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SCREENING OF LACTIC ACID BACTERIA FROM SUDANESE FERMENTED FOODS FOR BACTERIOCIN PRODUCTION

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ABSTRACT

Forty isolates of lactic acid bacteria (LAB) were isolated from different types of fermented foods consumed in Sudan. Phenotypic tests revealed that all isolates were homofermentative LAB. Twenty-four isolates produced inhibitory substances primarily active against *Staphylococcus aureus* ATCC 2818 and *Escherichia coli* ATCC 29522. The inhibitory activity of 88% of enterococci and 58% of lactobacilli was recorded from meat isolates, whereas all activity of pediococcal isolates came from fermented milk isolates. The cell-free cultures of 18 isolates exhibiting inhibitory activity was chosen for further investigation such as sensitivity to proteolytic enzyme (pepsin), effect of heat treatment (60°C for 60min, 100°C for 20min and 121°C for 15min) and effect of pH (pH 2.0, pH 6.5, and pH 9.0). The inhibitory activity was eliminated upon treatment with pepsin. The bacteriocin-like substances lost their activity after heating at all temperatures used and at alkaline pH (9.0), whereas they were active at acidic pH (2.0). The antimicrobial activity of bacteriocin-like substances produced by the isolated LAB could prevent spoilage and/or pathogenic microorganism in Sudanese fermented food. Further study should be related with species identification of the producer strains and with the purification and characterization of these becteriocin-like substances in order to explore them in food industry.

Keywords: Antimicrobial activity, bacteriocin, dodery, lactic acid bacteria, Sudanese fermented foods

INTRODUCTION

Historically, fermentation is one of the oldest methods of food processing and preservation that has been developed by default rather than by design **(Stiles 1996)**. Through thousands of years the demands for the production and consumption of fermented foods has extremely increased and accordingly those foods occupied a substantial part of the diet worldwide. Recently, fermented foods and beverages are estimated to make up approximately 1/3 of the human diet **(van Hylckama Vlieg *et al.*, 2011)**. This explosion in the fermented foods production and consumption resulted from the increasing demand for nutritious, safe, natural, additives-free, and well-preserved foods **(Vaughan *et al.*, 1994)**. It is well established that fermentation enhances the nutritional quality of foods and contributes to food safety particularly under conditions where refrigeration or other foods processing facilities are not available **(Motarjemi 2002)** such as in arid and semi-arid rural areas in Sudan.

Lactic acid bacteria (LAB) are generally regarded as safe (GRAS), play an essential role in the majority of food fermentations and preservation, and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable, and bakery products. They contribute to the enhancement of the sensory, quality and safety features of these fermented foods **(Holzapfel & Wood, 2014)**. Their antimicrobial activity has been attributed to fermentation products such as organic acids, carbon dioxide, hydrogen peroxide, diacetyl and bacteriocins, among other, which can inhibit pathogenic and spoilage microorganisms, extending the shelf life and enhancing the safety of food products **(Piard & Desmazeaud, 1992)**. Bacteriocins are a heterogeneous group of low molecular- mass peptides, or proteins, with a bactericidal or bacteriostatic mode of action, in particular against closely related species **(De Vuyst & Vandamme, 1994)**. Since consumers are becoming increasingly health aware, the application of bacteriocins as a natural preservative in food has recently received considerable attention **(Papagianni & Anastasiadou, 2009)**.

Like other African countries, Sudan have a rich tradition in food preservation and processing technologies, and many indigenous fermented foods of animal or plant origin are still widely consumed and highly appreciated. The raw materials from which these foods are prepared include sorghum, pearl millet, dates, honey, milk, fish, meat, wild plants, marginal food crops and even skins, hooves, bones, caterpillars, locusts, frogs and cow urine **(Dirar 1993)**. The preparation and storage of fermented foods are strongly dictated by the ecology of a hostile environment of drought, desertification and recurrent food shortage **(Dirar 1993)**. However, the fermentation step is spontaneous, uncontrolled and usually with varied fermentation times, temperatures and microbiological profiles, resulting in products inconsistent in quality attributes **(Dirar 1993)**. Moreover, these fermented foods are still mainly prepared at the household level under poor sanitary conditions and marketed through informal routes. Consequently, many different processes derived by different types of microorganism could be expected. Add to this, there is a lack of knowledge about the microorganisms involved and their metabolic influence on flavour, hygienic safety and shelf life of these products. Therefore, the objective of this study was to initially identify and characterize the microorganisms involved in the indigenous fermentation of these products, and to provide the basic information for development of starter cultures with predictable functional characteristics that may lead to products with consistent safety, taste and quality, as well as improved marketability. In line with this concept, investigations on kinds of organisms dominate Sudanese fermented foods as well as the production of antimicrobial substances is claimed for future control of the fermentation process.

MATERIAL AND METHODS

Materials

Seven foods were either collected from local markets (Khartoum, Sudan) or prepared at home, representing different sources of fermented foods consumed in Sudan. These foods include; cereal-based fermented food such as sorghum dough (homemade), dairy products those were mish, fermented milk (homemade) and cheese (local markets), vegetable fermented foods such as cucumber and radish pickles (homemade), and meat products such as sausage and dodery (local markets).

Isolation and identification of lactic acid bacteria (LAB)

Ten grams of each fermented food sample were mixed with 90 mL sterile peptone water (Oxoid,Ltd., Basingstoke, the United Kingdom) and homogenized for 30 second in Lab- Blender. The samples were serially diluted and 100 µL of suitable dilutions was spread on De Man, Rogosa and Sharpe (MRS) agar plates (Oxoid, Basingstoke, Hampshire, United Kingdom). The plates were incubated for 3 days at 37 °C under anaerobic conditions. Bacterial colonies grown on the plates of the highest dilutions were individually picked up and streaked on fresh MRS agar plates to have pure single colonies. Then pure strains were either maintained on MRS agar slants media at 4°C for routine uses or stored at -20 °C in 50 % glycerol.

Forty presumptive LAB isolates were obtained from different sources of fermented foods, 25 isolates from sorghum dough, pickles, sausage, yoghurt, cheese and mish and 15 isolates obtained from Dodery (fermented cattle, sheep and goat bones). These isolates were identified phenotypically using the methods described by **Schillinger and Lucke (1987)**. For preliminary identification, following phenotypic tests were used: cell morphology, Gram reaction, catalase activity, growth at 10°C and 45°C, growth in the presence of 6.5 and 18% NaCl, production of CO_2 from glucose, and growth at pH 9.6.

Antagonistic activity detection

A total of 40 LAB were tested for their ability to produce inhibitory substances against two indicator organisms. The indicator organisms used were *Staphylococcus aureus* ATCC 2818 and *Escherichia coli* ATCC 29522 (obtained from the Department of Veterinary Microbiology, Faculty of Veterinary Sciences, University of Khartoum, Sudan). The inhibitory potential of 40 LAB isolates was investigated by using the agar-spot test method (**Schillinger and Lucke 1989)**. Briefly, overnight cultures of individual isolates were spotted (5µl) onto the surface of agar plates (MRS broth with 1.2% agar) and incubated at 37°C for 24h. Anaerobic conditions were used to minimize the formation of hydrogen peroxide and acetic acid. The spots in each plate were overlaid with about 7 mL of soft nutrient agar (0.75% agar) inoculated with 650 µL (10^{-1} dilution) of an overnight cultures of the indicator organisms. The plates were incubated for 24 h at 37°C and clear zones around the spots of the isolated LAB were estimated in mm.

Cell-free supernatant of 18 isolates that showed positive inhibition zones against *S. aureus* ATCC 2818 and *E. coli* ATCC 29522 in agar-spot test were prepared and tested by the agar-well diffusion assay as described by **Schillinger and Lucke (1989)**. Briefly, the isolates were cultivated individually in MRS broth media for 18 h at 37°C. Cell-free supernatant was obtained by centrifuging the cultures at 3000×g for 15 min at 4°C. The supernatants obtained were adjusted to pH 6.5 with 1N NaOH and were used for agar well diffusion assay. Petri dishes with 10 mL of nutrient agar (Oxoid, Basingstoke, Hampshire, United Kingdom) were prepared and inoculated with 0.1 mL of a 24 h old nutrient broth culture of individual indicator organisms. Once solidified, the dishes were stored for 2 h at 4°C. Four wells were made in the agar using sterile cork borer (Ø 6 mm). The wells were filled with 100 µL of the neutralized cell-free supernatants of the test isolates; then the inoculated plates were incubated for 24 h at 37°C. The clear zones around the wells in agar plates were then evaluated. Positive controls of the bacteriocin producing strain *Enterococcus faecium* BFE 2207 **(Yousif 2003)** was treated in the same manner.

Characterization of the inhibitory substances

The supernatants were also determined to be sensitive toward the action of proteolytic enzyme, pH and heat treatment. The sensitivity of inhibitory substances produced by the selected LAB isolates toward proteolytic enzyme was tested individually by adding pepsin (Merck-7189) to cell-free culture supernatants at final concentrations of 1mg/mL. The reaction mixtures were then incubated at 37 ºC for 1 h and the remaining activity of supernatants was determined by using the agar well-diffusion assay described previously. All enzyme-supernatant incubations were set at pH 3.0.

Cell-free culture supernatants of the selected isolates were subjected to heat treatment at temperatures of 60°C for 60 min, 100°C for 20 min, and autoclaving (121°C/15 min). The remaining activity of supernatants was determined by the agar well-diffusion assay as described above.

Stability at different pH values was tested by adjusting the pH of cell-free culture supernatants of isolates to pH 2.0, 6.5, and 9.0 with either 1N HCl or 1N NaOH. Agar well-diffusion assay described by **Schillinger and Lucke (1989)** was used to evaluate the residual activity. All determinations were carried out in triplicates.

RESULTS AND DISCUSSION

Isolation and phenotypic characterization of LAB isolated from Sudanese fermented foods

It is well understood that LAB, which grow as the adventitious microflora of foods or that are added to foods as starter cultures, are generally considered to be harmless, or even as advantageous for human health (as in the case of probiotics). In the current study, 40 strains were isolated from seven Sudanese fermented foods. All isolates were Gram-positive, catalase negative, non-endospore forming, and produced acid from glucose. They were thus presumptively identified as lactic acid bacteria. Among these, 57% (23 isolates) were cocci which occurred either single or in pairs with elongated coccid cell morphology and were able to grow in the presence of 6.5% NaCl, at pH 9.6, and at 45°C in MRS broth. These cocci were characterized as homofermentative enterococci. Thirty three percentage (13 isolates) were rods, occurring either singly or in pairs. They were unable to grow in the presence of 18%NaCl and at pH 9.6. Some of them were unable to grow in the presence of 6.5%NaCl and at 45°C and none of them produced gas from glucose when tested in MRS broth. These isolates were grouped as homofermentative lactobacilli. The remaining ten percentage (4 isolates) were cocci, occurring either in pairs or tetrads, were unable to grow in the presence of 18% NaCl, and at pH 9.6, but were able to grow in the presence of 6.5% NaCl and at 45°C in MRS broth. These cocci also exhibited well rounded cell morphology typical for pediococci, while other LAB cocci such as enterococci and *leuconostoc* exhibits a more elongated or coccid cell morphology. Accordingly, these cocci were supposed to be homofermentative pediococci. In accordance with these findings, the most important genera of LAB that are associated with food reported so far included *Lactococcus, Pediococcus, Enterococcus, Oenococcus, Leuconostoc, Lactobacillus, Carnobacterium* and *Weissella* **(Devirgiliis *et al*., 2013; Yousif 2003)**. Previous studies on microbial community in fermented foods from different sources (meat, fish, dairy products, fruits, vegetables, cereals) have demonstrated the dominance of most of these genera of LAB in such fermented foods **(Abdelgadir *et al*., 2001; Grosu-Tudor *et al*., 2014; Hwanhlem *et al*., 2011; Pringsulaka *et al*., 2012; Sulieman *et al*., 2006; Yang *et al*., 2012)**.

The isolation and screening of microorganisms from natural sources has always proved to be a successful way for obtaining industrially important strains or strains with valuable medical applications (**Yang *et al*., 2012)**. The majority of enterococcal isolates were mainly found in meat sources representing 83% (52% from dodery and 31% from sausages), 13% were isolated from dairy products (cheese and fermented milk), and 4% from pickles (Table 1). These *Enterococcus* strains are thus made up more than 50% of the genera isolated from all Sudanese fermented foods. Whereas, the majority of lactobacilli strains (39%) were isolated from mish, 23% from dodery, 15% from sorghum dough, 15% from pickles and the remaining 8% were isolated from sausages (Table 1). Although, lactobacilli were detected in most of the tested Sudanese fermented foods (sorghum, pickles, mish, sausages and dodery), there were however could not be found in cheese and fermented milk. The absence of lactobacillus strains in fermented milk and cheese could be attributed to the heat treatment of the milk before the preparation of these products that could eliminate the indigenous microflora of the milk. Previously, various lactobacillus strains have been isolated from other Sudanese fermented dairy products such as *rob* **(Abdelgadir *et al*., 2001)** and *garris* **(Sulieman *et al*., 2006)**. Although, the preparation of these fermented products is slightly differ from one area to the other, but in most cases raw milk is used **(Abdelgadir *et al*., 1998; Dirar 1993)**. On the other hand, in cheese and fermented milk both enterococci and pediococci were observed with the latter being exclusively found in these fermented products representing 100 % of the isolated pediococal strains (Table 2). The detection of pediococci and enterococci indicate the contamination of these products during the fermentation. It is likely that because of their natural occurrence as contaminants of raw meat and dairy products, thermotolerance, low toxicity, and their ability to acidify an environment, enterococci have become an important ingredient in fermented foods **(Lebreton *et al*., 2014)** and are widely distributed in these fermented foods. Enterococci are of importance in foods due to their involvement in food spoilage and fermentations, as well as their utilization as probiotics in humans and slaughter animals **(Franz *et al*., 2011)**. However, some strains among enterococci can cause nosocomial infections (**Franz *et al*., 2011)**. It is well known that lactobacilli represent a major LAB component within the complex microbiota of fermented foods obtained from cereal, meat, dairy, and vegetable sources **(Corsetti *et al*., 2007; Devirgiliis *et al*., 2013)**. Pediococci are also reported to inhabit on a great variety of vegetables, ripened cheeses, buttermilk, and a variety of processed meats **(Weiss 1992)**. They also play an important role as a member of some commercial starters for fermented foods **(Ratanaburee *et al*., 2013)**. The results of this study demonstrated that the distribution of LAB in Sudanese fermented food is mainly depend on the type of the raw material from which the food is prepared as well as the fermentation procedure.

Table 1 Distribution of LAB in Sudanese fermented foods.

Food Source	Isolated genera		
	Lactobacilli	Enterococci	Pediococci
Sorghum (SH)	[a] 2 (15%)	-	-
Pickles (PI)	2 (15%)	1 (4%)	-
Cheese (C)	-	1 (4%)	3 (75%)
Fermented milk (FM)	-	2 (9%)	1 (25%)
Mish (M)	5 (39%)	-	-
Sausages (SU)	1 (8%)	7 (31%)	-
Dodery (D)	3 (23%)	12 (52%)	-
Total	13 (33%)	23 (57%)	4 (10%)

[a], no. of isolates; -, not detected; (%), percentage of isolates from different sources.

Detection of antagonistic activity

A total of 40 LAB isolates of three genera, *Lactobacillus* (13 isolates), *Enterococcus* (23 isolates) and *Pediococcus* (4 isolates) were isolated from seven Sudanese fermented foods (sorghum dough, pickles, cheese, fermented milk, mish, sausage and dodery). Twenty-four isolates were found to produce inhibition zones against *S. aureus* and 21isolates inhibited the growth of both, *S. aureus* and *E. coli* (Table 2). These results revealed that all isolates exhibited varying degree of inhibitory activity against strains of *S. aureus* and *E. coli*, in many cases some isolates showed a higher antimicrobial activity than others did. These findings are in agreement with those obtained by **Bromberg *et al.* (2004)** who determined the production of bacteriocin-like substances by some LAB isolated from meat and meat products active against *S. aureus*. This result also confirms finding reported by **Ben Omer *et al.* (2006)** who screened 30 isolates of LAB isolated from ben saalga, a traditional fermented gruel from Burkina Faso, and found that about 40% of isolates, which produced antimicrobial substances, were active against *S. aureus* and 30% of isolates were active against *E. coli*.

The sensitivity of indicator organism *S. aureus* and *E. coli* toward different LAB isolates is shown in Figure 1. In this regard, almost 70% of enterococci showed high antagonistic activity against *S. aureus* in the agar spot test method, while 74% were found to be active against *E. coli*. Whereas, 50% of lactobacilli exhibited inhibitory activity when tested against *S. aureus* and *E. coli*. However, only 25% of pediococci were active against *S. aureus*, but less than 10% activity was observed against *E. coli*. **Hwanhlem *et al.* (2011)** reported that LAB isolated from Thai fermented fish showed wide zones of inhibition against *E. coli* and *S. aureus*. **Balla *et al.* (2000)** reported that the enterococcal bacteriocins (enterocins A, B, P, L50, Q and 1071) approved to be strong inhibitors of foodborne pathogens such as *S.aureus*. **Ben Omer *et al.* (2006)** concluded that some lactobacilli have ability to produce inhibitory substances active against pathogenic bacteria. **Bhunia *et al.* (1988)** reported that the pediocin AcH a bacteriocin produce by *Pediococcus acidilactici* xz was active against several food spoilage bacteria and foodborne pathogens including *S. aureus.* Outstandingly, the utilization of bacteriocins or bacteriocin-producing LAB associated with foods may provide a novel means of preserving foods and beverages from detrimental effects of spoilage and/or pathogenic bacteria.

In order to exclude the possibility of inhibition by low pH resulted from organic acids that produced by the isolated LAB, the cell-free culture supernatants were neutralized with 1N NaOH before use in the well-diffusion assay. Out of 18 isolates, only two isolates (SU1, D9) produced inhibition zones on agar in well diffusion assay (data not shown) against both indicator microorganisms *E. coli* and *S. aureus*. **Schillinger & Lucke (1989)** assumed that some factors such as aggregation, non-diffusible bacteriocins, protease inactivation and concentration effects could lead to false negative results in the well-diffusion assay. Our results indicated that these two strains might produce bacteriocins, whereas, the antimicrobial activity of other strains may be due to acidifying effect of organic acids together with very low activity of bacteriocins. Since the neutralization of the supernatant extract resulted in loss of this activity. The food source, fermentation condition and isolation media might be important for the successful isolation of bacteriocinogenic LAB, because many bacteriocin-producing LAB have been isolated from meat products, such as fermented meat and fish **(Pringsulaka *et al*., 2012)**. In our study, these two bacteriocin-producing strains (D9 and SU1) have been isolated from fermented meat products.

Table 2 Antagonistic activity of isolated LAB against *S. aureus* ATCC 2818 and *E. coli* ATCC 29522

Isolates	Source	Genus	Indicator strain	
			S. aureus	*E. coli*
SH1	Sorghum	*Lactobacillus*	-	-
SH2	Sorghum	*Lactobacillus*	+ +	+ +
SU1	Sausage	*Enterococcus*	+ +	+ +
SU2	Sausage	*Enterococcus*	+	+
SU3	Sausage	*Enterococcus*	+ +	+
SU4	Sausage	*Enterococcus*	+ +	+ +
SU5	Sausage	*Enterococcus*	+ +	+ +
SU6	Sausage	*Enterococcus*	+	+ +
SU7	Sausage	*Enterococcus*	-	-
SU8	Sausage	*Lactobacillus*	+ +	+
C1	Cheese	*Pediococcus*	-	-
C2	Cheese	*Pediococcus*	+	+
C3	Cheese	*Pediococcus*	+	+
C4	Cheese	*Enterococcus*	+ +	+
FM1	Fermented milk	*Enterococcus*	+ +	+
FM2	Fermented milk	*Enterococcus*	+	+
FM3	Fermented milk	*Pediococcus*	+ +	+
M1	Mish	*Lactobacillus*	+	+
M2	Mish	*Lactobacillus*	+	+
M3	Mish	*Lactobacillus*	-	-
M4	Mish	*Lactobacillus*	+ +	+ +
M5	Mish	*Lactobacillus*	+	+
PI1	Pickle	*Lactobacillus*	+	+
PI2	Pickle	*Lactobacillus*	+ +	+ +
PI3	Pickle	*Enterococcus*	+	+
D1	Dodery	*Enterococcus*	+ +	+ +
D2	Dodery	*Enterococcus*	+	+ +
D3	Dodery	*Lactobacillus*	+ +	+ +
D4	Dodery	*Lactobacillus*	+ +	+ +
D5	Dodery	*Enterococcus*	+ +	++
D6	Dodery	*Enterococcus*	+ +	+ +
D7	Dodery	*Enterococcus*	+ +	+ +
D8	Dodery	*Enterococcus*	+ +	+ +
D9	Dodery	*Enterococcus*	+ +	+ +
D10	Dodery	*Enterococcus*	-	-
D11	Dodery	*Enterococcus*	+ +	+ +
D12	Dodery	*Enterococcus*	+ +	+ +
D13	Dodery	*Enterococcus*	+ +	+ +
D14	Dodery	*Lactobacillus*	+ +	+ +
D15	Dodery	*Enterococcus*	+ +	+ +

- = no inhibition zone, + = inhibition zone less than 2.5 mm, ++ = inhibition zone over 2.5 mm. SH: Sorghum dough, C: Cheese, FM: Fermented milk, M: Mish, P: Pickles, SU: Sausage, D: Dodery.

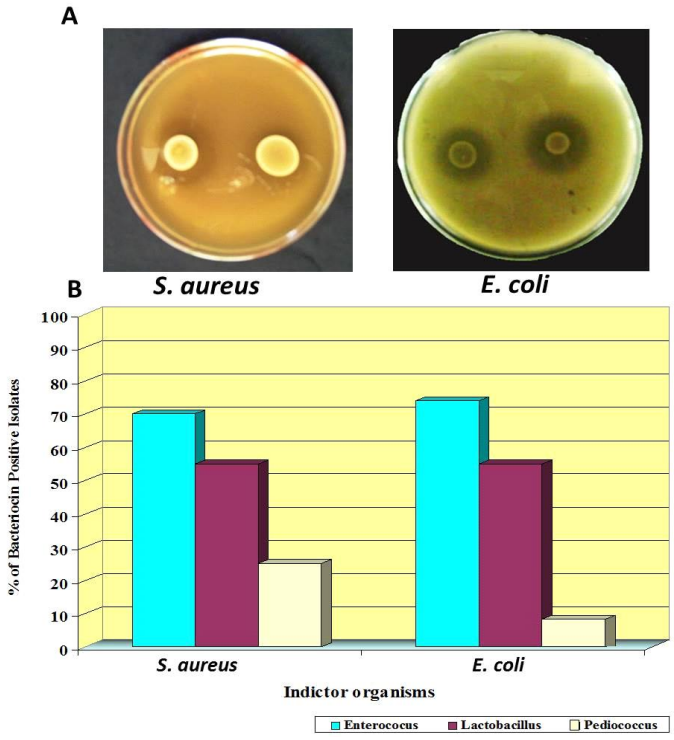

Figure 1 Inhibitory activity of the isolated LAB against indictor organisms *S. aureus* ATCC 2818 and *E. coli* ATCC 29522 as assayed by agar-spot method.

The distribution of positive becateriocin producing isolates among different genera of LAB isolated from various Sudanese fermented foods is shown in Figure 2. The results illustrates that about 88% of enterococci isolated from fermented meat products (dodery and sausage) and 12 % of fermented dairy products (fermented milk and cheese), showed inhibitory activity against target indicator strains *E. coli* and *S. aureus* (Fig. 2A). It seems, that enterococci obtained from meat products had higher inhibitory activity against both Gram -

positive and Gram- negative indicator organisms. On the other hand, all isolated lactobacilli exhibited varying degree of inhibitory activity against *S. aureus* and *E. coli*. Among the culture of lactobacilli about 44% of doddery isolates, 14% of sausage isolates, 14% of mish isolates, 14% of sorghum isolates, and 14% of pickle isolates produced inhibitory substances against the indictor strains used in this study (Fig. 2B). Lactobacilli from meat products again (doddery and sausage) expressed high percentage (58%) of inhibitory activity against both Gram- positive and Gram-negative indicator organisms. These results indicate that type and nutritional composition of the raw material from which fermented food is prepared play a critical role in the development of bacteriocin producing strains. This could be linked to the fact that the nutritious food considered as an ideal media for the growing of many fermenting and spoilage microorganisms. Thus, fermenting LAB apply different strategies to suppress the growth of other microorganism in such nutritious food. One of these strategies is the formation of antibacterial proteinaceous compounds known as bacteriocins. To date, many types of bacteriocins have been isolated and identified from various LAB. Enterocins have been isolated from strains originating from different sources, including foods (cheese, meat, fish, and vegetables), animals, and humans. The detection of enterococcal isolates with antagonistic activity against *S. aureus* and *E.coli* agrees with many data available on the ability of enterococci to produce antimicrobial compounds mainly bacteriocins, which are active against various spoilage and pathogenic bacteria **(Yousif 2003)**. **Aslim *et al.* (2005)** found that all *Lactobacillus* strains isolated from Turkish dairy products were able to produce bacteriocin-like substances against number of spoilage and pathogenic bacteria. **Khalil *et al.* (2009)** found that 19 strain of *Lactobacillus spp.* isolated from fermented vegetables products were able to produce bacteriocins that active against *S. aureus*. **Ogunshe *et al.* (2007)** concluded that a total of 50 bacteriocin-producing *Lactobacillus* strains isolated from some Nigerian indigenous fermented foods (cereal base) were active against several food borne pathogenic bacteria such as *Escherichia coli*. The results of inhibitory activity of pediococci show that all pediococci isolated from fermented milk were active against *S. aureus* and *E.* coli (data not shown). These results were similar to that obtained by **Savadogo *et al*. (2004)** who found that pediococci isolated from Burkina Faso fermented milk were able to produce inhibitory substance that active against *S. aureus* and *E. coli.* **Yousef *et al.* (1991)** reported that *Pediococcus* inoculants or purified pediocin could function as a bio-preservative to eliminate gram-positive pathogenic bacteria in cooked meats during extended refrigerated storage.

Characterization of inhibitory substance

The sensitivity of the antibacterial substances produced by 18 isolates of LAB toward pepsin was determined in controlled and reproducible conditions. The results demonstrated that all the compounds were inactivated by the proteolytic enzyme, which indicates their proteinaceous nature (Table 3). In general, the inhibitory compounds produced by these isolates presented different patterns of sensitivity. All of them were completely inactivated by pepsin. Only one isolate was resistant to the enzyme treatment (isolate D13). These results strongly suggest that the inhibitory activity in supernatants of isolates is due to a proteinaceous compound, which allows considering it a bacteriocin. Pepsin inhibited the antagonistic activity of 95% of the isolates. The results were similar to those obtained by Bromberg *et al*. (2004) who found that pepsin inhibited the antagonistic activity of 90% of the antibacterial substances produced by lactic acid bacterial strains isolated from meat and meat products. Sensitivity to pepsin was shown in other bacteriocins such as enterocin 416KI **(Schillinger & Lucke, 1989)**, plantaricin 35d **(Messi *et al*., 2001)**, and sakacin A **(Sabia *et al*., 2002)**.
As illustrated in Table 4, heating the cell free supernatants of the isolated LAB at 60°C for 60 min, at 100°C for 20 min, and at 121°C for 15 min completely abolished the inhibitory activity of the supernatants of all tested isolates. These results demonstrated that the heat liable bacteriocins might be responsible for the inhibitory activity of the cell free supernatants of the isolated LAB. Based on these results the bacteriocins produced by the isolated strains in the current study might be considered to belong to class III bacteriocins. In the literature, LAB bacteriocins have been grouped into three main classes: class I, the small (< 5 kDa) heat-stable peptides that are extensively modified after translation, resulting in the formation of lanthionine or methyllanthionine; class II, the other small (< 10 kDa) and heat-stable peptides; and class III, are large and heat-labile protein bacteriocins **(Nes *et al*., 2007)**. The later types of bacteriocins are much less studied compared to other classes (class I and II bacteriocins) and accordingly only few reports concerning their isolation and characterization are available **(Grosu-Tudor *et al*., 2014; Sabia *et al*., 2014; Vaughan *et al*., 1994)**.

Table 3 Sensitivity of inhibitory substances produced by LAB isolates to treatment with proteolytic enzyme.

Isolates	Genus	Sensitivity to Pepsin	
		Untreated	treated
Control (*Enterococcus faecium* BFE 2207)	*Enterococcus*	+	-
(SH2)	*Lactobacillus*	+	-
(SU1)	*Enterococcus*	+	-
(SU4)	*Enterococcus*	+	-
(SU5)	*Enterococcus*	+	-
(SU8)	*Lactobacillus*	+	-
(FM1)	*Enterococcus*	+	-
(FM2)	*Enterococcus*	+	-
(FM3)	*Pediococcus*	+	-
(D1)	*Enterococcus*	+	-
(D3)	*Lactobacillus*	+	-
(D6)	*Enterococcus*	+	-
(D7)	*Enterococcus*	+	-
(D9)	*Enterococcus*	+	-
(D11)	*Enterococcus*	+	-
(D12)	*Enterococcus*	+	-
(D13)	*Enterococcus*	+	+
(D14)	*Lactobacillus*	+	-
(D15)	*Enterococcus*	+	-

SH: Sorghum dough, SU: Sausage, FM: Fermented milk, D: Dodery.

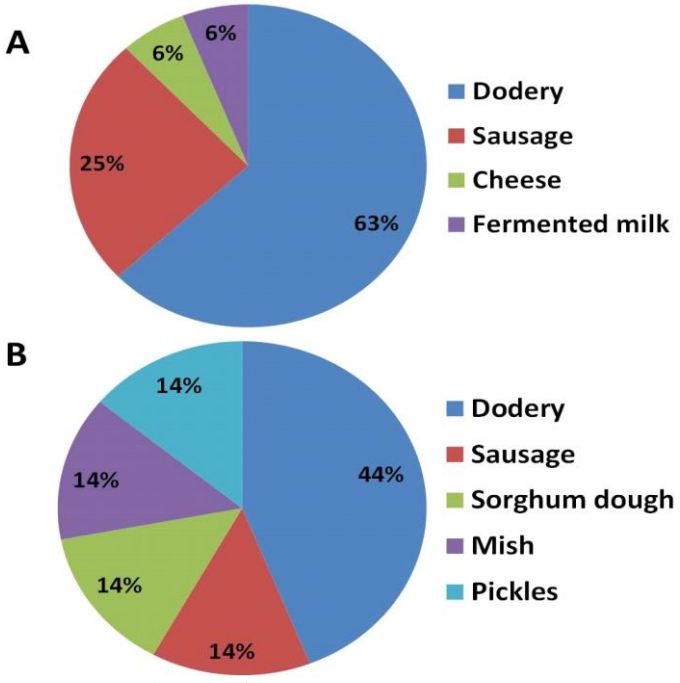

Figure 2 Sudanese fermented foods showing positive bacteriocin producing LAB isolates of the genus *Enterococcus* (A) and *Lactobacillus* (B).

Results on the stability of the antimicrobial substances in the cell free supernatants of the isolated LAB at different pH values are presented in Table 5. All the inhibitory substances showed activity at pH 2. However, elevating the pH toward alkaline condition appeared to diminish the inhibitory activity of the cell free extracts of most of the isolated LAB. With exception of the cell free extract of the isolate D9 and SU1, all supernatant extracts of the isolated strains showed no any inhibitory activity against the indicator microorganisms at pH ≥ 6.5. This results indicated that the antimicrobial activity of other strains might be due to the production of organic acids, since the pH neutralization resulted in the loss of this effect as mentioned above for agar diffusion assay. Interestingly, in the agar diffusion assay only these two isolates (D9 and SU1) showed inhibition zone against both *E. coli* and *S. aureus*. Similar low numbers of bacteriocin-producing strains have been reported by other authors for strains isolated from fresh-cut vegetable products **(Yang *et al*., 2012)** or from milk and meat products **(Sezer & Guven, 2009)**. Such bacteriocins with acidic condition preference at very narrow pH spectrum might find applications as highly selective antibacterial agents under very low pH conditions. It is well documented that most of the bacteriocins

identified so far express great activity at low pH. These results are comparable with those reported by **Messens and De Vuyst (2002)** who stated that many LAB and bacteriocins display greater antibacterial activity at lower pH values (pH 5 and below).

Table 4 Effect of heat treatment on inhibitory substances produced by LAB isolates.

Isolates	Genus	Activity of bacteriocin-like substances			
		Unheated	60°C/ 60min	100°C/ 20 min	121°C/ 15 min
Control (*Enterococcus faecium* BFE 2207)	*Enterococcus*	+	-	-	-
(SH2)	*Lactobacillus*	+	-	-	-
(SU1)	*Enterococcus*	+	-	-	-
(SU4)	*Enterococcus*	+	-	-	-
(SU5)	*Enterococcus*	+	-	-	-
(SU8)	*Lactobacillus*	+	-	-	-
(FM1)	*Enterococcus*	+	-	-	-
(FM2)	*Enterococcus*	+	-	-	-
(FM3)	*Pediococcus*	+	-	-	-
(D1)	*Enterococcus*	+	-	-	-
(D3)	*Lactobacillus*	+	-	-	-
(D6)	*Enterococcus*	+	-	-	-
(D7)	*Enterococcus*	+	-	-	-
(D9)	*Enterococcus*	+	-	-	-
(D11)	*Enterococcus*	+	-	-	-
(D12)	*Enterococcus*	+	-	-	-
(D13)	*Enterococcus*	+	-	-	-
(D14)	*Lactobacillus*	+	-	-	-
(D15)	*Enterococcus*	+	-	-	-

SH: Sorghum dough, SU: Sausage, FM: Fermented milk, D: Dodery.

Table 5 Effect of pH on inhibitory substances produced by LAB isolates

Isolates	Genus	Activity of bacteriocin-like substances			
		Unheated	pH 2.0	pH 6.5	pH 9.0
Control (*Enterococcus faecium* BFE 2207)	*Enterococcus*	+	+	-	-
(SH2)	*Lactobacillus*	+	+	-	-
(SU1)	*Enterococcus*	+	+	+	-
(SU4)	*Enterococcus*	+	+	-	-
(SU5)	*Enterococcus*	+	+	-	-
(SU8)	*Lactobacillus*	+	+	-	-
(FM1)	*Enterococcus*	+	+	-	-
(FM2)	*Enterococcus*	+	+	-	-
(FM3)	*Pediococcus*	+	+	-	-
(D1)	*Enterococcus*	+	+	-	-
(D3)	*Lactobacillus*	+	+	-	-
(D6)	*Enterococcus*	+	+	-	-
(D7)	*Enterococcus*	+	+	-	-
(D9)	*Enterococcus*	+	+	+	+
(D11)	*Enterococcus*	+	+	-	-
(D12)	*Enterococcus*	+	+	-	-
(D13)	*Enterococcus*	+	+	-	-
(D14)	*Lactobacillus*	+	+	-	-
(D15)	*Enterococcus*	+	+	-	-

SH: Sorghum dough, SU: Sausage, FM: Fermented milk, D: Dodery.

CONCLUSION

Sudanese fermented foods contained a group of LAB that produced antimicrobial bacteriocin-like active compounds, which showed inhibitory activity against *S. aureus* ATCC 2818 and *E. coli* ATCC 29522. Enterococci were observed for their highest incidence of antagonistic activity, especially those obtained from Dodery (63%). The antimicrobial activity of bacteriocins produced by LAB isolated in this study could potentially act as barrier to spoilage and/or pathogenic microorganism in such foods.

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