strongly inhibited the growth of the tested clinical strains. Recently, Benmoussa et al. (2016) used four methods for extraction of the volatile compounds from Tunisian *T. vulgaris* leaves. The results showed that *T. vulgaris* essential oils had antibacterial effect against multi-drug resistant bacteria, and solvent-free microwave extraction method was efficient giving high antimicrobial activity which was attributed to 17 volatiles compounds, and high monoterpense hydrocarbons content. Thus, it was postulated that solvent-free microwave extraction (SFME) is the best alternative method of essential oils extraction. Moreover, Sakkas et al. (2016) studied the efficacy of five essential oils (Basil, chamomile blue, origanum, thyme, and tea tree oil) on 3 g negative and positive clinical isolates (*Acinetobacter baumannii, E. coli, K. pneumoniae* and *P. aeruginosa*) using broth macrodilution method; antibacterial activity against this multi-drug resistant isolates was poor.

The aim of this study was to investigate the antibacterial activity of *Boswellia* sp. and *Thyme* sp. extracts on *Pneumonia* clinical isolates.

MATERIALS AND METHODS

Sample collection and essential oils extraction

Thyme leaves and *Boswellia* gums were collected from local markets in Dammam, Saudi Arabia, dried and ground into powder. 30 g of ground leaves or powdered gum was dissolved in 100 ml of 96% ethanol; similarly, 30 g of ground leaves or powdered gum was extracted in 100 ml of distilled water, and the last group of extraction was prepared in 100 ml of 1:1 water and 96% ethanol mixture. Extracts were placed three days at room temperature. Thereafter, oils were extracted by rotary evaporation at 60°C for 24 h for ethanol evaporation. The extracts were filtered using disposable membrane bacterial filters to get sterile extracts.

Bacterial strains

Standard strains of *Pneumoniae* were used; *Streptococcus pneumoniae* ATCC and *K. pneumoniae* ATCC. In addition, 20 clinical isolates of *S. pneumoniae* and 5 of *K. pneumoniae* were isolated from different patient's materials. Standard strains of *E. coli* ATCC, *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains were obtained from Laboratory of Microbiology of King Fahad Hospital in Khobar City.

Screening of antibacterial effects

This study used well diffusion assay technique (Chung et al., 1998); and 0.1 ml of overnight bacterial culture strain was inoculated into trypticose soy broth. Overnight culture inoculum was spread over blood agar by using L-shape spreader and sterile swaps. Holes were made by using sterile 5 mm diameter cork porer; 25 µl of different extracts were added in each hole, and was inoculated with a loopful of the test organism previously diluted to 0.5 McFarland turbidity standards seeded on the medium. Plates were then incubated at 37°C for 24 h; sterile water and solvents were used as control along with a combination of *thyme* and *Boswellia* (1:1), and diameter of inhibition zone was estimated in mm.

Minimum inhibitory concentration assay

Bacterial isolates showed susceptibility to the extracts, their

Table 1. Screening for the best solvent of essential oils using inhibition zone (mm) method on *pneumonia* standard isolates (ATCC).

Solvent extracts	Ethanol (96%)	H_2O	Ethanol : H_2O (1:1)
Thymus sp.	4 (*S. pneumoniae*) 3.2 (*K. pneumoniae*)	3 (*S. pneumoniae*) 2 (*K. pneumoniae*)	15 (*S. pneumoniae*) 7 (*K. pneumoniae*)
Boswellia sp.	*9 (S. pneumoniae)* 5 *(K. pneumoniae*)	*34 (S. pneumoniae)* *R (*K. pneumoniae*)	*30 (S. pneumoniae)* 8 (*K. pneumoniae*)

*R= Resistant.

antibacterial response was determined using extracts at dilutions 0.25, 0.5, 0.75 µg/ml and sterile solvent as control treatment. Minimum inhibitory concentration (MIC) was determined as described by Sienkiewicz et al. (2012), and was read in µ/ml after overnight incubation at 37°C. All experiments were made in replicate.

Chemical analysis

Chemical analysis of thyme and frankincense was performed using (HPLC) chromatography and Agilent device (1100HPLC). Phenolic compounds were estimated at 280 nm wavelength, and flavonoid analysis carried out using the following condition: degazer auto-sampler, qauternery pumb and column cabin at 35ºC; the fragmentation column was zorbox ODS of 5 µm 4.6 × 250 mm dimensions. The flow rate of the mobile phase was 1 ml/min as described by Pascale et al. (1999) and Pirjoet et al. (2000).

RESULTS AND DISCUSSION

Results presented in Table 1 indicate that *Thymus* and *Boswellia* extracts from ethanol water mixture (1:1) gave the highest antibacterial effect against the tested bacteria (*S. pneumoniae* and *K. pneumoniae*) as measured by diameter of inhibition zone. These results are not in accordance with the findings of Hasson et al. (2011), in which methanolic extracts of *Boswellia* gave the highest antibacterial activity than water extracts. However, Costal et al. (2012) reported that the highest extraction yield for *Thymus lotocephalus* was achieved with water than water ethanol mixture. Zielinski and Kozlowsk (2000) proposed that higher extraction yield were achieved with water compared to water/ethanol mixture and ethanol alone. This can be explained on the basis that proteins and carbohydrates are more soluble in water than in ethanol and aqueous mixtures of ethanol.

Table 2 shows the results of antibacterial activity of *Boswellia* and *thymus* extracts on *pneumoniae* isolates and standard ATCC isolates, using well diffusion and their MIC. *S. pneumoniae* (ATCC), was used as control isolates, and was more sensitive to *Boswellia* extracts than *K. pneumoniae* (ATCC) reference strain. *K. pneumoniae* (ATCC) gave 8 and 7 mm zones of inhibition with *Boswellia* and thyme extracts, respectively. Results indicate that the inhibition zone ranged from 2 to 30 mm; 12 *S. pneumoniae* isolates were sensitive to *Boswellia* extracts, which equals 60% of the tested isolates. MIC of four *S. pneumoniae* isolates was 0.75 µg/ml, and five isolates had 0.5 µg/ml MIC. 30% from the tested bacterial isolates were resistant to *Boswellia* extract. However, six isolates of *S. pneumoniae* (represent 30% of isolates) were sensitive to thymus extracts and the rest were resistant. Inhibition zones of *S. pneumonia* isolates ranges from 1 to 12 mm, and MIC values of the sensitive isolates were 0.25 µg/ml for 3 isolates and 0.75 µg/ml for 2 isolates, with one isolate having 0.5 µg/ml MIC. Results of Table 2 also show that six *S. pneumoniae* isolates (30% of isolates) were sensitive to *Boswellia* and thyme (1:1) mixed extract, and gave inhibition zones ranging from 2 to 7 mm; whereas isolates numbers 4 and 8 were more sensitive to *Boswellia* extracts, and the rest of tested isolates were resistant. No effect of thymus extracts on any of *K. pneumoniae* clinical isolates was observed. Results indicate that *Boswellia* extract has higher antimicrobial activity than thyme extract.

Al-Saidi et al. (2012) reported on the antibacterial activity of omanilubans (oleo-gum resins of *Boswellia*) essential oils against both Gram-positive and Gram negative. Results of this investigation indicated that *Boswellia* volatile oils were more active slightly against Gram positive than Gram negative bacteria. These results are in accordance with those of other investigators (Al-Saidi et al., 2012). The resistance of some of the Gram-negative bacteria could be due to the more hydrophilic outer membrane containing lipopolysaccharide (LPS), and the outer membrane acting as a penetration barrier for macromolecules and hydrophobic compounds (Nikaido, 1996). Raja et al. (2011) reported that the lack of antibacterial activity of active compounds of *Boswellia* sp. against Gram-negative bacteria might be attributed due to the presence of lipophilic outer membrane in Gram-negative, which composed primarily of lipopolysaccharide molecules and forms hydrophilic permeability barrier providing protection against the effects of highly hydrophobic compounds. This is probably the explanation of the resistance of Gram-negative bacteria to lipophilic acetyl- keto- β-boswellia acid (AKBA). Sienkiewicz et al. (2012) concluded that thyme oil has strong antibacterial

Table 2. Antibacterial effect of *Boswellia* and *Thymus* extracts on *pneumoniae* isolates by well diffusion and MIC method.

Pneumonia isolates	*Boswellia* sp.		*Thymus* sp.		B:T
	I. Z (mm)	MIC (µg/ml)	I.Z (mm)	MIC (µg/ml)	I:Z
S.p.(ATCC)	30	0.25	15	0.75	R
K.p.(ATCC)	8	0.75	7	0.75	R
S.P.1	R	-	R	-	R
S.P.2	R	-	R	-	R
S.P.3	4	0.75	R	-	R
S.P.4	8	0.25	R	-	16
S.P.5	12	0.50	R	-	R
S.P.6	2	0.50	R	-	R
S.P.7	R	-	4	0.25	3
S.P.8	22	0.25	3	0.25	17
S.P.9	19	0.25	3	0.25	2
S.P.10	R	-	1	0.50	3
S.P.11	4	0.75	3	0.75	R
S.P.12	R	-	R	-	R
S.P.13	11	0.50	R	-	R
S.P.14	1	0.75	R	-	R
S.P.15	4	0.50	12	0.75	R
S.P.16	5	0.50	R	-	R
S.P.17	R	-	R	-	R
S.P.18	7	0.25	R	-	2
S.P.19	R	-	R	-	R
S.P.20	R	-	R	-	R
K.p.1	R	-	R	-	R
K.p.2	R	-	R	-	R
K.p.3	12	0.75	R	-	R
K.p.4	R	-	R	-	R
K.p.5	R	-	R	-	R

I:Z, Inhibition zone by mm; S.P. = *Streptococcus pneumonia*; K.P. = *Klebseilla pneumonia;* MIC = Minimum inhibitory concentration (µg/ml); B:T = *Boswellia* and *Thyme* mixed extract.

properties against many pathogenic bacteria, with this activity due to the high content of phenolic compounds with antibacterial properties, such as thymol and carvacol, which constitute more than 40% of the oil ingredients. Similarly, Al-bayati (2008) reported that combinations of two essential oils of *T. vulgaris* and *Pimpinella anisum* possessed high inhibitory activities on *K. pneumoniae* and other tested bacteria through synergistic action. These results are different from our results, since most *K. pneumoniae* and *S. pneumoniae* strains were resistant against mixed extracts of Boswell and thyme. This could be due to the limited number of tested bacterial isolates. Similarly, Javed et al. (2015) studied the antibacterial activity of *Boswellia sacra* and *Nigella sativa* essential oils and found that *B. sacra* essential oil gave the lowest zone of inhibition against *K. pneumoniae* (13 mm / 2.5 µg/ml). These results agree with results of this research, since the application of *Boswellia* extract recorded 12 mm inhibition zones against *K. pneumoniae*. Application of mixture of two essential oils of *B. sacra* and *N. sativa* gave high zone of inhibition (23 mm) against *K. pneumoniae*; however, the results here indicate that mixed extracts of *Boswellia* and thyme gave 17 mm zone of inhibition against *S. pneumoniae* (isolate number 8). Moreover, no susceptibility was reported against *K. pneumoniae* clinical isolates. Similarly, Al-bayati et al. (2008) reported that *K. pneumoniae* was resistant to methanol extract of *T. vulgaris* and *P. anisum.* It has frequently been reported that Gram positive bacteria were more sensitive to the essential oil than Gram negative bacteria, which could be due to the presence of hydrophilic outer membrane that blocks the penetration of hydrophobic essential oil into target cell membrane. The antibacterial activities of methanol extract of *T. vulgaris* essential oil could be associated with the presence of phenolic compounds like carvacrol, thymol, γ-terpinene and p-cymene, which have antibacterial properties (Essawi and Sour, 2000).

Table 3. Thyme and frankincense flavonoids concentrations (ppm) using HPLC.

Flavonoids	Flavonoids (ppm)	
	Thyme	***Boswellia***
Luteolin	--	4.188
Narengin	975.92	7.55
Rutin	118.2	3.3873
Hisperidin	1418.6	10.847
Rosmarinic	604.07	5.3906
Quercetrin	92.229	1.2138
Quercetin	53.745	5.7968
Hispertin	38.467	12.058
Kampferol	175.63	1.8054
Apegnin	101.09	1.5162
7-OH-ydroxyflavone	115.63	1.8481
Total	3693.581	55.6012

Table 4. Thyme and frankincense phenolic compounds concentrations (ppm) using HPLC.

Phenolic compounds	Phenolic compounds (ppm)	
	Thyme	***Boswellia***
Gallic	2.90	1.09
Pyrogallol	177.87	33.09
Amino-benzoic-4	6.30	0.51
OH-Tyrosol-3	3.74	1.12
Protocatchuic	5.62	2.98
Catechein	84.37	9.05
Chlorogenic	36.10	3.16
Catechol	105.50	10.22
Epicatechein	24.31	6.33
Caffeine	55.12	2.87
P-OH-benzoic	70.33	4.67
Caffeic	71.49	4.56
Vanillic	84.61	8.21
P-coumaric	17.76	6.51
Ferulie	70.72	3.02
Iso-ferulic	11o.56	4.58
Reversetrol	15.65	2.68
e-vanillic	877.47	147.65
Ellagic	164.71	49.91
Alpha-coumaric	56.93	16.82
Benzoic	254.32	102.94
methoxy--3,4,5 cinnamic	16.25	14.26
Coumarin	34.83	6.04
Salycillic	61.45	21.42
Cinnamic	24.80	4.04
Thymol	52.87	41.47
Total	2485.58	509.2

Similarly, Nazzaro et al. (2013) reported that rod-shape bacteria are generally sensitive for essential oils with antibacterial properties, but in this study, most *K. pneumoniae* isolates which are rod-shaped showed resistant response against the tested extracts. Many bioactive compounds such as thymol, carvacol, flavoinds, biphenyls and aliphatic phenols are found in thymus species (Nadia et al., 2013) while Sakkas et al. (2016) noted that the inhibitory effects of thyme are due to thymol, p-cymene and linalool compounds. Most of the antimicrobial activity in *Thymus* essential oil appears to be associated with phenolic compounds (thymol and carvacol); however, in some cases an elevated concentration in these components does not necessarily increase the antimicrobial properties (Rota et al., 2008). This result could explain the differential antibacterial activity of *Boswellia* and thyme extract, since thyme extract was efficient at low rate of bioactive concentration compared with thyme extract. The chemical composition of essential oil in plants of genus *Thyme* is species and chemotype dependent (Nabavi et al., 2015). This result may explain why thyme extract did not show strong antimicrobial activity despite the high rate of antibacterial properties.

Results of Tables 3 and 4 show the flavonoid and phenolic compounds concentrations in thyme and frankincense using HPLC chemical analysis methods. Results showed that phenol and flavonoid concentrations in thyme are higher than in frankincense (2485.6 and 509.2 ppm phenol content) and (3963.6 and 55.6 ppm flavonoid), respectively. Despite the higher concentrations of flavonoid and phenolic compounds in thyme than those of *Boswellia* (Tables 3 and 4), *Boswellia* showed higher antimicrobial activity than Thyme, except *K. pneumoniae* isolate No. 3 which was sensitive. Results also show that the application of aqueous and alcohol extracts of thyme and *Boswellia* gave high inhibitory action compared to aqueous extracts, which may be related to increased inhibitors concentrations. Al-Aubadi et al. (2011) reported that alcohol extracts displayed antibacterial activity because alkaloids are dissolved easily in ethyl alcohol than in water. The inhibitory effect of thyme and *Boswellia* is attributed mainly to their contents of phenolic and flavonoid contents, such as carvacol (15%) and thymol (40%) (Alves-Silva et al., 2013). The inhibitory action of these compounds against the bacteria cell depends on their concentrations and composition. The target of these compounds is degradation of the cell wall, increased membrane fluidity and inhibition of a membrane-embedded enzyme (Nazzaro et al., 2013). Phenolics compounds can penetrate into the phospholipids layer of the bacterial cell wall, bind to proteins and block their normal functions. Because of their lipophilic nature, essential oils and their compounds can influence the percentage of unsaturated fatty acids and their structure (Burt and Reinders, 2003). Tables 3 and 4 indicate that essential oils of thyme and *Boswellia* contain acidic components such as benzoic acid (254.3 ppm in thyme and 102.9 ppm in *Boswellia*), e-vanilli acid (47.9 ppm in

thyme and 147.7 ppm in *Boswellia*), allagic acid (164.7 ppm in thyme and 49.9 ppm in *Boswellia*). These acids can change membrane proteins then block their normal functions (Nazaro et al., 2013). Similarly, essential oils are rich in phenolic compounds that can affect structure and unsaturated fatty acids of bacteria cell wall (Sakkas et al., 2016). Results also show that thyme and *Boswellia* contain flavonoid compounds such as hispertin (38.5 ppm in thyme and 12.1 ppm in *Boswellia*), narengin (975.9 ppm in thyme and 7.5 ppm in *Boswellia*). Flavonoid compounds hydroxyl group bind to amino group of cell proteins, leading to formation of non-dissolved proteins, an action that consequently leads to cell death (Nazzaro et al., 2013).

Conclusion

The results of this study demonstrate the effectiveness of *Thyme* sp. and *Boswellia* sp. extracts against *pneumoniae* clinical isolates. *S. pneumoniae* showed more sensitivity to *Boswellia* extracts than *K. pneumoniae*. Thus, further studies is required for new compounds from natural sources that have antimicrobial activity against different pathogenic bacteria especially gram negative and multi drug resistant bacteria.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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