Influence of vitamins and osmolites on growth and bacteriocin production by *Lactobacillus salivarius* CRL 1328 in a chemically defined medium

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Abstract: The aim of this study was to analyze the influence of vitamins, glycerol, and salts on the growth and bacteriocin production by *Lactobacillus salivarius* CRL 1328, a human vagina isolate, by using a chemically defined medium to determine the optimal conditions for salivaricin production. The single omission of D-biotin, thiamine, *p*-aminobenzoic acid, folic acid, or cyanocobalamin did not affect the bacterial growth, whereas the removal of nicotinic acid, riboflavin, and pyridoxal produced a decrease of about 30% in the growth rate. Maximum salivaricin activity was observed after the addition of 5 or 10 g/L of NaCl. On the basis of the nutritional requirements and the levels of salivaricin production, a new optimized and simplified defined medium (SDM–NaCl) for *L. salivarius* CRL 1328 bacteriocin production was formulated. The kinetics of salivaricin production in SDM–NaCl and in the complex media LAPTg revealed that bacteriocin production was growth linked. A combination of tricine – sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine–SDS–PAGE), Lumitein protein gel staining, and a bioassay for antibacterial activity indicated that the molecular mass of salivaricin CRL 1328 is about 4.5 kDa. The partially purified bacteriocin, obtained from SDM–NaCl after concentration, allowed for the design of a relatively simple method for the recovery of a biologically active protein.

Key words: bacteriocin, *Lactobacillus*, salivaricin, vagina, lactic acid bacteria.

Résumé : Le but de cette étude était d'analyser l'influence des vitamines, du glycérol et des sels sur la croissance et la production de bactériocines par *Lactobacillus salivarius* CRL 1328, un isolat vaginal humain, en utilisant un milieu de culture chimique défini, afin de déterminer les conditions optimales de production de salivaricine. L'omission simple de D-biotine, de thiamine, d'acide *p*-aminobenzoïque, d'acide folique ou de cyanocobalamine n'affectait pas la croissance bactérienne, alors que le retrait d'acide nicotinique, de riboflavine et de pyridoxal diminuait le taux de croissance d'environ 30 %. L'activité maximale liée à la salivaricine était observée après l'ajout de 5 ou 10 g/L de NaCl. Sur la base des besoins nutritionnels et des niveaux de production de salivaricine, un nouveau milieu défini optimal et simplifié (SDM–NaCl) a été formulé pour la production de salivaricine par *L. Salivarius* CRL 1328. Les cinétiques de production de salivaricine dans le milieu SDM-NaCl et dans le milieu complexe LAPTg ont révélé que la production de la bactériocine était liée à la croissance. La combinaison d'une électrophorèse sur gel de polyacrylamide Tricine–SDS, d'une coloration protéique sur gel par la Lumitein et d'un essai biologique de l'activité antibactérienne a indiqué que le poids moléculaire de la salivaricine de CRL 1328 est d'environ 4,5 kDa. La bactériocine partiellement purifiée du milieu SDM–NaCl concentré a permis d'élaborer une méthode relativement simple de récupération de la protéine biologiquement active.

Mots-clés : bactériocine, *Lactobacillus*, salivaricine, vagin, bactérie lactique.

[Traduit par la Rédaction]

Introduction

Lactobacilli are the dominant microorganisms of the vagina in women of childbearing age, playing a pivotal role in preventing colonization by undesirable organisms, including those responsible for bacterial vaginosis, urinary tract infections, aerobic vaginitis, and sexually transmitted diseases (Vitali et al. 2007). They may act by stimulating the immune system, by competing with other microorganisms for adherence to the vaginal epithelium, and by producing inhibitory metabolites such as organic acids, H_2O_2, or bacteriocins (Reid and Bruce 2006). Bacteriocins are a heterogeneous family of generally small, heat-stable peptides with potent antimicrobial activity that are produced by many bacterial species, including some probiotic strains (Cotter et al. 2005). Bacteriocins have potential biomedical applications, providing valuable alternatives to antibiotics for the treatment of human and animal infections (Twomey et al. 2002).

Salivaricin CRL 1328 is a heat-stable bacteriocin produced by *Lactobacillus salivarius* CRL 1328, a strain isolated from human vagina (Ocaña et al. 1999). This bacteriocin is active against potentially urogenital pathogenic bacteria such as *Enterococcus faecalis*, *Enterococcus faecium*, and *Neisseria gonorrhoeae* (Ocaña et al. 1999). The growth and bacteriocin production kinetics by *L. salivarius* CRL 1328 in the com-

Received 9 September 2008. Revision received 3 November 2008. Accepted 20 November 2008. Published on the NRC Research Press Web site at cjm.nrc.ca on 31 March 2009.

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Table 1. Composition of the complete chemically defined medium (complete CDM), the chemically defined medium containing NaCl (CDM–NaCl), and the new optimized defined medium (SDM–NaCl).

	Concentration (g/L)		
Constituent	Complete CDM	CDM–NaCl	SDM–NaCl
Glucose	10	10	10
Sodium acetate	5	5	5
KH_2PO_4	3	3	3
K_2HPO_4	3	3	3
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2
$MnSO_4 \cdot 4H_2O$	0.05	—	—
$FeSO_4 \cdot 7H_2O$	0.02	—	—
NaCl	—	10	10
Tween 80	1	1	1
L-Alanine	0.10	0.10	0.10
L-Arginine	0.10	0.10	0.10
L-Asparagine	0.20	—	—
L-Aspartic acid	0.20	0.20	0.20
L-Cysteine	0.20	0.20	0.20
L-Glutamine	0.20	—	—
L-Glutamic acid	0.20	0.20	0.20
Glycine	0.10	0.10	0.10
L-Histidine	0.10	0.10	0.10
L-Isoleucine	0.10	0.10	0.10
L-Leucine	0.10	0.10	0.10
L-Lysine	0.10	0.10	0.10
L-Methionine	0.10	0.10	0.10
L-Phenylalanine	0.10	0.10	0.10
L-Proline	0.10	0.10	0.10
L-Serine	0.10	0.10	0.10
L-Threonine	0.10	0.10	0.10
L-Tryptophan	0.10	0.10	0.10
L-Tyrosine	0.10	0.10	0.10
L-Valine	0.10	0.10	0.10
Nicotinic acid	0.001	0.001	0.001
Pantothenic acid	0.001	0.001	—
Pyridoxal	0.002	0.002	0.002
Riboflavin	0.001	0.001	0.001
p-Aminobenzoic acid	0.01	0.01	—
Folic acid	0.001	0.001	—
Cyanocobalamin	0.001	0.001	—
D-Biotin	0.01	0.01	—
Thiamine	0.001	0.001	—
Adenine	0.01	0.01	0.01
Guanine	0.01	0.01	0.01
Inosine	0.01	—	—
Xanthine	0.01	0.01	0.01
Orotic acid	0.01	0.01	—
Uracil	0.01	0.01	0.01
Thymine	0.01	—	—

plex de Man–Rogosa–Sharpe (MRS) or LAPTg media have been the subject of previous investigations (Ocaña et al. 1999; Juarez Tomas et al. 2002). The optimal growth conditions were recorded in MRS broth, whereas the maximum bacteriocin activity was obtained in LAPTg at 37 °C with an initial pH of 6.5 (Juarez Tomas et al. 2002). However, for investigating the effect of different nutrients and salts on bacteriocin production, a chemically defined medium (CDM) is required. A CDM containing a carbohydrate source, mineral salts, amino acids, vitamins, and nucleic acid bases is therefore more suitable to investigate the influence of nutrients on the growth and the basic needs for the synthesis of bacteriocin in lactic acid bacteria. This study was undertaken to analyze, using a CDM, the influence of different culture medium components, such as vitamins, nitrogen sources, and salts, as well as the effect of osmotic stress conditions, on the growth and salivaricin production by *L. salivarius* CRL 1328. This investigation allowed us to optimize a new simplified defined medium (SDM–NaCl) for salivaricin CRL 1328 production. In addition, the molecular mass of the partially purified salivaricin CRL 1328, obtained from SDM–NaCl after concentration, was assessed by a combination of Tricine–SDS–PAGE and a bioassay for its antibacterial activity.

Materials and methods

Microorganisms, media, and growth conditions

The bacteriocin-producing *L. salivarius* CRL 1328 and the indicator strain *E. faecalis* UNT were obtained from the CERELA Culture Collection, Tucumán, Argentina, and the Instituto de Microbiología of the Universidad Nacional de Tucumán, Argentina, respectively. Both strains were originally isolated from human vagina (Ocaña et al. 1999).

Growth and salivaricin CRL 1328 production were tested in a CDM (pH 6.5) adapted from that described by Hebert et al. (2004) (Table 1). Media were sterilized by filtration through a cellulose acetate membrane (0.22 μm pore size; Millipore, Billerica, Massachusetts). Working cultures of *L. salivarius* were propagated in LAPTg (Raibaud et al. 1973) at 37 °C for 16 h under quiescent aerobic conditions. To eliminate carry-over nutrients, the cells were harvested by centrifugation (8000*g*, 15 min), washed twice in sterile saline solution (8.5 g/L NaCl), and resuspended in this solution to the original volume. This cell suspension was used to inoculate the different media at an initial optical density at 560 nm (OD_{560}) of 0.07. Bacterial growth was monitored by determining the OD_{560} and the data obtained were correlated with the dry cell mass. Cells were harvested by filtration (0.22 μm) and dried at 120 °C to constant mass. A change of 1 unit of optical density was shown to be equivalent to an increase of 0.35 g dry cell mass per litre.

When indicated, the CDM was supplemented with NaCl (5, 10, or 20 g/L), 10 g/L $CaCl_2$, 50 g/L glycerol, or high (10-fold) concentrations of each of 18 amino acids.

The following terms were used to describe the relationship between medium components and growth, as determined by the single omission technique. A constituent was considered essential if its omission caused less than half maximum growth rate of the positive control, stimulatory when in its absence the growth rate was between 50% and 80% of that observed in complete CDM, and nonessential when the growth rate was 80% (or more) of that obtained in the complete CDM.

Table 2. Effects of salts and glycerol on the growth parameters, water activity, and salivaricin production by *Lactobacillus salivarius* CRL 1328 grown on chemically defined medium (CDM).

				Salivaricin CRL 1328 production (AU/mg protein)	
Additive	Growth rate (h^{-1})[a]	OD_{max} (560 nm)[b]	a_w	Exponential growth phase[c]	Stationary growth phase[d]
None	0.43±0.02	1.47±0.08	0.976±0.005	144.1±8.0	288.3±11.5
$CaCl_2$ (10 g/L)	0.42±0.03	1.35±0.06	0.976±0.005	143.5±8.5	291.2±12.8
NaCl (5 g/L)	0.43±0.02	1.39±0.07	0.971±0.005	585.5±15.3*	1171.4±35.1*
NaCl (10 g/L)	0.42±0.02	1.42±0.06	0.970±0.005	589.8±21.8*	1174.3±32.6*
NaCl (20 g/L)	0.34±0.01*	1.05±0.05*	0.963±0.005	592.5±16.5*	596.5±21.5*
Glycerol (50 g/L)	0.39±0.02*	1.19±0.06*	0.963±0.005	288.3±12.3*	576.3±17.9*

Note: Note: *$p \leq 0.05$ vs. basal CDM. AU, arbitrary units; a_w, water activity.

[a]Values are the means and standard deviations from three independent experiments.

[b]Growth rate was estimated by following turbidimetry at 560 nm; values obtained in the stationary phase represent the OD_{max}.

[c]Samples were taken after 4 h of growth.

[d]Samples were taken after 12 h of growth.

Table 3. Effects of single omissions of vitamins from the complete CDM–NaCl on the growth of *Lactobacillus salivarius* CRL 1328.

Omitted vitamin	Growth rate (h^{-1})[a]	OD_{max} (560 nm)[b]
None	0.43±0.02	1.47±0.08
Nicotinic acid	0.31±0.02*	0.80±0.05*
Pantothenic acid	0.42±0.02	1.39±0.06
Pyridoxal	0.30±0.01*	0.79±0.06*
Riboflavin	0.29±0.01*	0.70±0.04*
Folic acid	0.44±0.02	1.41±0.07
D-Biotin	0.44±0.03	1.40±0.07
Cyanocobalamin	0.43±0.02	1.35±0.06
Thiamine	0.42±0.02	1.39±0.07
p-Aminobenzoic acid	0.43±0.02	1.37±0.08

Note: Note: *$p \leq 0.05$ vs. CDM–NaCl. CDM–NaCl, chemically defined medium with 10 g/L NaCl.

[a]Values are the means and standard deviations from 3 independent experiments.

[b]Growth rate was estimated by following turbidimetry at 560 nm; values obtained in the stationary phase represent the OD_{max}.

Water activity (a_w) measurements

Water activity of the culture media was measured at 40 °C by using a Thermoconstanter TH200 instrument with a TH temperature controller (0–50 °C setting range), a BSK sensor, and a RTD-200 transmitter with a humidity range of a_w from 0.005 to 1.000 (Novasina, Zürich, Switzerland). The humidity equilibrium was determined until the measurements remained unchanged (after 48 h).

Bacteriocin activity

At regular time intervals, samples were withdrawn aseptically from the culture medium to determine bacteriocin activity by an agar-well diffusion assay (Jack et al. 1995). The supernatant fluids were separated by centrifugation (8000*g*, 10 min, 20 °C), adjusted to pH 6.5 with 2 N NaOH, and treated with 1000 U/mL of catalase (Sigma-Aldrich, St. Louis, Missouri) to exclude the antimicrobial effects of organic acids and hydrogen peroxide, respectively. The supernatant was then sterilized through a filter (0.22 μm pore size; Millipore) and 2-fold dilutions of the cell-free supernatant were made in sterile fresh LAPTg medium. Aliquots (25 μL) of the diluted samples were poured into the 4 mm holes of the LAPTg agar plates (1% agar), which contained 1×10^6 CFU/mL of the indicator strain *E. faecalis* UNT in log growth phase. The plates were incubated for 5 h at room temperature and then for 24 h at 37 °C. An arbitrary unit (AU/mL) was defined as the reciprocal of the highest dilution that produced an inhibition halo. Specific activity was expressed as that unit per milligram of protein.

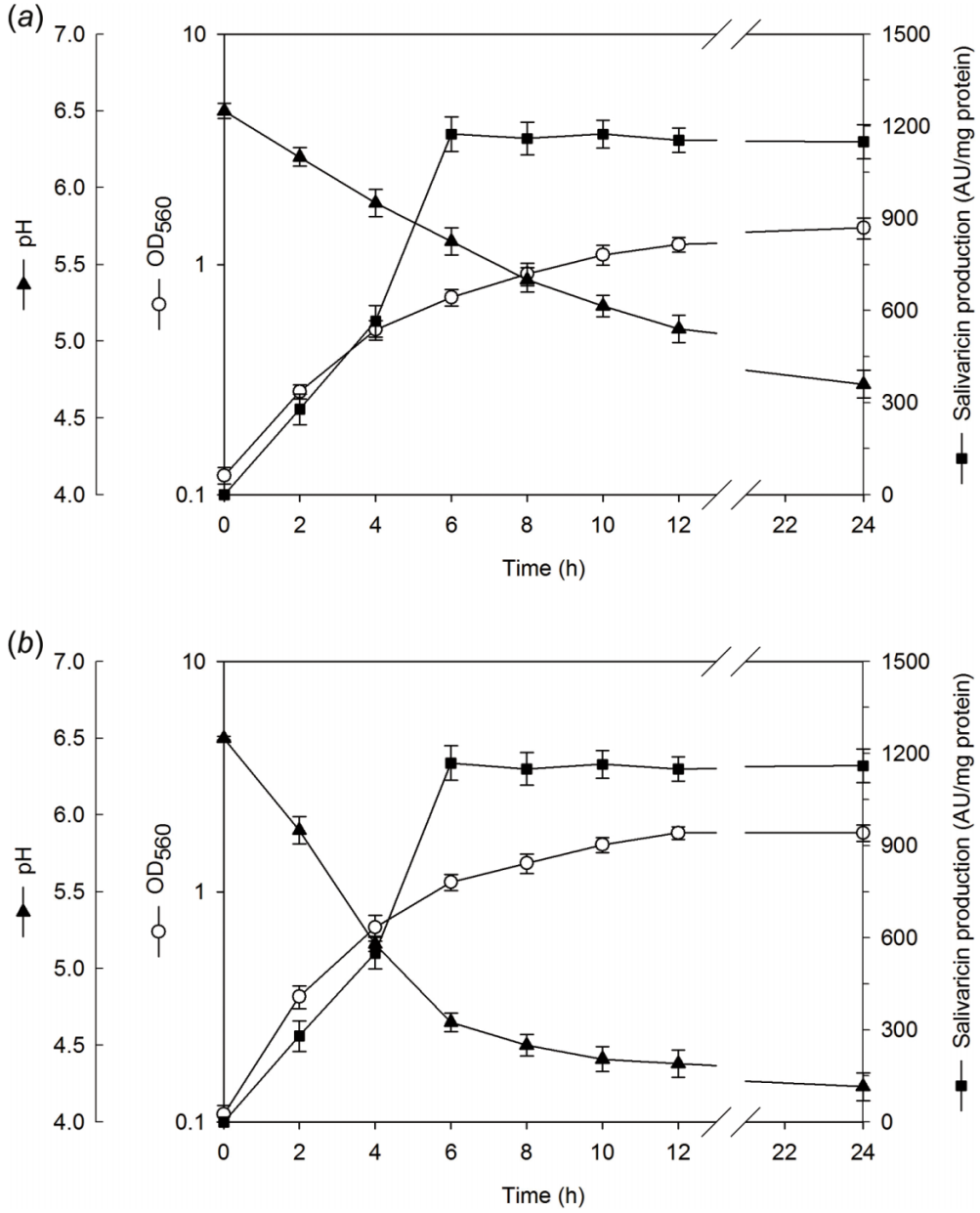
Protein determination

Protein concentration was determined by using a Quant-it protein assay kit (Invitrogen, Buenos Aires, Argentina) according to the manufacturer's instructions.

Molecular mass determination

Lactobacillus salivarius CRL 1328 was harvested from SDM–NaCl cultures by centrifugation (8000*g*, 10 min, 4 °C) after 16 h of fermentation at 37 °C. The supernatant fraction, adjusted to pH 6.5 and treated with catalase as described above, was filtered through 0.22 μm filters (Millipore). This material was referred to as cell-free crude supernatant fluid. Salivaricin CRL 1328 was concentrated by ultrafiltration by using filter units with a nominal molecular mass limit of 3000 Da (Centricon-3 concentrators; Amicon, Beverly, Massachusetts). The samples were then loaded on 2 parallel SDS gels, along with polypeptide SDS–PAGE molecular mass standards (Bio-Rad Laboratories, Hercules, California). The gels were prepared with a 6% acrylamide–bisacrylamide stacking gel and a 17% separating gel for Tricine–SDS–PAGE (Schagger and von Jagow 1987). Separation was performed at 100 mA for 6 h by using a vertical slab gel apparatus, the Mini PROTEAN 3 (Bio-Rad). One of the gels was stained either with Coomassie Brilliant Blue R-250 or Lumitein protein gel stain (Biotium, Hayward, California). The other gel was used for activity detection: the gel was first soaked for 2 h in fixation solution (50% methanol, 10% acetic acid), rinsed with distilled water 6 times during 15 min, and placed onto 1% LAPTg agar seeded with the in-

Fig. 1. Kinetics of salivaricin production during the growth of *Lactobacillus salivarius* CRL 1328 in SDM–NaCl (*a*) and LAPTg (*b*). Cell growth (○), pH (▲), and bacteriocin activity (■). SDM–NaCl, new optimized and simplified defined medium.



dicator strain. The plate was later incubated at 37 °C for 24 h. The molecular mass was estimated by comparison of the mobility of the inhibition zone with that of the molecular mass markers (polypeptide molecular mass standards; Bio-Rad) run simultaneously.

Statistical analysis

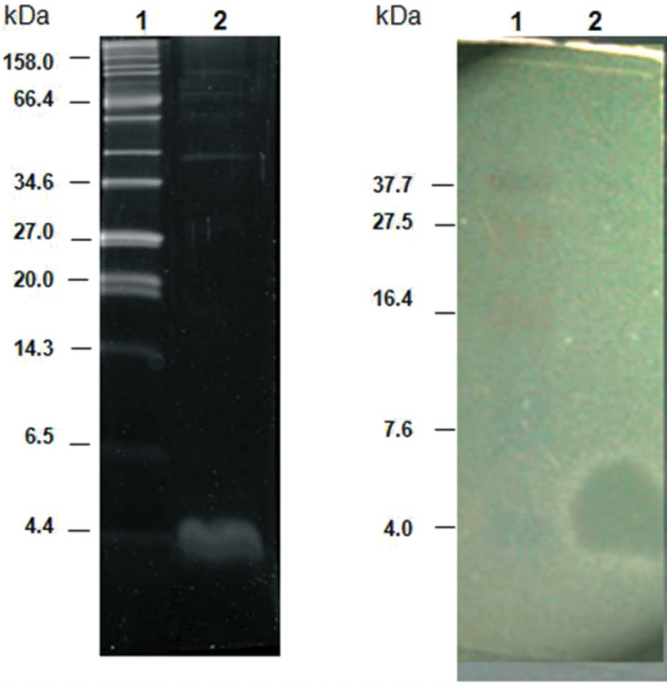
All the assays were carried out in triplicate, and results were expressed as mean values with standard deviations. Statistical analyses were performed by using Minitab 14 software (State College, Pennsylvania). Comparisons were accomplished by the analysis of variance general linear model, followed by Tukey's post hoc test, and $p < 0.05$ was considered as differences being statistically significant.

Results

Influence of vitamins, salts, and osmotic conditions on the growth and salivaricin CRL 1328 production by *L. salivarius* CRL 1328

To determine the effect of $CaCl_2$ and NaCl on the growth and bacteriocin production by *L. salivarius* CRL 1328, a series of omission and addition experiments were made. Previously, we observed that several compounds of the original CDM described by Hebert et al. (2004), including $MnSO_4$, $FeSO_4$, asparagine, glutamine, inosine, orotic acid, and thymine, did not have any effect on *L. salivarius* CRL 1328 cell growth and bacteriocin production (unpublished data). Therefore, these compounds were omitted from the original

Fig. 2. Detection of salivaricin CRL 1328 and its activity in a Tricine–SDS–PAGE. (*a*) Lumitein staining; lane 1, protein marker (New England Biolabs, Beverly, Massachusetts); lane 2, cell-free crude supernatant concentrated by ultrafiltration (cutoff of 3 kDa). (*b*) Bacteriocin activity as visualized by an activity overlaid assay using *Enterococcus faecalis* UNT as the indicator strain. Lane 1, kaleidoscope prestained polypeptide standards (Bio-Rad Laboratories, Hercules, California); lane 2, cell-free crude supernatant concentrated by ultrafiltration. Molecular masses (in kDa) of reference proteins are indicated.



CDM (Table 1). The cell growth was not affected upon the addition of $CaCl_2$ or NaCl up to 10 g/L, whereas increasing the amount of NaCl to 20 g/L resulted in a decreased growth rate (Table 2) and in a longer lag phase. The salivaricin production, evaluated at the exponential and stationary phases of growth, was maximal in the presence of 5 and 10 g/L NaCl, obtaining an approximately 4-fold increase in the bacteriocin production compared with complete CDM (Table 2). On the other hand, the addition of $CaCl_2$ had no effect on salivaricin production (Table 2). Although, NaCl at 20 g/L was the most adverse compound on the cell growth, the a_w obtained with this osmolite was similar to the values with 50 g/L glycerol. However, the salivaricin production in both osmotic conditions was similar (Table 2).

Based on the obtained results, CDM containing 10 g/L NaCl (CDM–NaCl) was chosen to analyze the influence of vitamins and different nitrogen sources on the growth and salivaricin production by *L. salivarius* CRL 1328 (Table 3). The omission of pantothenic acid, D-biotin, thiamine, *p*-aminobenzoic acid, folic acid, or cyanocobalamin had no effect on the cell growth (μ = 0.43 h^{-1}) or on salivaricin production (Table 3). The removal of nicotinic acid, riboflavin, and pyridoxal resulted in a decreased growth rate (μ = 0.30 h^{-1}), whereas the bacteriocin production was not affected (Table 3). When all vitamins were removed from the CDM–NaCl, the maximum specific growth rate and salivaricin production were reduced around 40% (data not shown). On the basis of these nutritional requirements and the values of salivaricin production, a new optimized and simplified defined medium (SDM–NaCl) for *L. salivarius* CRL 1328 bacteriocin production was formulated (Table 1).

To determine the effect of the nitrogen source on the growth and salivaricin production, *L. salivarius* CRL 1328 was grown on SDM–NaCl supplemented with higher concentrations of free amino acids (10-fold). The addition of high concentrations of free amino acids (10-fold) had no effect on the growth (μ = 0.43 h^{-1}) or on salivaricin production (1170 UA/mg protein).

In view of the obtained results, the growth kinetics and the salivaricin production by *L. salivarius* CRL 1328 in SDM–NaCl and in the complex medium LAPTg were compared (Fig. 1). The specific growth rate in LAPTg (μ = 0.52 h^{-1}) was higher than that observed in SDM–NaCl (μ = 0.43 h^{-1}). In contrast, the specific salivaricin production in both media was similar; that is, showing a concomitant increase with cell growth up to the mid-exponential growth phase and remaining in high levels until the stationary growth phase (Fig. 1).

Characterization of salivaricin CRL 1328 activity by Tricine–SDS–PAGE

The cell-free culture supernatant (640 AU/mL) was concentrated 16 times by ultrafiltration (10 240 AU/mL) and the salivaricin activity was detected after Tricine–SDS–PAGE (Fig. 2). In the discontinuous Tricine–SDS–PAGE analysis, the purified bacteriocin gave a major band, which corresponded to an estimated molecular mass of approximately 4.5 kDa (Fig. 2*a*). In the post-electrophoretic detection analysis, this band was active against the indicator strain *E. faecalis* UNT (Fig. 2*b*).

Discussion

Some *Lactobacillus* species play a major role in maintaining a healthy vaginal ecosystem. Through the production of H_2O_2, organic acids, and bacteriocins, lactobacilli prevent the establishment and overgrowth of other bacteria (mainly pathogens) that could otherwise create an altered vaginal microflora; for example, as in the case of bacterial vaginosis (Reid and Bruce 2006). Although the availability of antibiotics to treat these infections has significantly improved the health and well being of women, their overuse has contributed to the emergence of antibiotic-resistant bacteria. Salivaricin CRL 1328, a bacteriocin produced by *L. salivarius* CRL 1328, could be used as an alternative to the application of antibiotics either in a prophylactic or therapeutic way to prevent urogenital infections. For the potential commercial use of bacteriocins in both clinical and industrial applications, the optimization of their production is of fundamental importance. Therefore, to determine the optimum culture conditions for bacteriocin production, a chemically defined medium was used. Stern et al. (2006) used a minimal medium containing K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4$, glucose, histidine, and arginine for the growth and bacteriocin production by *L. salivarius* NRRL B-30514. However, *L. salivarius* CRL 1328 was not able to grow in the presence

of histidine and arginine as the only nitrogen sources (data not shown). Our observations are supported by phenotypic predictions obtained from the genome sequence information available for *L. salivarius* UCC118, which revealed auxotrophy for 8 amino acids (Claesson et al. 2006). The SDM–NaCl optimized in this work, containing 18 amino acids and 3 vitamins, supported a sustained growth at a reasonably high rate for *L. salivarius* CRL 1328. The levels of salivaricin produced in SDM–NaCl were similar to those obtained in LAPTg (1171 AU/mg) (Table 2). In this medium, the production of salivaricin CRL 1328 displayed a primary metabolite kinetics, as it was produced during the exponential growth phase (Fig. 1), remaining at high levels at the stationary growth phase. The fate of bacteriocin activity in the stationary phase is bacteriocin-dependent. Whereas for some bacteriocins the activity decreases slightly (Moretro et al. 2000), for others there is an almost complete loss of activity (Lejeune et al. 1998) or are shown to be stable (Nilsen et al. 1998).

Some vitamins, including nicotinic acid, riboflavin, and pyridoxal, were stimulatory for the growth, although no effect on salivaricin production was observed. Geshnizgani and Onderdonk (1992) reported that the supplementation of a chemically defined medium with vitamins provides more suitable conditions for the growth of vaginal microorganisms in vitro. Moreover, Reid et al. (1998) demonstrated that *Lactobacillus rhamnosus* GR-1 survived better than the uropathogenic strains *Escherichia coli* and *Enterococcus faecalis*, when it was co-incubated in a combined nutrient composition of vitamins and lactose.

The production of bacteriocins by *L. salivarius* has been reported previously (Flynn et al. 2002; Pilasombut et al. 2006; Barrett et al. 2007); the 2-component class II bacteriocins ABP-118 (produced by a human intestinal probiotic strain *L. salivarius* UCC118), FK22 (from a chicken intestine isolated *L. salivarius* K7), and salivaricin P (from a porcine intestinal probiotic strain *L. salivarius* DPC6005) have been characterized at the molecular level (Flynn et al. 2002; Pilasombut et al. 2006; Barrett et al. 2007). In addition, *L. salivarius* NRRL B-30514 produced a bacteriocin OR-7 of which the amino acid sequence was consistent with that of class IIa bacteriocins and had antibacterial activity against the gram-negative *Campylobacter jejuni* (Stern et al. 2006). In the present study, a combination of Tricine–SDS–PAGE, Lumitein protein gel staining, and a bioassay for antibacterial activity indicated that the molecular mass of salivaricin CRL 1328 is around 4.5 kDa (Fig. 2). The partially purified bacteriocin, obtained from SDM–NaCl after concentration, allowed for the design of a relatively simple method for the easy recovery of the biologically active protein. This would facilitate the purification of salivaricin CRL 1328, allowing ongoing investigations that will lead to a better understanding of the mode of action and the interaction between the endogenous bacteria of the vagina.

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT). *Lactobacillus salivarius* CRL 1326 was licensed to Probiotical–Anidral for industrial applications.

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