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Bacteriocin-like Substance Produced by *Lactobacillus salivarius* subsp. *salivarius* CRL1384 with Anti-*Listeria* and Anti-*Salmonella* Effects

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Abstract: *Lactobacillus salivarius* subsp. *salivarius* CRL1384 was isolated from the crop of a broiler chick and it was selected because of its lactic acid and other inhibitory substances production against *Listeria monocytogenes, Listeria* spp.*, Enterococcus hirae, Salmonella enterica* serovarieties Gallinarum, Pullorum, Enteritidis and Typhimurium. The antimicrobial substance showed to be different from organic acids and hydrogen peroxide. It was produced by *L. salivarius* CRL1384 not only in the presence of glucose but also of complex carbohydrates such as sucrose, maltose, molasses and brown sugar. Besides, this strain was bile salt resistant and maintained its property in the presence of 0.3% w/v. The chemical nature of the compound revealed that it was heat resistant (121°C for 15 min) and completely lost its activity after treatment with different proteolytic enzymes. It was not affected by lipase but α-amylase reduced its action and this effect was carbohydrate dependent. A bacteriocin solution of 200 AU mL^{-1} had a bactericidal effect on both *List. monocytogenes* and *Sal.* Enteritidis cells after 24 h of contact. The information for this bacteriocin production is encoded at chromosomal level. All these properties are very interesting and valuable and could turn this strain into a potential component of a poultry probiotic supplement.

Key words: Pultry, *Salmonella, Listeria monocytogenes, Lactobacillus salivarius*, probiotic, bacteriocin

Introduction

Lactic acid bacteria are an important part of healthy intestinal microflora of different hosts because they may inhibit pathogenic or spoilage bacteria (Piard and Desmazeaud, 1991,1992) through many mechanisms. In particular, *Lactobacillus salivarius* strains have been employed in probiotic supplements or competitive exclusion cultures not only for humans but also for chickens principally because of their high lactic acid production (Aiba *et al.*, 1998; Jin *et al.*, 1996; Miyamoto *et al.*, 2000; Weinack *et al.*, 1985). However, little is known about their bacteriocin synthesis ability; few articles report bacteriocin production by this micro-organism and none of them is of avian origin (Aiba *et al.*, 1998; Arihara *et al.*, 1996; Robredo and Torres, 2000; Ocaña *et al.*, 1999).

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Listeriosis and salmonellosis are very important infectious diseases for man, animal husbandry and food industry. Many strains of this bacterial genus have become highly resistant to antibiotics and natural alternatives are needed (Bailey *et al.*, 1990; Roberts and Wiedmann, 2003). In our group we are looking for lactic acid bacteria isolated from chickens that may inhibit pathogenic microorganisms with impact on human and poultry health. Our selection criterion was the antimicrobial substances production in the presence of glucose or more complex carbon sources, such as prebiotic molecules, in order to develop a poultry symbiotic supplement (Audisio, 1999; Audisio *et al.*, 1999a, b; Audisio *et al.*, 2000, 2001). Thus, around 200 *Lactobacillus* strains were isolated from the intestinal tract of commercial and free-range broiler chickens in Tucumán, Argentina and they were screened for the production of antimicrobial activity against *Listeria monocytogenes* and *Salmonellae* strains. These were avian host-specific (*Sal.* Gallinarum and Pullorum) and non host-specific (*Sal.* Enteritidis and Typhimurium) that are very important for human health. Only ten strains were found with those properties and one of them was studied in more detail in this study. We analyzed the antimicrobial spectrum of the substance synthesized by *Lactobacillus salivarius* CRL1384 against those pathogens and we tried to determine its physicochemical properties. Besides, we studied its growth and metabolism products in the presence of complex carbohydrate molecules such as brown sugar, molasses, sucrose and maltose.

Materials and Methods

Strains, Culture Media and Growth Conditions

Lactobacillus salivarius CRL1384 was isolated from the crop of a 1 week-old broiler chick (Audisio, 1999; Audisio *et al.*, 1999a) and it was identified by biochemical tests, carbohydrate fermentation pattern (APICH50) and 16S rRNA sequence. It was activated in LAPTg medium (*25*) and incubated at 37°C both under microaerophilic (about 7% v/v O_2 and 14% v/v CO_2 concentration) and/ or aerobic conditions to determine which atmosphere was the best for lactobacilli growth.

A list of the indicator strains tested for sensitivity is shown in Table 1. They were propagated in brain-heart-infusion broth (BHI, Difco) at 37°C. When an agar medium was required, 1.5% w/v granulated agar (Anedra) was added to the broth medium.

All strains were kept at 20°C in skim milk (9% w/v) with the addition of yeast extract (0.5% w/v).

Detection of Inhibitory Activity

Antagonistic substances from *L. salivarius* CRL1384 cell cultures were studied both in liquid an in solid media according to the following techniques: the well diffusion assay (Tagg and McGiven, 1970) and by the spot-on the lawn methods (Audisio 1999; Harris *et al.*, 1989; Lewus and Montville, 1991).

Antimicrobial Substance Physicochemical Characterization

To determine heat resistance, an aliquot of sterile CFS was heated to 121 °C for 15 min in an autoclave while another aliquot without thermal treatment was used as control. Then, the heated sample was cooled and assayed with the control one to determine antimicrobial activity.

The effect of the following proteolytic enzymes on the antimicrobial molecule was determined: trypsin, papain, pronase E, α-chymotrypsin, pepsin, nagarase and proteinase K (Sigma). Besides, lipase and α-amylase (Sigma) action was analyzed. All enzyme solutions were prepared to a final

Table 1: Indicator strains and their sensitivity to non treated cell-free supernatants of *Lactobacillus salivarius* CRL1384

Indicator strain	Source†	Inhibition zone (diameter in mm)‡
Enterococcus hirae 8043	ATCC	4
Enterococcus avium 31/96	Inst. Malbrán	-
Enterococcus faecium 1385	CRL	-
Lactobacillus spp. MCA18	CA	-
Salmonella enterica serovar Gallinarum 91/91	INTA	-
Salmonella enterica serovar Pullorum 91/91	INTA	-
Salmonella enterica serovar Enteritidis 90/390	INTA	-
Salmonella enterica serovar. Typhimurium 89/43	INTA	-
Listeria monocytogenes ScottA	IHT	12
Listeria innocua 7	INRA	12
Listeria spp. 00/270	Inst.Malbrán	4
Listeria spp. 01/2000	Inst.Malbrán	4
Listeria spp. 99/316	Inst.Malbrán	4
Listeria spp. 99/218	Inst.Malbrán	4
Listeria spp. 01/198	Inst.Malbrán	4
Listeria spp. 01/01	Inst.Malbrán	4
Listeria spp. 00-3/364	Inst.Malbrán	4
Listeria spp. 01/155	Inst.Malbrán	4
Listeria spp. 99/207	Inst.Malbrán	4
Listeria spp. 01/198	Inst.Malbrán	4
Klebsiella spp.	CA	-

†ATCC, American Type Culture Collection; CRL, Centro de Referencia para Lactobacilos; IHT, Karlsruhe, Germany; INRA, Unité de Recherches Laitiéres et Génetique, France; Inst. Malbrán, Instituto de Microbiologúa "Dr. Carlos Malbrán", Bs. As., Argentina; INTA, Instituto Nacional de Tecnología Agropecuaria, Balcarce, Bs. As., Argentina; CA, Dr. Carina Audisio (INIQUI-Universidad Nacional de Salta).
‡Determined by the well-difussion assay, -, no inhibition

concentration of 1 mg mL^{-1} in phosphate buffer (pH 7.00, 0.05 M), except for lipase and α-chymotrypsin which were resuspended in buffer Tris-HCl (pH 8.00, 0.05 M) added with CaCl$_2$ 0.01 M. In all assays, inhibitory action was tested against *List. monocytogenes* ScottA by the well-diffusion assay.

Antimicrobial Substance Titer Determination

It was determined by using a serial twofold dilution method and was expressed in arbitrary units per milliliter (AU mL^{-1}) (Daba *et al.*, 1991). *List. monocytogenes* ScottA was selected as the indicator strain because it was the most sensitive among all examined strains.

Effects of Different Carbon Sources and Bile Salts on Antimicrobial Substance Production

LAPTg broth without glucose (LAPT) was employed as a base medium. It was supplemented with sucrose, maltose, molasses and brown sugar to a final concentration of 1%w/v. Solutions at 20% w/v of sucrose, brown sugar, molasses and maltose were prepared with distilled water and sterilized by filtration (maltose) and autoclaving (sucrose, brown sugar and molasses). The chemical composition of molasses and brown sugar were analyzed by HPLC (High Pressure Liquid Chromatography) as in a previous work (Audisio *et al.*, 2001). They mainly contained sucrose and fructose and glucose to a lesser extent.

In order to determine the effect of α-amylase on the bacteriocin production in presence of different carbohydrates, aliquots of different CFS obtained from cultures of *L. salivarius* with molasses, brown sugar or sucrose, were treated with this enzyme to a final concentration of 1 mg mL^{-1}.

Bile salts were added to LAPT to a final concentration of 0.3% w/v since this amount may be considered similar to that present in healthy chicken intestine (Jin *et al.*, 1998).

Listeria and Salmonella Response

Bacteriocin action against *List. monocytogenes* ScottA and *Sal.* Enteritidis 86/360 cells was determined according to different techniques. One of them was similar to that proposed by Vlaemynck *et al.* (1994). The other method was the microplate technique (Daba *et al.*, 1993; Toba *et al.*, 1991) with modifications. Briefly, 200 µL bacteriocin (100 AU mL^{-1}) were placed in different wells and 20 µL of an overnight indicator strain culture (~ 10^9 CFU mL^{-1}) were added. The microplates were incubated at 25°C for 24 h. After this period, wells were observed for positive (turbidity) or negative pathogen growth. Viability of *Listeria* and *Salmonella* cells was analyzed in those cases that presented a weak turbidity to determine the bacteriocin effect.

SDS-PAGE

Twenty microliter samples were mixed with 7 µL of the following buffer solution: 20% glycerol, 1% bromophenol blue, 4.6% SDS, 1.5% Tris base and 10% ß-mercaptoethanol and heated at 100°C for 5 min. Twenty microliters, in duplicate, of each sample were analyzed by SDS-PAGE on a 5 and 20% uniform pore gel (Schägger and von Jagow, 1987). After 3 h electrophoresis at 75 V, the gel was removed and assayed for molecular weight estimation and biological assay. Molecular weight was determined by staining with Coomassie brilliant blue (CBB). A molecular weight marker kit (MW 200.0-2.5 kDa from Invitrogen) was used for the standard proteins. To determine biological activity, the gel was fixed for 30 min in a 20% isopropanol and 10% (w/v) acetic acid mixture and then washed in distilled water for 1 h with continuous stirring. A biological analysis was carried out according to Bhunia *et al.* (1987). Treated gel, as it was described below, was aseptically placed on BHI agar seeded with *List. monocytogenes* ScottA and the plate was incubated at 30°C for 24 h and examined for inhibition zones.

Plasmid Analyses

An overnight culture of *L. salivarius* CRL1384 in LAPTg broth (5 mL) was centrifuged (6,000 g for 10 min at 4°C); cells were recovered and plasmid presence was analyzed according to Bruno-Bárcenas *et al.* (1998).

Statistical Analyses

All analyses were carried out according to the Tukey test and they were considered significant at p<0.05 level. The assays were performed in triplicate.

Results and Discussion

Lactobacillus salivarius sub sp. salivarius CRL1384 was characterized as belonging to this genus and species by biochemical tests, carbohydrate fermentation patterns (APICH50, Biomérieux, France) and on the basis of its 16S rRNA sequences. Its cell-free supernatants, without any treatment, were studied according to the well-diffusion assay and some of them inhibited the animal and human pathogenic strains listed in Table 1.

Among the Gram-positive pathogens, *Listeria monocytogenes* ScottA and *Listeria innocua* 7 were more sensitive to this substance than the other *Listeria* spp. tested (Table 1). Therefore, the inhibiting effects seem to be a function of the bacterial strain rather than of the genus or species. This is an interesting result because it is well known that *Listeria* strains, in general, are very resistant to low pH (Roberts and Wiedmann, 2003; McLauchin *et al.*, 2004). In this case, lactic acid would not be the only inhibitory molecule involved. To a lesser degree *Enterococcus hirae* ATCC8043 and *Enterococcus avium* 31/96 were affected.

The Gram-negative pathogens revealed a very important fact. Many negative results by the well diffusion assay (Table 1) were observed when crude (final pH 4.0-4.5) supernatants of *L. salivarius* grown in broth were used. Completely different results were noticed when the lactobacilli cells produced their metabolites in a solid medium. *Salmonella enterica* serovar. Gallinarum, Pullorum, Enteritidis and Typhimurium were totally inhibited when the spot-on the lawn method was assayed. Obviously, in this case, both lactic acid and the other substances were present, but the same thing happened when the crude supernatant (pH 4.0-4.5) was tested by the well-diffusion technique in preliminary assays (Table 1).

The results obtained with the well diffusion assay agree with those of Lewus and Montville (1991). They hold that this method could give false negatives, probably due to a resistance of the agar media to the diffusion of some bacterial metabolites. In our experiments, different culture conditions may be the reason for the differences observed in the techniques assayed. As *L. salivarius* may produce a higher amount of the antibacterial substance in the solid medium, it may be concentrated near the producer colony or continuously excreted over the entire course of the antagonism test. Another feasible explanation could be a smaller effective concentration of the antagonistic substance in the cell-free supernatant due to an interaction between this substance and the bacterial surface.

The chemical nature of the antagonistic molecule, different from lactic acid or hydrogen peroxide, was proved because neither the cell-free supernatant at pH 6.0 nor the catalase treatment reduced the antibacterial effect observed for the non treated supernatant.

Taking into account all the results observed, we decided to characterize physicochemically the antimicrobial substance produced by *L. salivarius*. The molecule was shown to be of proteinaceous nature by protease inactivation (trypsin, papain, pronase E, α-chymotrypsin, proteinase K). As lipase did not significantly modify its inhibitory effect, we inferred that no lipidic regions were related to the active site. Besides, this peptide was highly resistant to heat because it retained full antibacterial activity (200 AU mL^{-1}) after being heated at 121°C for 15 min.

Due to all these characteristics this substance was considered to be a bacteriocin or a bacteriocin like substance (Piard and Desmazeaud, 1992; Tagg *et al.*, 1976; Jack *et al.*, 1995). These results coincide with those of other authors who report on bacteriocins synthesized by strains of *L. salivarius*, but none of the bacteria was of avian origin (Arihara *et al.*, 1996; Ocaña *et al.*, 1999). There is also information of a bacteriocin produced by *L. salivarius* isolated from pigs but it does not present anti-*Listeria* effects (Robredo and Torres, 2000).

All the results presented above were observed when *L. salivarius* CRL1384 was grown in the presence of glucose as the main carbon source. However, we were interested in the effect of more complex carbohydrate molecules on the antimicrobial properties of this lactobacillus, because we had already observed very significant results with another bacteriocin producing strain (Audisio 1999; Audisio *et al.*, 1999, 2001). Besides, other authors had mentioned the importance of a carbon source in bacteriocin synthesis (Pitt and Gaston, 1988).

Table 2: Effect of different carbon sources and bile salts concentrations on the bacteriocin production by *Lactobacillus salivarius* CRL1384

Medium			TITER[‡] (AU mL^{-1})
LAPT[†]			160
Supplemented	Carbohydrate % (w/v)	Bile salts % (w/v)	
Brown sugar	2	-	160
	2	0.3	160
Sucrose	2	-	80
	2	0.3	160
Molasses	2	-	160
	2	0.3	80
Maltose	1	-	160
	1	0.3	200
Glucose	1	-	160
	1	0.4	80

[†]Base medium without any carbon source, [‡]Titer was determined by the well-diffusion assay using *Listeria monocytogenes* ScottA as indicator strain

When *L. salivarius* grew in the presence of 1 or 2% w/v of molasses, brown sugar, maltose or sucrose, no differences were observed in its growth rate (data not shown). In all cases, after 12 h of incubation at 37°C the log number of viable microorganisms was 8.82±0.08. Besides, bacteriocin production remained without significant changes (Table 2). These results are quite different from those observed with *Ent. faecium* CRL1385 bacteriocin synthesis (Audisio *et al.*, 2001). In this case, the nature of the carbon source was the key for the bacteriocin synthesis.

Unexpected results appeared when bacteriocin, produced in presence of different carbohydrates, was treated with α-amylase from different origins. The antagonistic effect disappeared when bacteriocin was produced in cultures with molasses, brown sugar or sucrose, but it remained without significant changes in the presence of maltose or glucose. To discard a problem of contamination of our α-amylase stock, perhaps with a protease molecule, two different α-amylase samples were used and the same results were obtained. These results suggested that a carbohydrate moiety could be involved in the active site of this bacteriocin and therefore more specific structural studies would be necessary.

It is interesting to remark that even though *L. salivarius* produces its bacteriocin in the presence of 0.3% w/v of bile salts in all the situations studied, the amount is variable and dependent on the carbon source employed (Table 2). Thus, the greatest titer (200 AU mL^{-1}) was obtained when the carbon source was maltose. Considering that maltose is the main product of starch digestion by β-amylase in the chicken gastrointestinal tract, the results obtained in this last experiment suggested that *L. salivarius* CRL1384 could be included in a probiotic formula to protect chickens against pathogen infection by *Listeria* and *Salmonella* strains.

The mode of action of the bacteriocin on *List. monocytogenes* ScottA cells and on *Salmonellae* strains was studied in detail. *List. monocytogenes* cells lost around two log of their viability after 2 h of contact with two bacteriocin concentrations (100 and 200 AU mL^{-1}) at 30°C. However, as no changes in the optic density of this cell-suspension were registered during the period tested, a lytic action was discarded. A bactericidal effect was observed in both cases against this Gram-positive pathogen (Fig. 1). These results were confirmed by the microplate technique and no significant modifications on the cell-viability were observed during a longer period of contact (24 h instead of 2 h) at 30°C. The viable cell number decreased from 9.14±0.05 to 6.48±0.12.
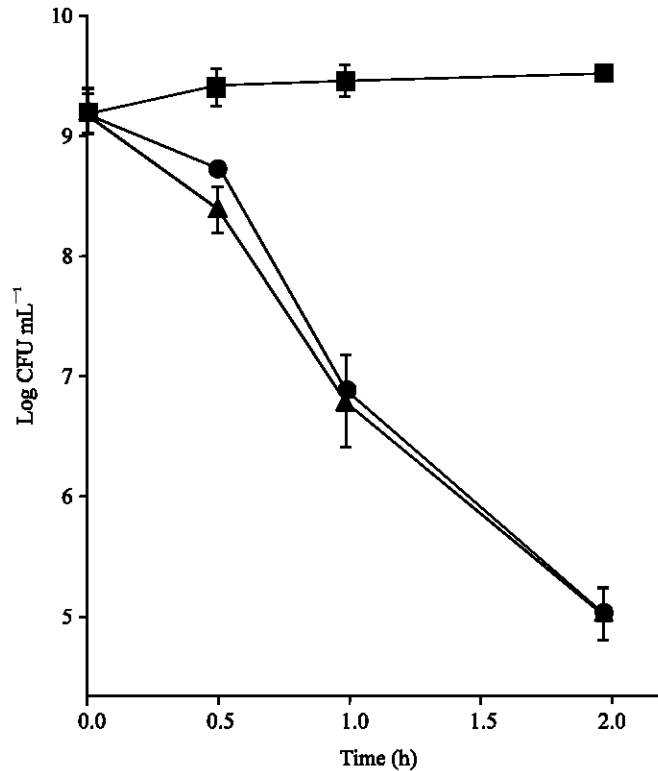
Fig. 1: Mode of action of bacteriocin synthesized by *L. salivarius* CRL1384 on resting cells of *List. monocytogenes* ScottA (■, without and, in contact with ●, 100 AU mL⁻¹ and ▲, 200 AU mL⁻¹)

On the other hand, the addition of 100 AU mL⁻¹ to a cell-suspension of *Sal.* Enteritidis resulted in a drop in the log number of viable cells; initially it was 9.32±0.16 and after 24 h of contact, 4.65±0.49.

The SDS-PAGE analysis of different crude samples confirmed the presence of a unique band with biological activity against *List. monocytogenes* ScottA. Its molecular weight was estimated around 6 kDa by Coomassie blue tinction of the gel (data not shown). This value correlated quite well with the thermostability that this bacteriocin presented and coincides with those of other authors who proposed that many bacteriocins of different lactic acid bacteria are relatively small molecules of uncomplicated structure (Ennahar *et al.*, 2001).

L. salivarius CRL1384 did not present any plasmids with the techniques employed in this work. Therefore it could be inferred that the information for its bacteriocin production is encoded at chromosomal level. This result is very significant because it shows that the property by which *L. salivarius* CRL1384 was selected is stable.

Conclusions

Lactobacillus salivarius CRL1384, a strain of avian origin, synthesizes a bacteriocin with a broad inhibitory spectrum. Its action is directed to *Listeria monocytogenes* ScottA, *Listeria* spp.

and *Salmonellae* strains. Bacteriocin production was maintained in the presence of bile salts and complex carbohydrates and was stimulated in cultures with maltose. *L. salivarius* CRL1384 could be included in a probiotic formula to protect chickens against potential infection by *Listeria* or *Salmonella* strains.

Acknowledgements

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