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RESEARCH ARTICLE

ISOLATION OF LACTIC ACID BACTERIA AND THEIR ANTIBACTERIAL SPECTRUM AGAINST GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

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ABSTRACT

Hundred and eighteen Lactic acid bacteria were isolated from indigenous sources including milk, yogurt, vegetables, meat, fermented dough and infant feces. On screening these isolates by stab and agar well method, were found them to produce antimicrobial substances inhibitory against other LAB strains. They also exhibited a broad spectrum of antagonistic activity against gram negative i.e *E. coli, S. typhi, S. dysenterae, K. pneumonia* and *P. vulgaris* and gram positive pathogens i.e *S. pyogenes, S. fecalis, S. pneumonae, S. aureus, B. subtilis, B. cereus* and *C. diphtherae.* Only 23 amongst these elaborated bacteriocin like inhibitory substance (BLIS) while the rest showed antagonism mainly due to organic acids. One of the strongest BLIS producing strain of lactobacillus LBT-36 isolated from fermented tomatoes was partially purified by ammonium sulphate precipitation and the purified protein was subjected to SDS-PAGE which gave a single band. Therefore, these strains after further essential investigations could be selected as good candidates for commercial use as bio-presevatives and can also contribute in improving human health by using them as Probiotics in different foods.

INTRODUCTION

Lactic Acid Bacteria (LAB) are widely used as starter cultures in fermentation and are well known in preventing spoilage of a variety of foods including milk, meat and vegetables. Since decades LAB have been known to ferment dairy products converting lactose to lactic acid which also proves to be antimicrobial. Also bacteriocin production is one of the significant function of a number of LAB genera including *Lactobacillus, Lactococcus, Pediococcus, Leuconostoc* and *Carnobacterium* (Zhu *et al*., 2000). The antimicrobial activity exhibited by many of the strains of genus *Lactobacillus* could be the combined effect of lactic acid, bacteriocin and H2O2 production where lactic acid lowers the pH and makes the environment undesirable for the survival of pathogens. Since most of the bacteriocins produced by species of genus *Lactobacillus* are classified as Class II bacteriocins and being heat stable membrane active peptides, inhibits the growth of many pathogens (Aslim *et al.,* 2005). Generally, bacteriocins are known to be ribosomally synthesized antimicrobial compounds and those secreted by Lactic acid bacteria are mostly small peptides ranging between 3-6 kDa in size, mostly active against food spoilage organisms because of which they are accepted as food preservative and are also reported to kill closely related bacteria (Kleanhammer, 1993; Topisirovic *et al.,* 2006). Amongst a number of bacteriocins produced by lactic acid bacteria, nisin, diplococcin, acidophilin, bulgarican, lactocin and plantaricin are the popular ones but still only nisin has been used commercially and studied extensively (Savadogo *et al.,* 2006). This antimicrobial property of Lactic acid bacteria has enabled researchers to use them and their end products as a substitute in place of antibiotics for treating GIT infections (Savadogo *et al.,* 2004). Considering the number of useful properties attributed to LAB, scientists have developed immense interest in using LAB for therapeutic purposes. Many members of genus *Lactobacillus* and *Bifidobacterium* have proved to be effective in treating gastrointestinal disorders caused by *Clostridium difficile* (Lee *et al.,* 2002). They are well known in keeping a healthy environment within the gastrointestinal tract (GIT) due to the production of inhibitory substances like Lactic acid along with other organic acids, H2O2, and bacteriocin. Whereas, other health improving effects are manifested by means of adhesion to intestinal surfaces, thus providing less chance for the adhesion of pathogens. LAB also play a significant role in enhancement of immune system (Voravuthikunchai *et al.,* 2006; Osmanagaoglu *et al.,* 2005), reduction of lactose intolerance and rota virus diarrhea symptoms in children (Lazlo *et al.,* 2004). Some of the health benefits are due to its antagonistic effects exerted on several pathogens infecting the GIT such as *Salmonella, Shigella* and *Helicobacter* and also being of therapeutic importance against colon cancer (Maragkoudadis

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et al., 2005). Moreover, the health promoting properties of members of genus *Lactobacillus* can be elaborated by using it as a Probiotic, which is defined by Guarner and Shaafsma (1998) as "living microorganisms, which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition". *L. acidophilus* and *L. casei* after essential assessments are being used as efficient probiotics (Kleanhammer and Kullen, 1999; Mercenier *et al.,* 2003). For use in the foods, probiotic microorganisms not only should be capable of surviving passage through the digestive tract but also should have the capability to proliferate in the gut (Sgouras *et al.,* 2004). Our present study after careful assessment acknowledges the broad spectrum antimicrobial effect of our LAB isolates but it's still too early to recommend them for biopreservation until complete characterization of these strains is conducted. Although, acid production by LAB facilitates fermentation of many foods but the bacteriocin production plays a significant role in preservation due to its antibacterial activity against food pathogens. Therefore, future research should be focused on the mechanism of action of LAB and their bioactive agent and emphasis should be given on their characterization so that they could be commercially accepted as natural food preservatives to extend the shelf life of foods.

MATERIAL AND METHODS

Sources of lab Isolates and Indicator Bacteria.

For the isolation of Lactic acid bacteria (LAB) samples of milk, yogurt, vegetables, wheat dough and meat samples were collected from indigenous sources while feces samples were collected from healthy infant. Pure cultures of Gram positive and Gram negative bacteria used as indicator strains were

B. subtilis, B. cerus, C. diphtherae, S. fecalis, S. aureus, S.pneumonae S. pyogenes and *K. pneumonae, E. coli, S. dysenterae, P. aeroginosa, P. vulgaris, S. typhi* respectively, and were obtained from the culture collection unit of the Department of Microbiology, University of Karachi. All LAB isolates were maintained as frozen stocks at –20°C in MRS broth (Oxiod) containing 15% v/v glycerol. Whereas, all the Gram positive and negative indicator strains were stored in Nutrient broth (Merck) with 15 % v/v glycerol at -20°C. Cultures were propagated at least twice before use.

Isolation of Lab

Isolation of LAB was carried out following the standard plate count method where milk and yogurt samples were diluted serially in PBS (pH-7.4) while meat and vegetable and feces samples were homogenized in Ringers solution then serially diluted in PBS. Each diluted sample (100µl) was transferred on to prepoured MRS agar plates and uniformly spread on the surface of agar with the help of a sterile spreader. Discrete colonies of the isolates were sub-cultured to get a pure culture on the MRS agar plates. Altogether 118 isolates from different samples were examined macroscopically for colonial features and microscopically for cell morphological appearance. The isolates were also tested for catalase negative reaction.

Screening of the lab isolates for antibacterial activity

Preparation of crude culture filtrate

Pure cultures of all the LAB isolates were grown individually for 24 hrs at 37°C in MRS broth. The culture broths were centrifuged at 11291.8 × g for 15 min at 4□C. The clear supernatants obtained were used for the detection of antimicrobial activity in the following forms:

a. Cell free cultural supernatant (CFCS), filter sterilized by membrane filtration via filters (0.45 pore size) (Iwaki, Japan).
b. Neutralized cell free cultural supernatant (NCFCS), filter sterilized supernatant, adjusted to pH-6.5 by 1M NaOH.

Assays for antibacterial activity

The LAB isolates after preliminary identification were investigated for antibacterial activity against other LAB strains by stab and agar well method. To demonstrate the activity by stab method, the cultures were stabbed on prepoured MRS agar plates and incubated for 24 hrs at 37°C. Next day the cultures were exposed to chloroform for 15 min to kill the producer and then were overlaid with MRS soft agar seeded with indicator LAB strain, the plates were incubated at 37 °C for 24 hrs and zone of inhibition were observed and measured. All those LAB strains able to show activity by stab method were also screened by agar well method for the production of bacteriocin (Tagg and Mc Given, 1971). The CFCS (100 µl) were poured into the wells (diameter 9mm) made in MRS agar plates. The plates were then overlaid with soft agar containing indicator strain. The plates were kept at 4 °C for 30 min for diffusion of supernatant into the agar and then incubated at 37°C for 24 hrs. Plates were observed for the zone of inhibition.

LAB isolates were also screened for inhibitory activity against a wide range of gram positive and gram negative indicator strains (Table 1). The strains showing inhibitory activity were tested for bacteriocin production by agar well method (as described above) by using both the supernatants (CFCS & NCFCS). Wells were made of the same diameter on nutrient agar plates overlaid with nutrient soft agar seeded with gram positive and gram negative indicator strains. The wells were filled with 100 µl supernatant from producer strains the plates were kept at 4 °C for 30 min for proper diffusion of CFCS and NCFCS into the agar and then incubated at 37°C for 24 hrs.

Partial purification of crude cfcs of *Lactobacillus*, lbt-36.

Ammonium sulphate precipitation

The partially purified bacteriocin of LBT-36 was abtained as follows:

The producer strain was propagated in MRS broth at 37˚C until early stationary phase. The cells were removed by

centrifugation at 11291.8×g for 15 min at 4˚C. Cell free cultural supernatant was collected and was adjusted to pH 6.5 with 1M NaOH to exclude the antimicrobial effect of organic acid. Ammonium sulphate (80%) was added to the Neutralized CFCS and mixed, kept stirred overnight at 4°C on a magnetic stirrer (Dunn, Labortechnik GMBH). The mixture was centrifuged at 11291.8×g for 30min at 4°C, pellets were resuspended in 25ml of PBS (pH 7.4). Further the precipitates were exhaustingly dialyzed (12 kDa cut off) against the same buffer with several changes with fresh PBS and stirring on a magnetic stirrer for overnight at 4˚C. The PBS was removed to concentrate the proteins, by immersing the dialysis tube in 15% solution of PEG -6000 (Merck) for overnight with gentle stirring. The concentrated, partially purified sample containing the bacteriocin was collected and stored at -20ºC for further use.

The antagonistic activity of the concentrated sample as well as the filtrate was checked for the presence of bacteriocin activity against *K. pneumonae, B. subtilus* and *S. typhi* by agar well method.

SDS-PAGE

Following the method described by Laemmli (1970) for Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS -PAGE), a 12.5% concentrated resolving gel and 5.2% stacking gel was run in duplicate. To determine the apparent molecular weight and number of bacteriocins produced by LBT- 36, the partially purified sample was loaded to the gel with the high molecular weight and broad range markers (Bio-Rad Catalog no. 161-0304) supplied with a constant current of 25mA for 90 min. One of the gels was stained with Commasie Brilliant blue concentrate (Sigma, B-8647) for 45 min and then de-stained for overnight to visualize the emerging bands. The weight of the bacteriocin peptide was assessed by comparing the distance traveled by the peptide and the standard molecular weight markers. The other gel was kept unstained for overlaying with sensitive bacteria to see the activity of the inhibitory protein on gel by the method described by Bhunia *et al.,* 1992 and examined for zone of inhibition.

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RESULTS

Cultures of Lactic acid bacteria isolated from different sources were identified up to genus level on the basis of their cultural, morphological characteristics and a negative Catalase test. Amongst our entire LAB isolates the predominant genera were *Lactobacillus* and *Lactococcus*, majority of which were isolated from milk and yogurt samples. Out of the 118 LAB strains when tested for antibacterial activity, 71 exhibited antibacterial activity against other LAB strains by stab and agar well method with CFCS. (Fig.1). We found 67% producers from milk, 50% from yogurt, 40% from vegetable, 50% from wheat dough, 83%from meat and 20% from infant feces (Fig.1.). Although the Neutralized cell free cultural supernatant (NCFCS) obtained from the LAB isolates exhibited varying degree of inhibitory activity against bacteria other than LAB. Of the tested isolates, twenty three turned out to be producers of bacteriocin like inhibitory substance effective against Gram negative pathogens, while only eight amongst them exhibited inhibitory activity against Gram positive pathogens by agar well method (Table 1, 2; Fig.2a&2b).

Fig.1. Screening of LAB for antibacterial activity by Stab and agar well method

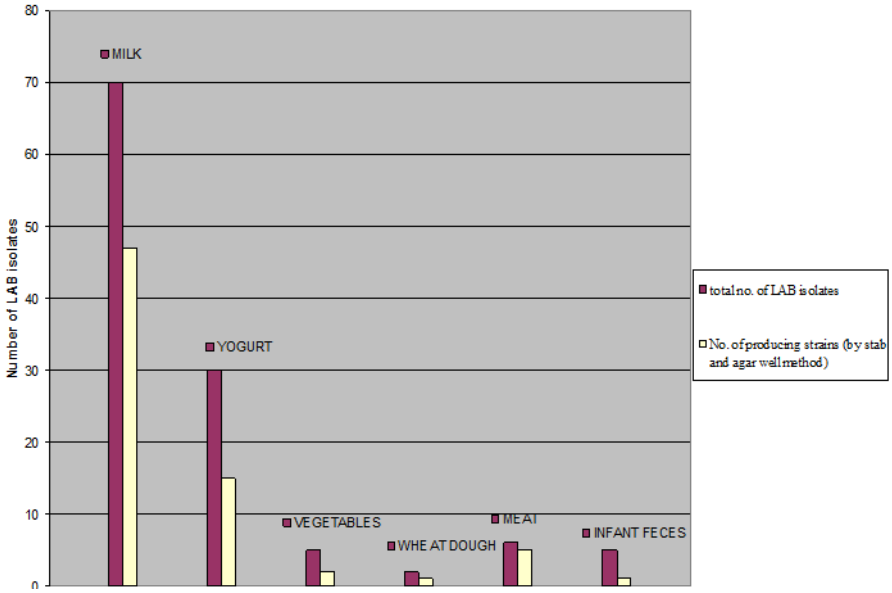

Table 1. Antagonistic acitivity of lab against gram positive bacteria by agar well method

LAB isolates as producers	Indicator strains						
	S.pyogenes	*S.fecali*	*S.pneumonae*	*B.subtilis*	*S.aureus*	*B.cereus*	*C.diphthe rae*
LBM-07							
CFCS	14	17	13	20	19	20	12
NCFCS	-	11	-	14	11	12	-
LBT-36							
CFCS	11	15	12	20	22	19	15
NCFCS	-	-	-	12	11	11	-
LBM-86							
CFCS	14	13	16	13	20	19	14
NCFCS	-	-	-	12	-	-	-
LBM-89							
CFCS	14	12	13	15	20	22	15
NCFCS	11	-	-	-	-	-	-
LCY-97							
CFCS	-	15	-	12	22	19	15
NCFCS	-	12	-	-	-	-	-
LBM-101							
CFCS	17	20	13	22	23	19	16
NCFCS	-	11	-	13	12	-	-
LBY-102							
CFCS	11	11	13	20	21	20	20
NCFCS	-	-	-	12	-	-	10
LBY-103							
CFCS	19	21	13	17	19	21	19
NCFCS	-	-	-	-	-	-	10

Activity in (mm) with well diameter (9mm) LBM-Lactobacillus isolate from milk
LBY-Lactobacillus isolate from yogurt
LCY-Lactococcus isolate from yogurt

Table 2. Antagonistic acitivity of lab against gram negative bacteria by agar well method

LAB Isolates as producers		Indicator strains Zone diameter(mm)				
		E.coli	*S.typhi*	*P.vulgarus*	*K.pneumonae*	*S.dysen*
LBM-07	CFCS	18	1	1	1	18
	NCFCS	14	5	6	3	14
LBM-09	CFCS	12	1	1	1	14
	NCFCS	10	5	9	2	-
LBM-24	CFCS	18	1	2	1	20
	NCFCS	12	3	0	3	16
LBM-25	CFCS	20	1	1	1	17
	NCFCS	13	4	2	2	-
LBM-27	CFCS	20	1	1	1	18
	NCFCS	-	4	4	2	11
LBM-32	CFCS	21	1	1	1	13
	NCFCS	13	8	3	2	11
LBW-35	CFCS	20	1	1	1	19
	NCFCS	14	7	7	4	-
LBT-36	CFCS	20	1	1	1	19
	NCFCS	-	7	9	4	12
LBM-38	CFCS	18	1	1	1	18
	NCFCS	-	7	4	3	-
LBM-49	CFCS	16	2	1	1	19
	NCFCS	12	1	8	5	-
LCM-52	CFCS	15	1	1	1	20
	NCFCS	-	8	9	3	11
LCM-82	CFCS	14	1	1	1	20
	NCFCS	-	7	4	2	11
LCY-83	CFCS	13	1	1	1	21
	NCFCS	12	8	2	1	12
LBM-86	CFCS	12	1	1	1	17
	NCFCS	-	8	3	4	-

Continue…….

LBM-88	CFCS	17	1	1	1	14
	NCFCS	-	9	5	4	11
LBM-89	CFCS	12	1	1	1	15
	NCFCS	-	4	9	9	12
LBM-92	CFCS	10	1	1	1	16
	NCFCS	10	2	4	6	-
LCM-94	CFCS	11	1	1	1	16
	NCFCS	11	2	2	6	11
LCY-97	CFCS	14	1	1	1	19
	NCFCS	-	8	3	4	11
LCY-98	CFCS	15	1	1	1	16
	NCFCS	10	7	4	4	-
LBM-101	CFCS	17	1	1	2	29
			7	7	0	
	NCFCS	12				-
LBY-102	CFCS	15	1	1	1	20
	NCFCS	12	7	6	9	11
LBY-103	CFCS	19	1	1	1	29
	NCFCS	10	9	8	9	-

Activity in mm with well diameter (9mm)

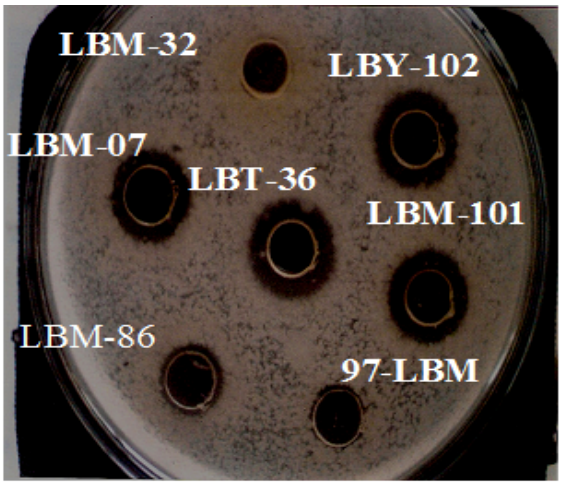

Fig.2a. Antagonistic activity of LAB isolates against *B.subtilis* by agar well method

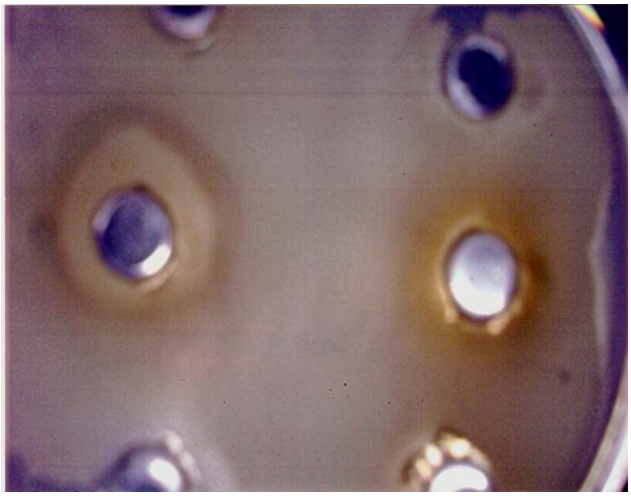

Fig. 3. Inhibition of *K.pneumonae* by the crude CFCS and the partially purified CFCS

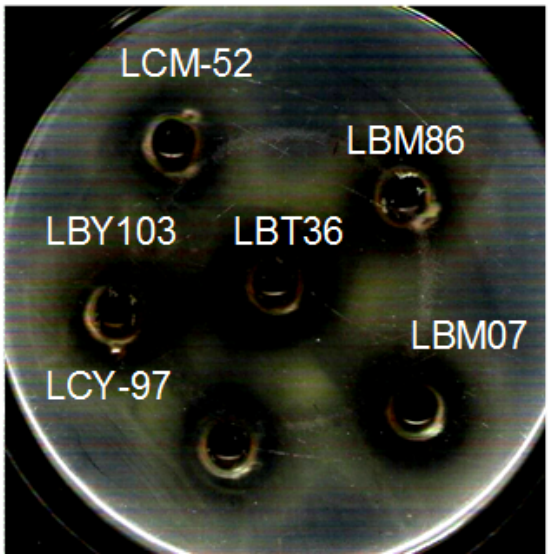

Fig. 2b. Antagonistic activity of LAB isolates against *E.coli* by agar well method

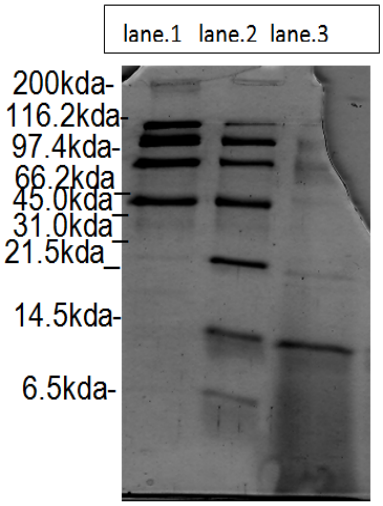

Fig.4. Peptide of 14.4kDa of the bacteriocin detected on SDS-PAGE.Lane 1:High mol.w markers; lane 2: Low mol. wt markers; lane3: bacteriocin peptide

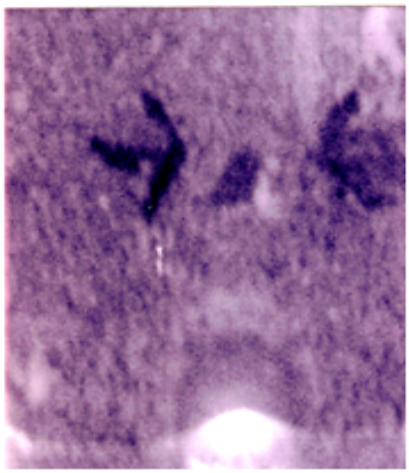

Fig.5. Inhibition of *K. pneumonae* by bacteriocin peptide on SDS-PAGE

The bacteriocin like inhibitory substance from LBT-36 after ammonium sulphate precipitation was checked against gram negative and positive indicators it seemed active only against *K. pneumonae* (Fig.3). This partially purified inhibitory protein, on SDS- PAGE gave one band of 14.4 kDa (Fig.4) and also showed inhibitory activity on gel (Fig.5).

DISCUSSION

The present study was primarily aimed to isolate strong bacteriocin producing LAB from indigenous sources. We found a significant number of producing strains from milk sample effective against other LAB strains. This owes to the fact that the numbers of milk samples screened were more as compared to other samples which provides a greater possibility of producing strains. The NCFCS of active milk isolates was tested against gram positive and negative bacteria where 64% were inhibitory towards gram negative while only 16 % were active against gram positive. As the activity of these LAB producers was by means of neutralized CFCS, hence it appears that these LAB cultures must have elaborated bacteriocin like substance because the inhibition by the crude CFCS is mainly due to organic acids or H_2O_2 or combined effect of both. The commercially used preservative nisin (bacteriocin) has been proved to be active against a wider range of Gram positive bacteria (Flores and Alegre, 2001).Whereas, a large number of our isolates displayed a much stronger antagonism against Gram negative rather than on Gram positive on the other hand Gilliland and Speck (1977) also reported a more pronounced inhibitory effect by *Lactobacilli* strains against Gram positive bacteria. It is also documented that bacteriocin of LAB can be antagonistic to various food borne pathogens such as, *S. aureus, L. monocytogenes, C. botulinum* and others (Delves-Broughton *et al*., 1996). The inhibition of *S. aureus, L. monocytogenes,* the two prominant food pathogens have also been reported by the bacteriocins elaborated by *L. sakei* CTC 494 and *L. salivarius* CTC 2197 (Silva *et al*., 2002). Also *L. casei*, *L. lactis*, and *L. acidophilus*, the commercially accepted *Lactobacillus* species, showed strong antagonism towards all *Salmonella* serotypes (Oyarzabal and Conner, 1995).

The bacteriocins and the bacteriocin like inhibitory proteins, although having similar site of action for inhibition differ in their mode of action which includes the mechanism in damaging the cell membrane and inhibiting the cell wall synthesis (Twomey *et al*., 2002). Another such study has provided us with evidence of Lactocins of *Lactobacillus* forming pores in the target cells due to the depletion of ATP (Li *et al*., 2005) and bacteriocin of *L. salivarius* CRL1328 showing a bacteriocidal mode of action against *E. feacalis* leading to vesiculization of the protoplasm and also pore formation (Ocana *et al*., 1999).

The *Lactobacillus* specie, LBT-36 seemed to have relatively strong antimicrobial activity and the bacteriocin elaborated by this specie after partial purification on SDS gel gave one band of 14.4 kDa of inhibitory peptide. Considering the molecular weight reported for bacteriocins produced by most LAB being 3-6kDa (Savadogo *et al.,* 2006), our bacteriocin has a higher molecular weight but bacteriocins with a higher molecular weight of 28 kDa and 37 kDa produced by *L. acidophilus* and *L. helveticus* have also been reported (Joerger and Klaenhammer, 1986). The activity of the bacteriocin produced by LBT-36 could have been of greater intensity after the antibacterial proteins in the CFCS got concentrated, but on the contrary the bacteriocin activity was not enhanced. This probably could be due to the presence of certain ingredients of the growth media which could not got rid of on partial purification. Such a phenomenon also correlates with the antagonism of Lactocin 160 produced by *L. rhamnosus* (Li *et al*., 2005). On the other hand, may be our isolate elaborated more than one bacteriocin, the other ones being of low molecular weight could not have been retained after dialysis which resulted in decrease in the activity. Hence the activity of the dialyzed sample may be only of the higher molecular weight protein.

Our study on the bacteriocins of LAB have still been very preliminary as far as the characteristic of bacteriocins are concerned and further research is required to investigate their physiochemical properties. The bacteriocin producing strains thus isolated and screened in future will also be assessed for their probiotic properties.

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