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Influence of growth medium composition on the bacteriocin activity of some lactic acid bacteria

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Abstract

Six lactic acid bacteria isolated from fermented foods, recently reported to produce bacteriocins, were grown in different media, including MRS broth containing commercial prebiotics or bacterial exopolysaccharides, as carbon source. Cow's milk was able to support a good growth and bacteriocin production for three strains, while the other three strains showed a higher activity compared with MRS medium, although the growth was much slower. MRS with an initial pH of 6.2 and containing glucose or sucrose as carbon source was, in general, the best synthetic medium for growth and bacteriocin production. Moreover, the use of buffered MRS resulted in a significant increase of the bacteriocin activity for all strains. Replacing the nitrogen source with tryptone or lactalbumin hydrolysate resulted in an increase of bacteriocin activity in some cases. Strains grown under mild stress conditions, such as low concentrations of NaCl or bile salts, showed a higher bacteriocin activity, too. Bacterial exopolysaccharides allowed a good growth of all strains and bacteriocin activities similar with the ones obtained when grown on control MRS medium. Very high activities were also detected in the case of Lactobacillus amylolyticus strains in the presence of lactulose and inulin, although the growth was much slower.

Keywords: bacteriocins, growth medium, lactic acid bacteria, prebiotics

1. Introduction

Many lactic acid bacteria (LAB), belonging to *Lactococcus, Lactobacillus, Enterococcus, Leuconostoc, Streptococcus*, and *Pediococcus* genera, have been shown to produce bacteriocins (DE VUYST & VANDAMME [1]). These are ribosomally synthetized proteins or peptides responsible, along with other compounds, of the antibacterial effect of the producing bacteria, having a great potential for the use in food industry, to prevent the food spoilage (DE VUYST & LEROY [2], BESHKOVA & FRENGOVA [3]), but also with clinical potential due to the activity against human and animal pathogens (PEREZ & al. [4]). During the last decades, an impressive number of reports on LAB-bacteriocins have been published, dealing with identification of new bacteriocins with unique properties, or with biochemical and genetic characterization of these compounds, indicating that there is still a lot to learn on this topic, but also due to the role that bacteriocins may play in the hygienic quality assurance of foods or as next-generation antibiotics targeting the multiple-drug resistant pathogens (PEREZ & al. [4], CINTAS & al. [5], MACWANA & MURIANA [6]). Despite the huge number of discovered bacteriocins, until now only nisin, produced by *Lactococcus lactis* has been legally accepted as biopreservative for certain types of food and a reasonable explanation is that newly described bacteriocins have to be fully characterized

before being aproved to be used in food industry. Moreover, a well established isolation and purification procedure should be provided for each new bacteriocin.

Although not completely understood, the environmental role of LAB bacteriocins seems to include: cell-to-cell communication, defense against other bacteria, and facilitation of horizontal gene transfer (SNYDER & WOROBO [7]). Under natural conditions it is very difficult to prove the role of bacteriocins in the defense of an ecological niche. However, under laboratory conditions it was clearly shown that bacteriocins inhibit the growth of other bacteria, especially closely related, which might be competitors for the same environment (SNYDER & WOROBO [7]). Moreover, it was shown that many bacteriocins are overproduced by bacteria subjected to various stress conditions (KANMANI & al. [8]), including the co-cultivation with other bacterial strains (MALDONADO-BARRAGAN & al. [9]). Furthermore, the composition of the growth media and the conditions of growth significantly influence the bacteriocins production (PEREZ & al. [4], CINTAS & al. [5], MACWANA & MURIANA [6], SNYDER & WOROBO [7], KANMANI & al. [8]). Although bacteriocins can be produced in the food matrix, during fermentation, they are produced in much higher amounts during *in vitro* fermentations, under optimal physical and chemical conditions. This is due to the fact that bacteriocin production follows primary metabolite kynetics, being directly affected by the cultivation conditions and biomass production (DE VUYST & LEROY [2]). Having all these in mind, it is important to get the most data concerning the optimal conditions for bacteriocin production, in order to get large amounts needed for controlling the growth of undesirable bacteria. On the other hand, these data could contribute to the reduction of production costs of these antibacterial compounds (PEREZ & al. [4]). Six LAB strains, namely: *Lactococcus lactis* 19.3, *Lactobacillus plantarum* 26.1, *Enterococcus durans* 41.2, *Lactobacillus amylolyticus* P40, *Lactobacillus amylolyticus* P50, and *Lactobacillus oris* P49, have been recently reported to produce bacteriocins (GROSU-TUDOR & al. [10]). The first three bacteriocins are heat stable, low-molecular mass peptides, with a wide inhibitory spectrum (including *Listeria monocytogenes* and *Staphylococcus aureus*), while the other three are heat sensitive, high-molecular mass proteins, with a very narrow inhibitory spectrum (GROSU-TUDOR & al. [10]). The purpose of this work was to study the influence of growth media on the production of bacteriocins by the six strains and to find some optimal growth conditions in order to recover increased amounts of bacteriocins. Furthermore, the growth and bacteriocin production were investigated in the presence of some prebiotics, namely lactulose and inulin, but also in the presence of some exopolysaccharides produced by LAB previously isolated from raw and fermented vegetables (results not published).

2. Materials and Methods

Bacteria and growth media

Lactococcus lactis 19.3, *Lactobacillus plantarum* 26.1, *Enterococcus durans* 41.2, isolated from dairy products (GROSU-TUDOR & al. [11]), *Lactobacillus amylolyticus* P40, *Lactobacillus amylolyticus* P50, and *Lactobacillus oris* P49, isolated from *bors* (GROSU-TUDOR & al. [10]) were used as bacteriocin-producing strains. *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901^T was used as an indicator strain, being sensitive to all six bacteriocins. Strains were maintained at -80°C in MRS broth (DE MAN & al. [12]), containing 25% (v/v) of glycerol as a cryoprotectant. To obtain fresh cultures from the freezed stocks, strains were propagated twice in MRS medium at the optimal temperature (28 or 37°C) before the experiments.

Bacteriocin quantification

Antibacterial activity was assayed quantitatively by an agar spot test (DE VUYST & al. [13]). After the growth, cells were removed from the bacterial cultures by centrifigation

(13,000 *g*, 10 min, 4°C) and serial twofold dilutions in water of the cell free culture supernatants (CFCS) were spotted (10 µl) onto fresh indicator lawns of *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901^T. For some experiments, bacterial cells were suspended in 0.1M NaCl, pH 2.0, and left for 1h at 4°C (YANG & al. [14]). After centrifugation, the pH value of the supernatant was adjusted to 6.5-7.0 before using them in the agar spot test, for the quantification of antibacterial activity of the bacteriocins adsorbed on the cells surface. The activity was defined as the reciprocal of the highest dilution which demonstrated complete inhibition of the indicator lawn and was expressed in activity units (AU) per millilitre of sample.

Bacteriocin production in different growth media and initial pH

MRS broth, BHI broth (Merck, Darmstadt, Germany), cows' milk (1.8% fat), and soy milk - both commercial - were used to grow the bacteriocin-producing strains. For MRS medium, besides the control (with initial pH of 6.2), three other variants were used, with three initial pH values: 3.0, 4.0, and 5.0, respectively.

Influence of medium composition on bacteriocin production

The six bacteriocin-producing strains were grown in MRS broth (the control) and in several modified MRS media, as follows: MRS with sucrose, fructose (both from Merck KGaA, Darmstadt, Germany), lactose, galactose, or mannose (all three from Sigma-Aldrich Chemie GmbH, Germany), all in a final concentration of 20 g/L, as the sole sugar source, instead of glucose (Merck); MRS prepared without organic nutrients and supplemented with tryptone (Sigma-Aldrich), lactalbumin hydrolysate (Sigma-Aldrich), or casaminoacids (Difco Laboratories Detroit Michigan, USA), all in a final concentration of 20 g/L; MRS prepared without Tween 80; MRS supplemented with NaCl, in a final concentration of 10, 20, and 30 g/L, respectively; MRS supplemented with $CaCO_3$ (20 g/L); MRS supplemented with bile salts (Sigma-Aldrich), in a final concentration of 1 and 2 g/L, respectively. *L. lactis* 19.3, *Lb. plantarum* 26.1, *Ent. durans* 41.2 were incubated in all these variants at 28°C, while *Lb. amylolyticus* P40, *Lb. amylolyticus* P50, and *Lb. oris* P49 at 37°C, as previously shown to be the optimal growth temperature for the bacteriocin production (GROSU-TUDOR & al. [10]). Experiments were done in triplicate and results are given as the mean of the three experiments.

Growth and bacteriocin production in the presence of prebiotics or LAB-exopolysaccharides

MRS medium, prepared without glucose, was supplemented with 20 g/L of inulin (Serva Electrophoresis GmbH Heidelberg, Germany), 20 g/L of lactulose (Merck), and mixtures of glucose and inulin, or glucose and lactulose (all in a concentration of 10 g/L), respectively. Additionally, three exopolysaccharides (EPS, results not published) isolated from *Leuconostoc mesenteroides* 406 (WOUTERS & al. [15]) and from two other LAB strains, P109 and P127 (not identified to species level), were used as the sole carbon source. EPS material was obtained by precipitation of the culture supernatant with acetone, purification by dialysis, and freeze-drying. In paralel, the bacteriocin-producing strains were co-cultivated (1% inoculum of each strain) with one of the EPS-producing strains and the bacteriocin activity was determined by the agar spot method.

3. Results

Bacteriocin production in different growth media and initial pH (Table 1)

The bacteriocin activities recovered from cultures obtained in MRS broth were, for most of the tested strains, much higher than the ones from cultures obtained in BHI broth, even if bacterial growth was similar. The bacteriocin activity of *L. lactis* 19.3 was the same in both media, while for strain *Lb. amylolyticus* P50, the growth was very poor in BHI medium, but the activity was higher then in MRS medium. Soymilk allowed the growth of the six

strains, with similar or even higher values for CFU/ml as compared with the ones obtained in MRS medium, but the activities of the corresponding bacteriocins were lower compared with the ones from MRS cultures. The growth and activities of *L. lactis* 19.3, *Lb. plantarum* 26.1, and *Ent. durans* 41.2 were similar in cow's milk with the ones in MRS broth.

Table 1. Growth and bacteriocin activities of the selected strains incubated in different growth media

Growth medium*	P40		P49		P50		19.3		26.1		41.2	
	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml
MRS/glucose 2%	2.9E11	12,800	1.3E9	6,400	7.6E10	12,800	2.7E12	25,600	1.1E13	400	2.3E13	400
BHI	2.2E9	800	1.1E7	<100	3.2E6	25,600	1.5E11	25,600	3.9E12	100	8.5E12	200
cow's milk	5.2E6[a]/ 3.1E6[b]	409,600[a]/ 204,800[b]	3.5E5	12,800	1.5E7[a]/ 3.2E7[b]	12.800[a]/ 25,600[b]	3.8E11	25,600	4.8E12	400	4.8E12	200
soy milk	1.3E13	3,200	1.8E9	400	2.9E12	6,400	6.7E12	3,200	2.4E10	100	4.1E11	100
MRS pH 3.0	<100	0	<100	0	<100	0	<100	3,200	1.1E6	0	7.1E6	0
MRS pH 4.0	1.7E4	0	2.6E4	0	1.9E6	0	2.5E6	3,200	1.1E11	200	3.6E12	100
MRS pH 5.0	7.5E10	400	1.1E8	400	2.1E8	400	2.1E6	12,800	4.1E12	400	1.1E13	200

P40 – *Lb. amylolyticus* P40; P49 – *Lb. oris* P49; P50 – *Lb. amylolyticus* P50; 19.3 – *L. lactis* 19.3; 26.1 – *Lb. planturum* 26.1; 41.2 – *Ent. durans* 41.2.
*Incubation was performed for 24h at: 28°C for strains 19.3, 26.1 and 41.2, and 37°C for strains P40, P49 and P50, unless otherwise stated
[a]Activities were measured after 48h of incubation at 28°C; [b]Activities were measured after 48h of incubation at 37°C

The three strains isolated from bors showed a very poor growth in cow's milk (about 10^5- 10^7 CFU/ml) comparing with MRS medium (10^9- 10^{11} CFU/ml). However, the inhibitory activity was slightly higher (in the case of strains P49 and P50) or much higher in the case of strain P40 (about 32 times higher when incubated at 28°C). The initial pH of the growth medium influenced the growth and bacteriocin production. At low initial pH (3.0 and even 4.0), the growth for most strains was neglectable and the activity was very low or not detected. When the initial pH was 5.0, the growth became much better, for most strains being close to the growth at normal pH (6.2). However, the bacteriocin activities remained very low, except for the strains of dairy origin, which reached values similar with the controls.

Influence of medium composition on bacteriocin production (Table 2)

Concerning the sugar source, glucose and sucrose proved to be the best options for recovering high yields of bacteriocins for all the strains. Other sugars (such as lactose or mannose) may be also used, in some particular cases, to increase the bacteriocin production. Replacing the organic substrate of the MRS broth with tryptone resulted in a doubling of bacteriocin activity for strains 19.3 and 41.2, while for the other strains it did not have any effect or resulted in a decrease of the activity. In the presence of lactalbumin hydrolysate, the bacteriocin activity increased for strains P40 and 41.2, while for the other strains, a slight decrease was observed. Casaminoacids had, in general, a negative effect on bacteriocin production. Tween 80 proved to be very important for the growth and bacteriocin production for the three strains isolated from bors; when omitted from the growth medium, the activity decreased upto 128 times, while the activity of the other three strains was not significantly affected. Under mild stress conditions, such as in the presence of 1% NaCl, most of the strains were stimulated to produce bacteriocins. This effect was maintained, for some strains, even in the presence of higher concentration of NaCl, upto 3% (for strain P40), while for other strains, the increase of NaCl concentration had a negative effect. The growth was also correlated with the salt concentration: the higher the concentration, the lower the CFU/ml values. The presence of bile salts in the growth media had a dramatic effect on the growth/survival of most of the six bacteria. However, the activity of the two *Lb. amylolyticus* strains isolated from bors were similar to the controls. When the pH of the culture was maintained approximately constant by the addition of $CaCO_3$ to the MRS medium, the bacteriocin activity was higher in all six cultures. The most evident increasing effect was observed for the two *Lb. amylolyticus* strains; an activity increase of over 500 times was detected in these cases.

Table 2. Influence of medium composition on growth and bacteriocin production

Growth medium*	P40		P49		P50		19.3		26.1		41.2	
	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml
MRS/glucose 2%	2.9E10	12,800	1.3E9	6,400	7.6E9	12,800	2.7E12	25,600	1.1E13	400	2.3E13	400
MRS/sucrose 2%	7.6E9	12,800	1.3E10	6,400	1.2E10	12,800	4.6E10	51,200	2.7E13	800	3.4E13	400
MRS/fructose 2%	3.3E10	12,800	3.6E10	<100	2.1E8	<100	2.5E10	25,600	2.1E13	800	5.1E13	800
MRS/lactose 2%	7.5E11	800	1.1E11	6,400	<100	<100	3.7E10	51,200	4.7E12	800	1.3E12	400
MRS/galactose 2%	1.1E11	400	1.6E9	<100	5.2E8	100	6.4E10	25,600	1.6E13	800	1.9E13	200
MRS/mannose 2%	2.8E11	25,600	1.5E8	6,400	1.4E9	6,400	2.7E12	25,600	1.4E13	800	5.4E11	800
MRS-tryptone	7.7E10	12,800	5.4E11	<100	1.2E8	6,400	1.5E12	51,200	2.4E13	400	5.4E13	800
MRS-lactalbumin hydrolysate	6.5E6	25,600	3.3E7	400	2.1E8	6,400	2.3E12	12,800	7.7E13	400	8.3E13	1600
MRS-casaminoacids	3.2E5	25,600	5.5E9	400	<100	6,400	8.9E10	25,600	5.1E12	100	1.3E13	100
MRS without Tween 80	1.4E5	<100	3.1E5	100	3.1E6	3,200	2.1E13	25,600	2.1E13	400	7.4E13	200
MRS + NaCl 1%	1.3E11	102,400	3.5E8	51,200	3.8E9	25,600	4.1E11	25,600	1.2E13	800	7.8E13	800
MRS + NaCl 2%	6.6E10	25,600	1.4E7	1,600	1.9E8	12,800	2.7E10	12,800	1.2E13	800	8.2E13	800
MRS + NaCl 3%	3.9E6	25,600	4.1E6	200	1.4E6	6,400	1.1E10	6,400	8.9E12	400	8.3E10	400
MRS + $CaCO_3$ 2%	8.6E6	6,553,600	1.5E6	25,600	2.3E6	6,553,600	8.1E12	204,800	8.3E13	1,600	1.2E14	1,600
MRS + bile salts 0.1%	<100	12,800	3.1E3	200	<100	12,800	1.1E7	1,600	3.4E10	0	2.1E10	0
MRS + bile salts 0.2%	<100	12,800	2.6E2	200	<100	12,800	4.3E6	3,200	1.4E9	0	1.1E9	0

P40 – *Lb. amylolyticus* P40; P49 – *Lb. oris* P49; P50 – *Lb. amylolyticus* P50; 19.3 – *L. lactis* 19.3; 26.1 – *Lb. planturum* 26.1; 41.2 – *Ent. durans* 41.2.
*Incubation was performed for 24h at: 28°C for strains 19.3, 26.1 and 41.2, and 37°C for strains P40, P49 and P50

Growth and bacteriocin production in the presence of prebiotics or exopolysaccharides isolated from LAB (Table 3)

When glucose was replaced by lactulose in MRS medium, the three strains of dairy origin grew well, similar with the control. The bacteriocin activity was also enhanced in these conditions for strain *L. lactis* 19.3. The strains derived from bors showed a poor growth on lactulose (10^5-10^7 CFU/ml, compared with 10^8-10^{11} CFU/ml on glucose).

Table 3. Growth and bacteriocin activities of the selected strains in the presence of commercial prebiotics or bacterial exopolysaccharides, as the sole carbon source

Culture conditions	P40		P49		P50		19.3		26.1		41.2	
	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml	CFU/ml	AU/ml
MRS/glucose 2%	2.9E11	12,800	1.6E8	6,400	5.3E9	12,800	2.7E10	25,600	8.9E14	400	8.7E14	400
MRS + lactulose 2%	3.5E5	25,600	5.4E7	3,200	1.8E7	51,200	8E11	102,400	3.2E13	400	8.2E13	200
MRS +lactulose 1% + glucose 1%	2E10	25,600	1.1E7	3,200	2.6E9	51,200	4.2E11	102,400	2.7E13	400	1.3E13	400
MRS + inulin 2%	1.6E9	12,800	5E5	25,600	5.4E7	51,200	1.2E11	12,800	1.1E10	100	6E10	200
MRS + inulin 1% + glucose 1%	2E9	12,800	2E6	6,400	4.5E9	25,600	4E11	51,200	2.8E13	800	3E13	400
MRS/EPS P109	2.1E11	12,800	2E8	800	2.8E10	6,400	1.6E10	12,800	2.1E14	200	2.4E14	400
MRS/EPS P127	1.6E10	25,600	1.4E8	800	2.2E9	12,800	2.1E9	25,600	7.5E13	400	1.1E13	800
MRS/EPS *Leuc. mesenteroides* 406	4.3E10	12,800	2.8E8	800	5.4E10	6,400	3.4E9	12,800	2E14	200	5.1E13	200

However, the activities of the two strains of *Lb. amylolyticus*, P40 and P50 were four times higher when grown on lactulose than the controls. The use of a mixture of glucose and lactulose resulted, in some cases, in a better growth, but the bacteriocin activity was the same as the one determined in the cultures obtained in the presence of lactulose solely. The growth on inulin was, for all the strains, much slower than the growth on glucose. The bacteriocin activity decreased for the dairy strains, while for the other three strains, an increase of activity was observed, especially for strain *Lb. amylolyticus* P50.

The growth and bacteriocin production were followed up in time for strains *L. lactis* 19.3 and *Lb. amylolyticus* P50 grown in MRS with inulin and lactulose, respectively, and compared with the cultures obtained in control MRS. *Lb. amylolyticus* P50 cells grew fast in control MRS and the maximum bacteriocin activity in the culture supernatant (12,800 AU/ml) was reached after 24 h of incubation (Fig. 1a).

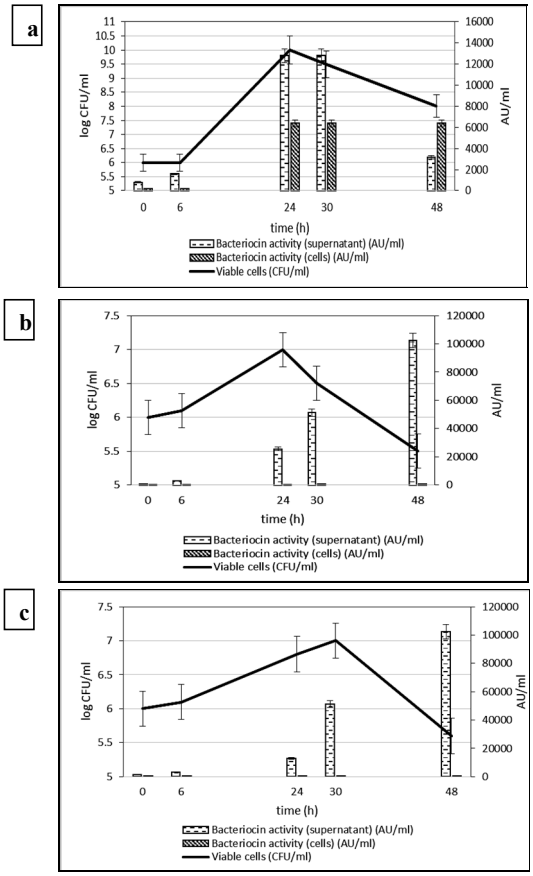

Fig. 1. Growth and bacteriocin production by strain *Lb. amylolyticus* P50 in normal MRS (a) and in the presence of inulin (b) or lactulose (c)

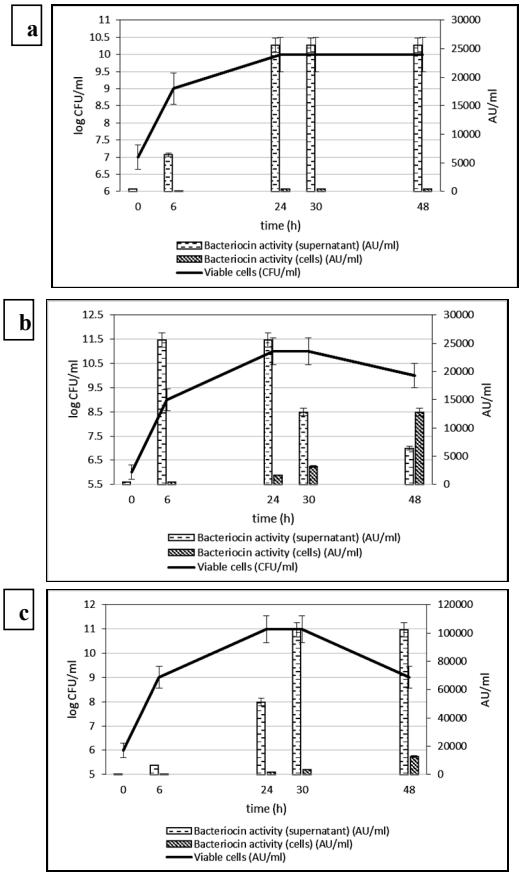

Fig. 2. Growth and bacteriocin production by strain *L. lactis* 19.3 in normal MRS (a) and in the presence of inulin (b) or lactulose (c)

The same activity was determined at 30 h of incubation, while at the end of experiment, at 48 h, it decreased to 3,200 AU/ml. On the other hand, the activity of the bacteriocins adsorbed on the cells was very low in the beginning, but increased to 6,400 AU/ml at 24 h and remained constant until 48 h. In the presence of inulin or lactulose (Fig. 1b,c), the growth was slower, but the maximum bacteriocin activity in the supernatant was 102,400 AU/ml, reached after 48 h of incubation. In these cases, the adsorbtion of bacteriocins on the cells was much lower, the highest activity being 400-800 AU/ml. In the case of *L. lactis* 19.3, cells were growing well, both in control MRS and in the presence of inulin or lactulose (Fig. 2). However, we observed that after reaching a maximum value, the viability of the cells decreased in the

media containing inulin or lactulose, while in the control MRS, it remained constant in time. The bacteriocin activity in the supernatant increased in MRS cultures to 25,600 AU/ml whithin the first 24h and remained constant (Fig. 2a). The adsorbtion of bacteriocins on the cells in this case was very low (200-400 AU/ml). In the presence of inulin, the maximum bacteriocin activity in the supernatant (25,600 AU/ml) was reached after 6 h of incubation, remained constant until 24 h and then decreased, to 6,400 AU/ml at 48 h. A concomitant increase in the activity of adsorbed bacteriocins was observed (Fig. 2b). When grown on lactulose, the activity in the supernatant increased slower, but reached a maximum of 102,400 AU/ml after 30 h of incubation. The activity of adsorbed bacteriocins also increased in time, but still remained very low, comparing to the bacteriocins in the supernatant (Fig. 2c). On the other hand, all six bacteriocin-producing strains were able to grow very well when one of the three EPS isolated from strains P109, P127 or *Leuc. mesenteroides* 406 was used as the sole carbon source. The bacteriocins activities were comparable with the ones of the controls (Table 3). Co-cultivation of the bacteriocin-producing strains with one of the three EPS-producing strains in MRS medium containing sucrose did not change significantly the bacteriocin activity; in the presence of strain P127, there was, however, a slight increase in the bacteriocin activity of *Lb. amylolyticus* P40, *Lb. plantarum* 26.1 and *Ent. durans* 41.2 (results not shown).

4. Discussions

Bacteriocins have long been proposed as a solution to the problems of food spoilage and food-borne infection in the food industry. Their under-utilization in this sector may be due to the lack of information of what bacteriocins can achieve in food systems, but also due to the poor characterization of these compounds (PEREZ & al. [4]). In this context, our study aimed to characterize six newly described bacteriocins, namely to correlate their activities with the growth media composition and to try to use some prebiotics or bacterial exopolysaccharides to support a good growth and bacteriocin production. Firstly, several growth media were used, both chemically defined media (MRS and BHI) and milk (cow's milk and soy milk), in order to select the best one for recovering the highest yields of bacteriocins. On the other hand, cow's milk and soy milk were used to investigate the possibility to use the strains as biopreservatives or as probiotics in dairy products and in vegetarian products, respectively. The six strains under study grew, in general, very well in MRS, BHI and soy milk. However, the highest bacteriocin production was in MRS medium. As expected, the three dairy strains showed a very good growth in cow's milk and similar bacteriocin activities with the ones in MRS. This result is very important if one would like to use a bacteriocin-producing strain as a starter culture or co-starter culture. Several studies have indicated that LAB are able to produce their bacteriocins in food matrices, and consequently display inhibitory activity towards sensitive food spoilage or pathogenic bacteria (LEROY & DE VUYST [2], DE VUYST & LEROY [16]). For the two *Lb. amylolyticus* strains a significant increase of the bacteriocin activities was observed in milk, although the growth was much slower than in MRS medium. However, they might be used in dairy industry, for instance in a combination with other proteolytic, acidifying strains. An important variable affecting bacterial growth and bacteriocin production is the pH value of the culture, including here the initial and final pH, the pH time course and pH drop generated in the culture media (PEREZ & al. [17]). The influence is also depending on the producing strain. In our case, all the tested strains showed the maximum bacteriocin production and the best growth at initial pH of 6.2 (control MRS). Lower values of the pH resulted in much lower bacteriocin yields or in the lack of growth and viability loss. This result is in accordance to other findings for strains of *Lactobacillus, Lactococcus* or *Enterococcus* (PEREZ & al. [17], SCHIRRU & al. [18]). On the other hand, the use of buffered MRS (by addition of 2% $CaCO_3$), resulted in a significant increase of the

bacteriocin activity for all six strains. Previous reports showed a different effect of a buffered growth media on bacteriocin production, depending on the strain and the type of bacteriocin (PEREZ & al. [17], YANG & RAY [19]). Several enzymes have been shown to be involved in the posttranslational processing of some bacteriocins and their activity is strongly influenced by the pH. In our study, the highest increase in bacteriocin activity in the buffered medium was observed for the two *Lb. amylolyticus* strains, producing bacteriocins that belong, most probably, to class III bacteriocins (GROSU-TUDOR & al. [10]), and for strain *L. lactis* 19.3, probably a nisin-producing strain. This is in accordance with previous reports, showing that posttranlational processing of some bacteriocins precursors, such as prenisin, occurs at higher pH values, of about 6.0 (YANG & RAY [19]). Although glucose was found to be the best carbon source for bacteriocin production by lactococci, lactobacilli and pediococci (PEREZ & al. [17]), the six strains under investigation showed similar bacteriocin activities when grown in the presence of glucose, sucrose or mannose, and for the three strains of dairy origin similar or even higher activities when grown on lactose. The type and concentration of the carbon source are very important for the bacterial growth and bacteriocin production, but the effect depend on the tested strain; sucrose, lactose, xylose, maltose or mannose have been recongized as good carbon sources for *L. lactis* and lactobacilli (CHINACHOTI & al. [20], TODOROV & DICKS [21], AKKOC & al. [22]). The same strain-dependent effect was observed in the case of nitrogen source used for bacterial growth. In general, the complex nitrogen sources (e.g. peptones, yeast and meat extracts) have been recognized as good sources for the production of different bacteriocins (PEREZ & al. [17]) and this was the case for three of our strains. However, the other three strains, *Lb. amylolyticus* P40, *L. lactis* 19.3 and *Ent. durans* 41.2 showed a preference for nitrogen sources derived from milk proteins instead of those of meat or yeast origin. The presence of Tween 80 in the growth medium was very important for the activity, especially in the case of the three large, heat-labile bacteriocins, produced by the strains isolated from bors. It was previously shown that Tween 80 has a positive effect due to the permeabilization of the cellular membrane that allows the diffusion of the bacteriocins from the cells to the culture media (HUOT & al. [23]). Although bacteriocins are primary metabolites, being directly correlated with bacterial growth, it was shown that a good growth is not enough for a good bacteriocin production (SCHIRRU & al. [18]) and that some bacteriocins are overproduced under some stress environmental conditions (KANMANI & al. [8]). For most of our six tested strains, the bacteriocin production was enhanced when they were grown in MRS medium containing small amounts of NaCl (1-2%), although the growth was slower in these conditions. Moreover, in the presence of small amounts of bile salts (0.1-0.2%), the growth was very slow or not detectable, but the bacteriocin activites of the two *Lb. amylolyticus* strains were still very high, making them suitable for inclusion in probiotic products, for instance. More studies are necessary to clarify if the high bacteriocin activity is due to an overproduction or a release of more bacteriocin molecules to the culture media. Finally, the effect of some commercial prebiotics and some LAB exopolysaccharides on the bacteriocin activities was investigated for the six selected strains. In the literature there are both reports on the improvement of bacteriocin production in the presence of certain prebiotics (e.g. raffinose, lactulose, trehalose, fructoligosaccharides) and reports showing the negative effect of other prebiotics (e.g. inulin) (GOMES & al. [24], CHEN & al. [25], VAMANU & VAMANU [26]). In our case, the effect of inulin and lactulose depended on the producing strain. Lactulose enhanced the bacteriocin activities for most of the strains, although the growth was not very good in the case of the three strains isolated from bors. Most of the strains were also able to grow in the presence of inulin, but the bacteriocin activities were usually lower, except for strains *Lb. amylolyticus* P50 and *Lb. oris* P49, when the activities increased 4 times. Certain prebiotics (in our case lactulose and inulin) can be,

therefore, used not only as good carbohydrate sources for growth, but also as promoters of bacteriocin production (CHEN & al. [25]). Our studies showed that growth of *Lb. amylolyticus* P50 in the presence of lactulose or inulin was slow and limited, but the bacteriocin production very high. It is possible that bacterial cells of this strain remain in a kind of latent phase, they do not use much energy for cell division, but they use more energy for bacteriocin production in these two media, less appropriate for their growth. The adsorbtion of bacteriocins on the cells surface was also limited in these prebiotic-media. On the contrary, in normal MRS, cells grew faster, but after 24 h of incubation, a significant part of the produced bacteriocins got adsorbed on the cells surface. It was shown that cells coverred with bacteriocins cease to grow, they do not divide and, most probably, they do not produce bacteriocins (DE VUYST & al. [27]). When grown in normal MRS, the highest bacteriocin activity of *L. lactis* 19.3 culture was observed in the end of the exponential growth phase. The activity in the supernatant remained constant for a long time, bacteriocins did not get adsorbed on the cells and the number of viable cells was also constant for a long time. A similar profile was observed for cells grown in the presence of lactulose, except that after 30 h of incubation, some bacteriocins got adsorbed on the cells and, consequently, the number of viable cells decreased. When grown in MRS medium with inulin, the bacteriocin was produced in high amounts in the early exponential phase, but after 24 h, the activity in the supernatant decreased a lot, due to the adsorbtion on the cells surface or due to a degradation (DE VUYST & al. [27]). Additionally, we found out that some exopolysaccharides isolated from selected LAB strains could be also a suitable choice for those bacteriocin-producing LAB strains that have the ability to ferment them. The six tested strains were able to grow very well on the three EPS used in the study and the bacteriocin activities were usually similar with the ones recorded in glucose-media.

5. Conculsions

Our results confirmed the fact that optimal conditions for bacteriocin production are not easily predicted because their regulation depends on complex parameters (LEROY & DE VUYST [28]). There are many key factors that affect bacteriocin production and the effects depend on the bacterial strain and the culture media. On the other hand, we have shown that several changes of the growth media can be made, in order to simplify them, but still to obtain high bacteriocin activities. Some of the tested strains may find application in probiotic or synbiotic products, as they are able to maintain good inhibitory activities in the presence of bile salts and at lower pH values. Moreover, their activities are similar or higher in the presence of some commercial prebiotics or LAB-exopolysaccharides.

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References

1. L. DE VUYST & E.J. VANDAMME eds., *Bacteriocins of lactic acid bacteria*. London Blackie Academic and Professional (1994).
2. L. DE VUYST & F. LEROY, Bacteriocins from lactic acid bacteria: production, purification, and food applications. *J Mol Microbiol Biotechnol* **13**, 194-199 (2007).
3. D. BESHKOVA & G. FRENGOVA, Bacteriocins from lactic acid bacteria: Microorganisms of potential biotechnological importance for the dairy industry. *Eng Life Sci* **12,** 419-432 (2012).
4. R.H. PEREZ, T. ZENDO, K. SONOMOTO, Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial Cell Factories* **13** (Suppl. 1), 11-53 (2014a).
5. L.M. CINTAS, M.P. CASAUS, C. HERRANZ, I.F. NES, P.E. HERNÁNDEZ, Review: Bacteriocins of Lactic Acid Bacteria. *Food Sci Technol Int* **7**, 281-305 (2001).

6. S.J. MACWANA & P.M. MURIANA, A 'bacteriocin PCR array' for identification of bacteriocin-related structural genes in lactic acid bacteria. *J Microbiol Meth* **88,** 197–204 (2012).
7. A.B. SNYDER & R.W. WOROBO, Chemical and genetic characterization of bacteriocins: antimicrobial peptides for food safety. *J Sci Food Agric* **94**, 28-44 (2013).
8. P. KANMANI, R.S. KUMAR, N. YUVARAJ, K.A. PAARI, V. PATTUKUMAR, V. ARUL, Application of response surface methodology in the optimisation of a growth medium for enhanced natural preservative bacteriocin production by a probiotic bacterium. *Nat Prod Res* **26**, 1539-1543 (2012).
9. A. MALDONADO-BARRAGAN, B. CABALLERO-GUERRERO, H. LUCENA-PADROS, J.L. RUIZ-BARBA, Induction of bacteriocin production by coculture is widespread among plantaricin-producing *Lactobacillus plantarum* strains with different regulatory operons. *Food Microbiol* **33**, 40-47 (2013).
10. S.S. GROSU-TUDOR, M.M. STANCU, D. PELINESCU, M. ZAMFIR, Characterization of some bacteriocins produced by lactic acid bacteria isolated from fermented foods, *World J Microbiol Biotechnol* **30**(9), 2459-2469 (2014).
11. S.S. GROSU-TUDOR, M. ZAMFIR, R. VAN DER MEULEN, L. DE VUYST, Isolation of novel homopolysaccharide producing lactic acid bacteria from Romanian raw milk and fermented dairy products. *Eur Food Res Technol* **237**, 609-615 (2013).
12. J.C. DE MAN, M. ROGOSA, M.E. SHARPE, A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130-135 (1960).
13. L. DE VUYST, R. CALLEWAERT, B. POT, Characterization of the antagonistic activity of *Lactobacillus amylovorus* DCE 471 and large scale isolation of its bacteriocin amylovorin L471. *Syst Appl Microb* **19**, 9-20 (1996).
14. R. YANG, M.C. JOHNSON, B. RAY, Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Appl Environ Microbiol* **58**, 3355-3359 (1992).
15. D. WOUTERS, S.S. GROSU-TUDOR, M. ZAMFIR, L. DE VUYST, Applicability of *Lactobacillus plantarum* IMDO 788 as a starter culture to control vegetable fermentations. *J Sci Food Agric* **93**(13), 3352-3361 (2013).
16. F. LEROY, L. DE VUYST, Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci Technol* **15**, 67-78 (2004).
17. R.A. PEREZ, E.A. GONZÁLEZ, A.T. AGRASAR, N.P. GUERRA, A review on some important factors affecting bacteriocin production by lactococci, lactobacilli and pediococci. *Current Biochemical Engineering* **1**, 9-24 (2014b).
18. S. SCHIRRU, L. FAVARO, N.P. MANGIA, M. BASAGLIA, S. CASELLA, R. COMUNIAN, F. FANCELLO, B.D. GOMBOSSY DE MELO FRANCO, R.P. DE SOUZA OLIVEIRA, S.D. TODOROV, Comparison of bacteriocins production from *Enterococcus faecium* strains in cheese way and optimised commercial MRS medium. *Ann Microbiol* **64**, 321-331 (2014).
19. R. YANG, B. RAY, Factors influencing production of bacteriocins by lactic acid bacteria. *Food Microbiol* **11**, 281-291 (1994).
20. N. CHINACHOTI, K. MATSUSAKI, K. SONOMOTO, A. ISHIZAKI, Nisin Z production by *Lactococcus lactis* IO-1 using xylose as a carbo source. *Biosci Biotechnol Biochem* **62**, 1022-1024 (1998).
21. S.D. TODOROV, L.M.T. DICKS, Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer produced by the fermentation of maize, barley and soy flour. *World J Microbiol Biotechnol* **20**: 643-650 (2004).
22. N. AKKOC, A. GHAMAT, M. AKCELIK, Optimization of bacteriocin production of *Lactococcus lactis* subsp. *lactis* MA23, a strain isolated from Boza. *Int J Dairy Technology* **64**, 425-432 (2011).
23. E. HUOT, C. BARRENA-GONZALEZ, H. PETITDEMANGE, Tween 80 effect on bacteriocin synthesis by *Lactococcus lactis* subsp. *cremoris* J46. *Lett Appl Bacteriol* **22**, 307-310 (1996).
24. B.C. GOMES, M.R. RODRIGUES, L.K. WINKELSTROTER, A. NOMIZO, E.C.P. DE MARTINIS, In vitro evaluation of the probiotic potential of bacteriocin producer *Lactobacillus sakei* 1. *J Food Prot* **75**, 1083-1089 (2012).
25. Y.S. CHEN, S. SRIONNUAL, T. ONDA, F. YANAGIDA, Effects of prebiotic oligosaccharides and trehalose on growth and production of bacteriocins by lactic acid bacteria. *Lett Appl Microbiol* **45**, 190-193 (2007).
26. E. VAMANU, A. VAMANU, Viability of the *Lactobacillus rhamnosus* IL1 strain in simulated gastrointestinal conditions. *Int J Pharmacol* **6**, 732-737 (2010).
27. L. DE VUYST, R. CALLEWAERT, K. CRABBE, Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology* **142**, 817-827 (1996).
28. F. LEROY, L. DE VUYST, Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. *Int J Food Microbiol* **72**, 155-164 (2002).