ANTIBACTERIAL ACTIVITY OF CELL FREE SUPERNATANT OF LACTIC ACID BACTERIA ISOLATED FROM FOOD SAMPLES AGAINST FOOD BORNE PATHOGENS

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ABSTACT

The ability to preserve foods using bacteriocin-producing lactic acid bacteria (LAB) isolated directly from foods is an innovative approach. Therefore, this study aimed at evaluating the efficacy of cell free supernatant of lactic acid bacteria isolated from food samples on selected spoilage and pathogenic microorganisms. A total of 48 food stuffs such as cucumber, fresh fish, nunu, meat, ogi, cheese and yoghurt were obtained aseptically and analyzed. Lactic acid bacteria were isolated using MRS agar and bacteriocin positive isolates were screened using disk diffusion assay against the test organisms. Antimicrobial activities of the cell free supernatants (CFS) of five of the best bacteriocin-producing lactic acid bacteria were tested against selected pathogens. The selected bacteriocin-positive isolates were identified using 16S rRNA gene sequencing. The highest inhibition zone of 40.5mm was recorded against *S. pyogenes* by *L. plantarum* with the least inhibition zone of 20.2mm recorded against *E. coli* by *L. acidophilus*. All the compounds produced by the lactic acid bacteria were fully inactivated by some of the proteolytic enzymes, which indicated their proteinaceous nature. The antimicrobial activity of the cell free supernatant of bacteriocins-producing lactic acid bacteria isolated in this work could serve as potential biopreservatives to control foodborne pathogens and spoilage bacteria.

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Key words: Cell free supernatant; Antimicrobial activity; Bacteriocin; Lactic acid bacteria; Foodborne pathogens

1. INTRODUCTION

More than 250 toxins and pathogens are known to be transmitted by food, and this list continues to grow steadily (Choffnes *et al.*, 2012; Tauxe, 2002). Several reports indicate that food-borne pathogens are one of the main causes of death in the world. For example, in the United States, 1,300 deaths are caused by 31 food borne pathogens each year, in addition to 56,000 hospitalizations and 9.4 million illnesses (Yang *et al.*, 2014). *Campylobacter* spp, *Clostridium perfringens*, *E. coli*, *L. monocytogenes*, Norovirus and *Salmonella* spp. are responsible for more than 90 percent of all symptomatic food related illnesses (Scallan *et al.*, 2011). Control of these food borne enteric pathogens is a real challenge for food industry and public health agency. Moreover, it is very difficult to protect safety of food chains due to resurgence of multidrug resistant strains of foodborne pathogens (Yang *et al.*, 2014).

Food borne diseases remain a major public health challenge in the African region, causing a significant burden of illness, disability and mortality. Outbreaks of food-borne diseases have been rarely reported for decades in Nigeria. These diseases have been associated with the consumption of foods like soft cheese, processed meat, beef, poultry or eggs and other related foods. Food borne diseases associated with pathogens such as *Salmonella enteritidis*, *Staphylococcus aureus*, *Campylobacter* spp and *Escherichia coli* have been reported in many parts of the world particularly in Australia, Canada, Japan, United States, European countries and in South Africa (Adak *et al.*, 2002; Bäumler *et al.*, 2000) and in Nigeria the situation is the same (Akinyemi *et al.*, 1998).

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One of the concerns in food industry is the contamination by pathogens, which are frequent cause of food borne diseases. Over the past decade, recurrent outbreaks of diarrhea, combined with the natural resistance of the causative agents, contributed to its status as hazard. The problem of selection of resistant bacteria to antibiotics (Kapil, 2005) and the increasing demand for safe foods, with less chemical additives, has increased the interest in replacing these compounds by natural products, which do not injure the host or the environment.

In order to achieve improved food safety against such pathogens, food industry makes use of chemical preservatives or physical treatments (e.g. high temperatures). These preservation techniques have many drawbacks which include the proven toxicity of the chemical preservatives (e.g. nitrites), the alteration of the organoleptic and nutritional properties of foods, and especially recent consumer demands for safe but minimally processed products without additives. To harmonize consumer demands with the necessary safety standards, traditional means of controlling microbial spoilage and safety hazards in foods are being replaced by combinations of innovative technologies that include biological antimicrobial systems such as lactic acid bacteria (LAB) and/or their metabolites (Nath *et al.*, 2013).

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The increasing demand for safe food has increased the interest in replacing chemical additives by natural products, without injuring the host or the environment. Biotechnology in the food-processing sector targets the selection, production and improvement of useful microorganisms and their products, as well as their technical application in food quality. The use of non-pathogenic microorganisms and/or their metabolites to improve microbiological safety and extend the shelf life of foods is defined as biopreservation (De Martinis *et al.,* 2001). Antagonistic properties of LAB allied to their safe history of use in traditional fermented food products make them very attractive to be used as biopreservatives (Caplice and Fitzgerald, 1999). The increasing interest in these compounds has stimulated the isolation of LAB with the

capability to produce bacteriocin and the characterization of many novel peptides (Deraz *et al.*, 2005). Therefore, this study aimed at evaluating the efficacy of cell free supernatant of lactic acid bacteria isolated from food samples on selected spoilage and pathogenic microorganisms.

2. MATERIALS AND METHODS

2.1 Sample collection

A total of 48 food samples were collected from different locations in Calabar. The collected samples included seven samples each of cucumber, ogi, meat and fresh fish, six samples each of yoghurt and natural milk (nunu) as well as eight samples of cheese were collected aseptically using sterile gloves and placed in food grade sampling bags. Samples were immediate placed in pre-cooled containers containing ice packs and then transported to the laboratory for analysis.

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2.2 Sample Analysis

2.2.1 Isolation of lactic acid bacteria

This was carried out using the methods of Downes and Ito (2001) and Von Schelhorn (1980) with little modifications. Fifty grams of the sample was weighed and placed in sterile blender previously sterilized with 70% alcohol and rinsed with deionized water. The samples were then blended aseptically and twenty-five grams of the product was placed in 225ml of sterile peptone in sterile flask for enrichment. The homogenates were vigorously shaken for 2 to 3 minutes from which further ten-fold dilutions up to 10^{-4} were prepared. Spread and pour plate methods were used as the samples were plated on de Mann Rogosa Sharpe (MRS) agar in triplicate and incubated anaerobically.

2.2.2 Purification and maintenance of microbial isolates

The isolates were purified repeatedly via subculturing using MRS agar. Pure cultures were preserved on nutrient agar slants overlaid with paraffin oil and store at an ambient temperature for cultural and biochemical characterization.

2.3 Characterization and identification of isolates

Pure cultures of selected LAB isolates were characterized as describe by Batt (1999). The following standard microbiological tests were used for characterization of isolates, microscopic examination of cell morphology, physiological tests, biochemical tests, cultural growth conditions and carbohydrates (sugar) fermentation profile. Identification was based on comparison of observed characteristics of isolates with those of lactic acid bacteria as described in the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

2.4 Sugar fermentation profile of the isolates using API CHL 50

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Isolates were identified using API 50 CHL system (bioMerieux). For primary species-specific identification, bacterial and fungal strains were subjected to API 50 CHL and API 20 AUX assay. Purified strains were cultured on MRS plates, after which grown colonies were cultivated in 5 ml of MRS both at 30°C. The result was read after 24 h and verified after 48 h. Fermentation of carbohydrates in the carbohydrate medium was indicated by a yellow colour, except for esculine (dark brown). Color reactions were scored against a chart provided by the manufacturer.

2.5 Test culture preparation

Foodborne pathogens (target organisms) use in this study included *Staphylococcus aureus, Escherichia coli, Salmonella typhi, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Klebsiella spp*. Pathogenic organisms were grown aerobically in tryptic soy broth supplemented with 0.6% (w/v) yeast extract (TSB/YE) at 37°C. The organisms were then maintained on agar slant overlaid with paraffin oil. Before each experiment, the

microorganisms were activated by successive sub culturing of each target organism. Culture of the target organism used in bioassay was standardized at approximately 5 x 10^7cfu/ml determined by standard pour plate method on appropriate media.

2.6 Preparation of cell free supernatants

Cell free supernatants were prepared based on methods by Schillinger *et al.*, (1996). The culture extract of the producer strains was obtained from 18 hours culture grown in MRS broth. The cultures were then centrifuged at 10,000 rpm for 5 minutes. The supernatants were adjusted to pH of 6.5 using 10N NaOH and filtered through 0.22 µm Millipore. The cell free supernatants were then used for antimicrobial activity.

2.7 Antimicrobial activity of cell free supernatants

Targeted food borne pathogens were diluted using 0.1% peptone water (PW) to get 0.5 McFarland Turbidity Standard. All targeted bacteria being used were freshly streaked onto Muller Hilton Agar using Kirby Bauer technique. Approximately 5 mm diameter size of wells were made in each plate. Immediately, 80µl of CFS from each isolate were transferred to each well separately. All plates were anaerobically incubated using candle jar for 24 hours. After incubation, the zones of inhibition were measured. The pathogenic bacteria without LAB cultures were used as control of experiments. All antimicrobial tests were carried out in triplicates and the mean diameter of inhibitory zones were obtained.

Another set of inoculated plates with paper discs impregnated with the cell free supernatants were prepared and incubated also anaerobically using candle jar for 24 hours.

2.8 Elimination of other inhibitors

The possibility that other inhibitors common to LAB may give inhibitory reactions appearing as bacteriocin activity was eliminated using the following assays as described by Muriana and Klaenhammer, (2006) and Garver and Muriana, (1994). To exclude the inhibition

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due to the presence of lytic bacteriophages, the reverse side technique was used according to Moreno *et al.*, (1999). Dialysis eliminated organic acids while the effects of hydrogen peroxide was excluded by addition of catalase (sigma LGI 026k 7049) in the concentration of 1mg per ml.

After the elimination process was concluded, antimicrobial activities of the cell free supernatants were repeated and the results obtained were recorded.

2.9 Proteinaceous nature treatments

The cell free supernatants were inoculated with the enzymes; α-chymotrypsin, trypsin and protease at 5mg/ml concentration to determine the effect of these enzymes on the activity of the bacteriocin. Supernatants mixtures in three different tubes according to the enzymes were incubated for 1 hour at 37°C. The mixtures were then heated in boiling water for 3 minutes to denature the enzymes. After incubation the bacteriocins activities were determined as described by Todorov and Dicks, (2006). Antimicrobial activities of the enzyme treated cell free supernatants was then carried out using agar well diffusion assay. About 80µl of treated cell free supernatants from each isolate were transferred to each well separately. All plates were anaerobically incubated using candle jar for 24 hours. After incubation, the zones of inhibition were measured. The pathogenic bacteria without LAB cultures were used as control of experiments. All antimicrobial tests were carried out in triplicates and the mean diameter of inhibitory zones were obtained.

RESULTS AND DISCUSSION

The results of the biochemical characterization and sugar fermentation profile of five of the best lactic acid bacteria isolated from samples used in this study are shown in Table 1. The

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API profiles of these isolated LAB enabled identification of G_1 as *Lactobacillus* spp, G_2 as *Lactococcus* spp, G_3 as *Leuconostoc* spp, G_4 as *Pediococcus* spp and G_5 as *Lactobacillus* spp respectively.

Table 1

Biochemical characterization of the bacterial isolates and API CHL 50 result

Isolate code	Gram reaction	Shape	Catalase	Motility	Oxidase	Fructose	Tehalose	Glucose	Lactose	Sucrose	Glycogen	Mannitol	Maltose	Xylose	Melizetose	Esculin	Sorbitol	Mannose	Raffinose	Arabinose	Rhamnose	Melibiose	Ribose	Salicin	Cellobiose	Probable organism
G_1	+	R	+	-	-	+	-	+	+	+	+	-	+	-	+	-	-	+	+	+	-	+	-	+	+	*Lactobacillus plantarum*
G_2	+	C	+	-	-	+	+	+	+	-	+	+	+	-	+	-	-	+	+	+	-	-	+	+	+	*Lactococcus* spp
G_3	+	C	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	*Leuconostoc* spp
G_4	+	C	+	-	-	-	-	+	+	-	+	+	+	-	+	-	-	-	-	+	-	+	-	-	+	*Pediococcus* spp
G_5	+	R	+	-	-	+	-	+	+	+	+	-	+	-	+	-	+	+	+	-	-	+	+	+	+	*Lactobacillus* spp

The results of the antimicrobial activities of the cell free cultures of the isolates are shown in Tables 2 and 3. Five of the best lactic acid bacteria isolates with inhibition zones of 20mm and above were selected and displayed on the Tables. Table 2 shows the results of the antimicrobial testing of the isolates against the test organisms using disc diffusion method. The highest inhibition zone of 42.5 mm was recorded by *Lactobacillus plantarum* spp (G_1) against *Staphylococcus aureus* while the least inhibition zone of 20 mm was recorded by *Pediococcus* spp (G_4) against *Klebsiella pneumoniae*. However, *Lactobacillus plantarum* and *Leuconostoc* spp (G_1 and G_3) were active only against *Staphylococcus aureus* and *Streptococcus pyogenes*

whereas *Lactococcus* spp, *Pediococcus* spp and *Lactobacillus* spp (G_2, G_4 and G_5) where active against *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. All the bacteriocin positive isolates were observed to have high antibacterial activity against *Staphylococcus aureus* and *Salmonella typhi* as compared to the other test organisms used.

Table 3 shows the results of the antibacterial activities of the cell free supernatants using agar well diffusion assay. The highest inhibition zone of 40.5 mm was recorded by *Leuconostoc* spp (G_3) against *Streptococcus pyogenes* while the least inhibition zone of 21.5 mm was recorded by *Pediococcus* spp (G_4) against *Klebsiella pneumoniae*.

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Table 2

Antimicrobial activity of cell free supernatant (CFS) of bacteriocin-positive isolates against test pathogens using disc diffusion method

Isolate code	Zone of inhibition in mm					
	Staphylococcus aureus	*Escherichia coli*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Streptococcus pyogenes*	*Pseudomonas aeruginosa*
G_1	42.5	-	-	-	42	-
G_2	-	28	34.5	25.6	-	29.5
G_3	41	-	-	-	41.5	-
G_4	-	27.5	34	20	-	29.5
G_5	-	31.5	32.5	30.7	-	28.5

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Table 3

Antimicrobial activity of cell free supernatant (CFS) of bacteriocin-positive isolates against test pathogens using agar well diffusion assay

Isolate code	Zone of inhibition in mm					
	Staphylococcus aureus	*Escherichia coli*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Streptococcus pyogenes*	*Pseudomonas aeruginosa*
G_1	37	-	-	-	37.5	-
G_2	-	24.5	32.5	28.5	-	25.4
G_3	33.8	-	-	-	40.5	-
G_4	-	27	31.5	21.5	-	26.5
G_5	-	28	32.5	26.5	-	25.9

Table 4 shows the results of the inhibitory activities after eliminating the effects of organic acid and hydrogen peroxide. It is observed from the results that elimination of organic acids and H_2O_2 from the cell free supernatant did not have any effect on the inhibitory activity of the culture.

Table 4

Inhibitory activity against test isolates after eliminating effect of organic acid and H_2O_2

Isolate code	Zone of inhibition in mm					
	Staphylococcus aureus	*Escherichia coli*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Streptococcus pyogenes*	*Pseudomonas aeruginosa*
G_1	28	-	-	-	27	-
G_2	-	21.8	23	22.8	-	20.4
G_3	23.9	-	-	-	21.5	-
G_4	-	20.5	21	22.8	-	19.4
G_5	-	20.9	25.9	23.2	-	24.8

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The zones of clearance (mm) recorded using disk diffusion assay shows that hydrogen peroxide and organic acids were not the source of inhibition. Table 4 shows that the highest inhibition zone of 28.0 mm was recorded by *Lactobacillus plantarum* (G_1) against *S. aureus* while the least zone of 19.4 mm was recorded by *Pediococcus* spp (G_4) against *Pseudomonas aeruginosa.*

The effect of proteolytic enzymes on bacteriocin activity is shown in Table 5.

Table 5

Action of proteolytic enzymes on the antimicrobial activity of the isolates against pathogens

	Zones of inhibition in mm					
Isolate code	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhi*	*Klebsiella pneumoniae*	*Streptococcus pyogenes*	*Pseudomonas aeruginosa*
G_1	-	-	-	-	-	-
G_2	-	-	-	-	-	-
G_3	-	-	-	-	-	-
G_4	-	-	-	-	-	-
G_5	-	-	-	-	-	-

Key

- : no activity after treatment

The results show that α-chymotrypsin, trypsin and protease enzyme had positive effect on bacteriocin activities of all the isolates thereby resulting in the complete inactivation of the activities of the compounds. The sensitivity of the antibacterial substances produced by all the isolates indicated that bacteriocin is proteinaceous in nature.

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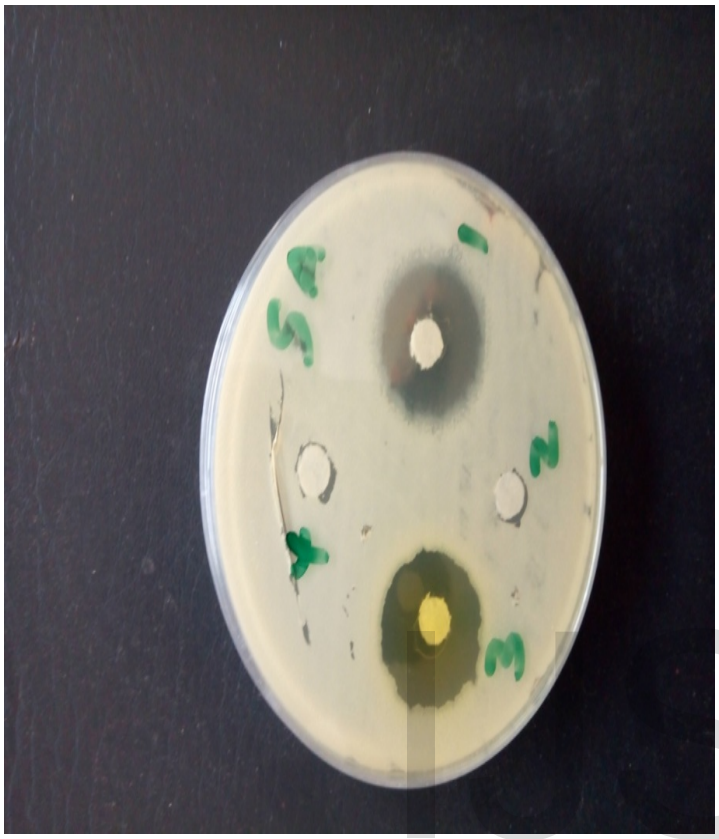

Plate 1: Antimicrobial activities of cell free supernatants against *Staphylococcus aureus*

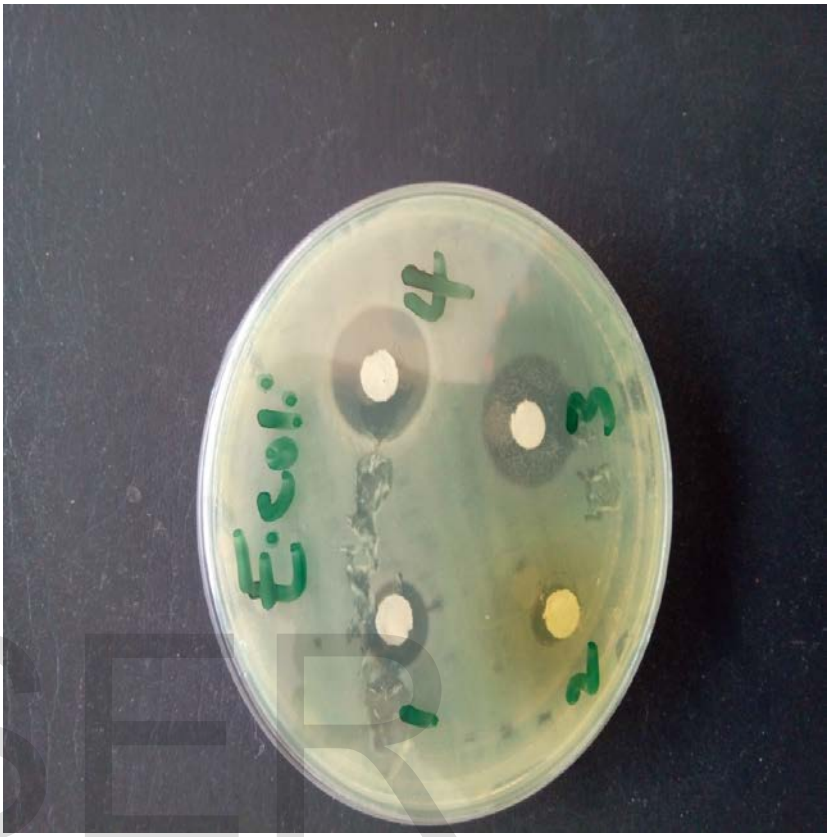

Plate 2: Antimicrobial activities of cell free supernatants against *Escherichia coli*

The extent by which Gram positive and Gram-negative indicator organisms were susceptible to the bacteriocin–like substances in this study is presented in Table 3 and 4. It was observed that partially purified bacteriocins produced by the isolates in this study inhibited *S. aureus, S. pyogenes, S. typhi, Klebsiella pneumonise, Pseudomonas aeruginosa* and *E. coli.* The isolates showed considerable zone of growth inhibition of all test microorganisms. This is consistent with results reported for other bacteriocins (De Martinis *et al.*, 2001; Ogunbanwo *et al.*, 2004). Tagg *et al.* (1976) suggested that bacteriocins usually have a narrow spectrum of activity inhibiting mostly Gram positive bacteria especially those closely related to the

producer organism however; the compounds produced by the isolates in this study were shown to be active against *E. coli, Salmonella typhi, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* This observation is at variance with some earlier works that reported activity against only Gram positive organisms (Tagg *et al.*, 1976; Schillinger and Lucke, 1987; Stevens *et al.*, 1991). However, a few bacteriocins from LAB with activity against Gram-negative bacteria have also been reported. For examples, bacteriocin produced by strains of *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from boza, a Bulgarian traditional cereal beverage has been reported to be active against *Pseudomonas aeruginosa, Enterococcus faecalis* and *E. coli* (Todorov and Dicks, 2006).

Previously, Oh *et al.* (2000) reported that crude bacteriocin from *Lb. acidophilus* 30SC were not capable to inhibit the growth of Gram negative bacteria, including *Klebsiella pneumoniae, E. coli* and *S. typhimurium.* However, in this study, three strains of the LAB isolated namely *Leuconostoc mesenteroides* strain M6, *Lactobacillus acidophilus* strain NK-S10, and *Pediococcus cellicola* strain: PMM-25 were highly capable to inhibit Gram negative bacteria such as *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli and Klebsiella pneumonia* as shown in Table 2 and 3. Savadogo *et al.* (2004) reported that action of the bacteriocins may be explain by the interaction with lipoteichoic acids, which are absent in Gram-negative bacteria, and these molecules would play the role of sites needed to produce the bacteriocinal effect. This last observation is in accordance with other reports and studies which stated that some bacteriocins produced by Gram-positive bacteria, regardless of its source, have a broad spectrum of activity against Gram positive microorganisms than did with Gram- negative (Savadogo *et al.*, 2004). Lima and Filho (2005) found that 265 out of 474 LAB produced bacteriocins which demonstrated activity against many Gram-positive organisms however the only Gram-negative susceptible organism was *Salmonella* spp. We believe that further

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understanding of the molecular foundation of the relationship between microbes (bacteriocin-producer) - microbes (inhibited-organism) should/may explain or at least clarify these findings.

After pH neutralization and H_2O_2 elimination, the CFS from the seven isolates had inhibitory effects on all the test organisms as presented in Table 4. Elimination of acid in cell free supernatant (CFS) does not have any effect on the inhibitory activity on the test isolates. Therefore, it can be concluded that the inhibitory action is caused by bacteriocin. This is in contrast to the work of Yang *et al.,* (2012) whereby after pH neutralization and H_2O_2 elimination, the CFS from the eight LAB isolates had inhibitory effects only on *L. innocua* but not the other test bacteria suggesting that organic acids and /or H_2O_2 produced by LAB had strong antimicrobial effects on bacteria tested.

Bacteriocins can be broken down by some proteolytic enzymes leading to a loss in their antimicrobial activity. In this study, the antimicrobial activity of the 5 best isolates was completely inactivated after treatment with the proteolytic enzymes trypsin, α-chymotrypsin and protease enzyme indicating that the inhibitory compounds, produced by these isolates are proteinaceous in nature which is consider as the main characteristic of any bacteriocin (Herreros *et al.,* 2005). The sensitivity of the antibacterial substances produced by lactic acid bacteria to α-chymotrypsin, trypsin, pepsin and protease was determined in controlled and reproducible conditions shown in Table 3. Similar behavior was observed by Khalil *et al.,* (2009) with a *Bacillus megaterium* 19 strain isolated from a mixture of fermented vegetable wastes. They found that pepsin and trypsin treatment inhibited the bacteriocin activity against *Staphylococcus aureus* more than *Salmonella typhimurium*. Cherif *et al*. (2001) used pepsin, papain, trypsin, chymotrypsin, proteinase K, lysozyme, catalase, DNase and RNase to treat thuricin 7, a bacteriocin produced by *Bacillus thuringiensis* BNG 1.7. They found that the inhibitory activity was only susceptible to proteinase K. Similar observation was reported by Hartnett *et al.,* (2002). Treatment of inhibitory compounds with catalase and α-amylase did not alter their activity, indicating that the inhibition recorded was not due to hydrogen peroxide and also

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that carbohydrate moieties if they exist in bacteriocins were not required for activity. Similar results were reported by Parente *et al.*, (1996) and Todorov and Dick (2006). However, some researchers have reported slight inactivation by α-amylase for group IV bacteriocins

Conclusion

The screening of lactic acid bacteria for the ability to produce bacteriocins is of great importance as the need for anti-pathogenic technology increases with a rapidly growing world population and increased emphasis on food safety. The results of this study indicated that some of environmental LAB are capable of synthesizing inhibitory substance(s) against some pathogenic/ spoilage bacteria. These inhibitory substances are proteinaceous in nature and act differently against the tested indicator bacteria. The number of Gram-negative bacteria, sensitive to these substances were more than Gram-positive; however, the Gram-positive bacteria were more susceptible to the same bacteriocin if compared to the Gram-negative. Also, it is noted that bacteriocins have lytic bactericidal mode of action which can prove helpful in combating food borne pathogens and help in maintaining food quality in dairy industries. The bacteriocin producers are recommended to food processing industries to be employed in biopreservation of food to enhance extension of shelf life of food products. Therefore, these strains would contribute to safety and organoleptic properties of food products.

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