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EXPERIMENTAL ARTICLES

Components of Fermentation Medium Regulate Bacteriocin Synthesis by the Recombinant Strain *Lactococcus lactis* subsp. *lactis* F-116

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Abstract—The regulation of the synthesis of bacteriocin produced by the recombinant strain *Lactococcus lactis* subsp. *lactis* F-116 has been studied. The synthesis is regulated by the components of the fermentation medium, the content of inorganic phosphate (KH_2PO_4), yeast autolysate (source of amine nitrogen), and changes in carbohydrates and amino acids. The strain was obtained by fusion of protoplasts derived from two related *L. lactis* subsp. *lactis* strains, both exhibiting a weak ability to synthesize the bacteriocin nisin. Decreasing the content of KH_2PO_4 from 2.0 to 1.0 or 0.5% caused bacteriocin production to go down from 4100 to 2800 or 1150 IU/ml, respectively; the base fermentation medium contained 1.0% glucose, 0.2% NaCl, 0.02% $MgSO_4$, and yeast autolysate (an amount corresponding to 35 mg % ammonium nitrogen). The substitution of sucrose for glucose (as the source of carbon) increased the antibiotic activity by 26%, and the addition of isoleucine, by 28.5%. Elevation of the concentration of yeast autolysate in the low-phosphate fermentation medium stimulated both the growth of the lactococci and the synthesis of bacteriocin. Introduction of 1% KH_2PO_4, yeast autolysate (an amount corresponding to 70 mg % ammonium nitrogen), 2.0% sucrose, and 0.1% isoleucine increased the bacteriocin-producing activity of the strain by 2.4 times.

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Key words: *Lactococcus lactis* subsp. *lactis*, bacteriocin, components of fermentation medium, synthesis regulation.

Many lactic acid bacteria are known to synthesize biologically active peptides or protein complexes, known as bacteriocins. Due to their antibiotic effects, bacteriocins are widely used as biological preservatives in the food industry [1, 2]. In 1996, the Council of Experts on Food Additives recommended the bacteriocin nisin for use as a food product preservative; it was given the designation of GRAS (Generally Recognized As Safe) [3]. Certain bacteriocins produced by the mesophillic lactococcus *Lactococcus lactis* subsp. *lactis* (nisins, lacticins, etc.) exhibit strain-specific differences in physicochemical properties, amino acid composition, molecular weight, and biological activity [1, 4–8]. Each strain within one species of lactococci is capable of forming one or more specific bacteriocins [1, 4]. The ability of the bacteria to form bacteriocins in the course of their vital activity is related to culturing conditions and the composition of the culture medium. Because lactic acid bacteria belong to chemoorganotrophs with considerably limited synthetic potential, their growth and development depends on the availability of factors, such as vitamins and particular amino acids [7, 9, 10]. The demand in such growth factors prompts researchers to elucidate what components of the fermentation medium affect the proliferation of lactococci and their ability to synthesize bacteriocins. Optimization of media for culturing bacteriocin-producing lactococci has been the subject of a series of reports [7, 9, 11–16]. On rare occasions, limiting the content of nitrogen and phosphorus in the fermentation medium or changing the carbon substrate results in overproduction of bacteriocins [11, 12, 15]. Free amino acids, which are directly involved in the formation of bacteriocins and proteinaceous antibiotics, play a significant role in the growth and development of these microorganisms [4–6]. The addition of such substances into the fermentation medium exerts effects comparable to those of the structural metabolic precursors of bacteriocins.

Research in this field focuses on targeted regulation of the biosynthesis of antibiotics, with a view to increasing their yield, obtaining more active combinations of known bacteriocins, or identifying new forms thereof. Methods of cell engineering, which have gained wide acceptance in recent years, make it possible to obtain hyperpoducers of antibiotics or strains capable of synthesizing structurally novel bacteriocins

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with a broad spectrum of antimicrobial effects. We reported previously that fusion of the protoplasts of two related strains of *L. lactis* subsp. *lactis*, which exhibited low nisin-synthesizing activity, allowed us to obtain a recombinant strain, F-116, the synthetic activity of which exceeded that of parental strains by 10–12 times [17]. A highly active bacteriocin was isolated from this strain; one of its fractions was similar to nisin in its biological activity and physicochemical properties, and another appeared as a new entity with no analogues among biologically active substances [18].

This work was designed to study the synthesis of the bacteriocin of the recombinant strain *Lactococcus lactis* subsp. *lactis* F-116, with emphasis on its regulation by components of the fermentation medium.

MATERIALS AND METHODS

The object of this study was strain F-116 of *Lactococcus lactis* subsp. *lactis*, which was obtained by fusion of protoplasts derived from two related strains of lactococci [17]. The strain was stored lyophilized in a household refrigerator. The lyophilized culture was reconstituted by sterile non-fat (skimmed) milk; the rate of casein clot formation was used as a measure of the physiological activity of the strain [10].

To obtain an inoculum, the culture from skimmed milk was reseeded into the inoculation medium, which contained 1% glucose, yeast autolysate (35 mg % of ammonium nitrogen), and tap water (pH 6.8–7.0). The culture was grown under stationary conditions at 28°C. Thereafter, the inoculum (OD_{540}, 0.14–0.19) was introduced in an amount of 5 vol. % into the base fermentation medium, which contained 20 g/l KH_2PO_4, 10 g/l glucose, yeast autolysate (35 mg % ammonium nitrogen), 2 g/l NaCl, and 0.2 g/l $MgSO_4$ (pH 6.8–7.0) [9].

The bacteria were cultured in flasks (each containing 250 ml of the medium) at 28°C for 30 h (stationary conditions).

Studies of the effects of nitrogen and phosphorus on bacteriocin synthesis involved two approaches: (1) fractional exclusion of components of the medium and (2) variation in the content of KH_2PO_4 (2 or 0.5%) and yeast autolysate (35, 50, or 70 mg % ammonium nitrogen), all other components remaining unchanged.

To study the effect of carbon source on biomass growth, pH kinetics, and the level of antibiotic activity, a series of carbohydrates was used, which contained D-arabinose, D-xylose, D-ribose, D-glucose, L-rhamnose, D-maltose, D-sucrose, D-mannose, D-lactose, D-galactose, raffinose, D-fructose, D-sorbitol, dulcitol, mannitol, glycerol, dextrin, and starch. The carbohydrates were added to the base fermentation medium to the amount of 1.0% each.

The effects of amino acids on bacterial growth and synthesis of bacteriocins were studied using DL-valine, DL-threonine, DL-leucine, L-serine, L-lysine, DL-cystine, L-glutamate, L-aspartate, L-isoleucine, L methionine, and L-cysteine, each of which was introduced into the base fermentation medium of the above composition in the amount of 0.1%.

Table 1. Effects of the ratio of KH_2PO_4 and yeast autolysate on the growth of *Lactococcus lactis* subsp. *lactis* F-116 and bacteriocin synthesis in the base fermentation medium after 12 h of incubation

Composition of the medium	Biomass, OD_{540} units	pH	Activity, IU/ml
Control (2% KH_2PO_4 + 35 mg % $[NH_4]^+$)	1.260	4.2	4100
1% KH_2PO_4 + 35 mg % $[NH_4]^+$	0.89	4.4	2800
1% KH_2PO_4 + 50 mg % $[NH_4]^+$	1.05	4.2	3180
1% KH_2PO_4 + 70 mg % $[NH_4]^+$	1.19	3.9	3300
0.5% KH_2PO_4 + 35 mg % $[NH_4]^+$	0.70	4.8	1150
0.5% KH_2PO_4 + 50 mg % $[NH_4]^+$	0.78	4.6	1270
0.5% KH_2PO_4 + 70 mg % $[NH_4]^+$	0.82	3.9	1336

The biomass of the lactococcus was determined nephelometrically ($\lambda = 540$ nm; $l = 1$ cm); pH was measured potentiometrically.

The bacteriocin-synthesizing activity was assessed as nisin production (judged by the suppression of growth of the indicator culture *Bacillus coagulans*, a thermophilic sporulating acid-resistant bacterium) [10]. A 1-day culture of *B. coagulans* was introduced into the agar medium as a suspension with a density of 10^9 cell/ml. Quantitative determination of the antibiotic activity was performed by measuring the zones of *B. coagulans* growth suppression with subsequent calculation involving a calibration plot for standard nisin solutions. Solutions of the preparation Nisaplin (Aplin & Barrett, United Kingdom), kindly provided by R.J. Evans, served as the standards (activity 1 000 000 IU/g [17]).

The experiment comprised three series of triplicate measurements. The results were processed statistically as described in [9].

RESULTS AND DISCUSSION

Culturing of the recombinant strain *L. lactis* subsp. *lactis* F-116 in base fermentation medium under stationary conditions resulted, after 12 h of growth, in an antibiotic activity of 4100 IU/ml and an OD_{540} equal to 1.26. Bacterial growth was associated with acidification of the medium to pH 4.2 (Table 1). The results of studies of the kinetics of lactococcus growth demonstrated that bacteriocin synthesis was paralleled by biomass accumulation and stopped at the end of the exponential growth phase or at the beginning of the steady-state phase (Fig. 1a).

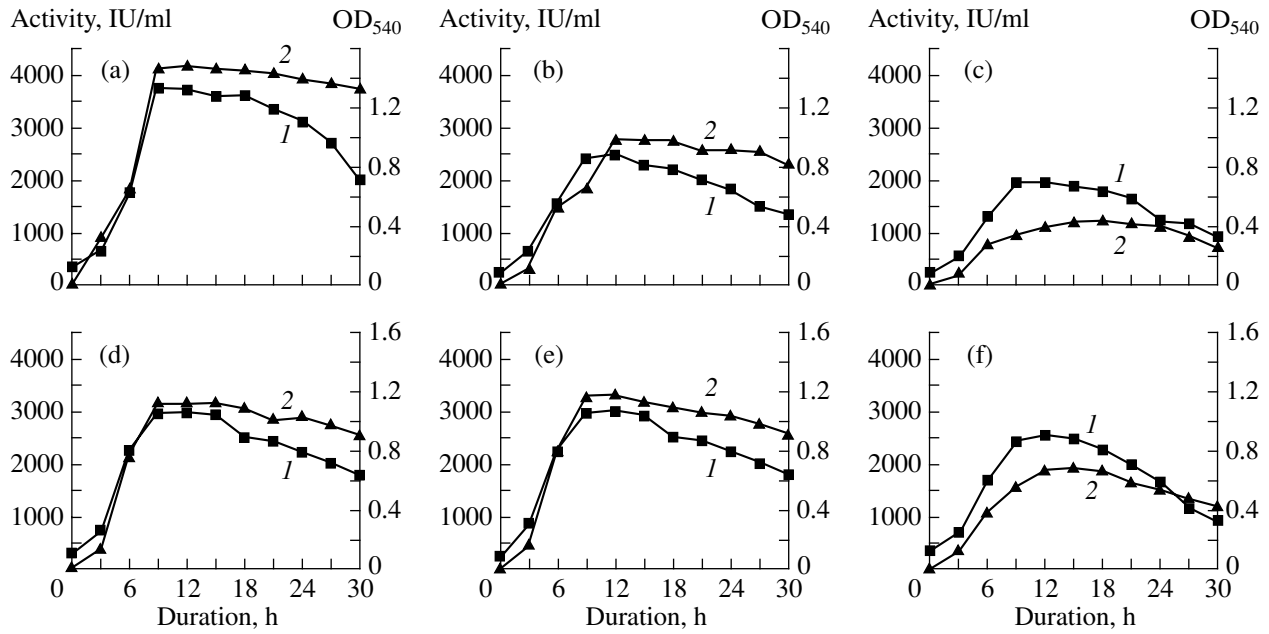

Fig. 1. Effect of the content of phosphate and yeast autolysate on the growth of *Lactococcus lactis* subsp. *lactis* F-116 (*1*) and bacteriocin synthesis (*2*) in the base fermentation medium containing (a) 2% KH_2PO_4 and 35 mg % $[NH_4]^+$, (b) 1% K_2HPO_4 and 35 mg %$[NH_4]^+$, (c) 0.5% KH_2PO_4 and 35 mg % $[NH_4]^+$, (d) 1% K_2HPO_4 and 50 mg % $[NH_4]^+$, (e) 1% K_2HPO_4 and 70 mg % $[NH_4]^+$, and (f) 0.5% K_2HPO_4 and 70 mg % $[NH_4]^+$.

When the content of inorganic phosphorus (KH_2PO_4) in the base fermentation medium was reduced from 2 to 1%, both the level of the antibiotic activity and the accumulation of the biomass decreased (to 2800 IU/ml and OD_{540} = 0.89, respectively). The acidification was also less pronounced under these conditions (the value of pH decreased to 4.4 in 12 h). Table 1 shows that further reduction of phosphate content to 0.5% suppressed both the growth of the bacteria (OD_{540} decreased 1.8 times) and the bacteriocin synthesis (by 72%). The extent of acidification of the medium is indicative of the formation of lactic acid, the extracellular metabolite of lactococci. The presence of phosphate in the culture medium is a prerequisite to glycolytic carbohydrate assimilation, which is characteristic of homoenzymatic lactic acid fermentation. In the absence of inorganic phosphorus, the process of fermentation is arrested at the first stage of 3-phosphoglyceraldehyde formation.

The study of the kinetics of the growth of strain F-116 in base medium with decreased phosphate content (1.0%) over a period of 30 h demonstrated that biomass accumulation attains its maximum level within 9 h (a decrease in OD_{540} from 0.14 to 0.89); this was paralleled by the elevation of antibiotic activity to 1900 IU/ml, and only by the end of the twelfth hour of incubation did it attain the maximum of 2800 IU/ml. After 12 h of growth, the exponential phase of growth was followed by the steady-state phase, which lasted for 3 h (Fig. 1b); further decrease in the optical density of the culture liquid was observed subsequently. The same growth cycle pattern and the associated kinetics of bacteriocin synthesis were also noted when the content of phosphates was reduced to 0.5%: the maximum value of biomass accumulation was recorded by 9 h of incubation, and bacteriocin-synthesizing activity attained its maximum value, equaling 1200 IU/ml, by 18 h. The decrease in the content of phosphates in the base fermentation medium caused bacteriocin synthesis to fall behind the growth of the producer biomass (Fig. 1c). Thus, phosphate deficiency suppressed the growth of the culture, the assimilation of carbohydrates, and the biosynthetic processes.

Our study of the regulation of bacteriocin synthesis in strain F-116 by the source of nitrogen involved three experimental settings, in which the content of yeast autolysate was increased from the base level of 35 mg % ammonium nitrogen to 50 and 70 mg %. In the low-phosphate base medium (1.0% KH_2PO_4), the level of biomass accumulation increased by 18 and 33.7%, respectively, whereas the corresponding increments of bacteriocin-synthesizing activity were of 13.6 and 17.9%. In the presence of the lowest phosphate concentration tested (0.5% KH_2PO_4), increasing the amount of yeast autolysate augmented the bacteriocin-synthesizing activity by 10.4 and 16.2%, respectively; this occurred in parallel with increased biomass accumulation (by 11.4 and 17.1% (Table 1)).

Table 2. Effects of carbohydrates on the development of *Lactococcus lactis* subsp. *lactis* F-116, grown in base medium containing 2% KH_2PO_4 and 1% carbohydrate for 12 h

Carbohydrate	pH	OD_{540} units	Activity, IU/ml
D-Arabinose	4.6	0.72	1900
D-Ribose	5.4	0.55	300
D-Xylose	6.6	0.35	0
D-Glucose	4.2	1.26	4100
D-Galactose	4.5	1.11	3100
D-Mannose	4.6	1.09	3000
L-Rhamnose	6.5	0.50	0
D-Fructose	6.3	0.37	0
D-Maltose	4.2	1.46	4350
D-Sucrose	4.0	1.52	5170
D-Lactose	4.3	1.11	3850
Raffinose	4.6	0.92	2000
Mannitol	6.6	0.42	0
D-Sorbitol	5.8	0.90	1400
Dulcitol	6.5	0.43	1000
Dextrin	6.3	0.68	250
Glycerol	6.8	0.23	0
Starch	6.8	0.23	0

Data on the growth dynamics of strain F-116, monitored over 30 h, demonstrated that, in base medium with decreased phosphate content (1.0 or 0.5% KH_2PO_4), increasing the level of yeast autolysate from 35 to 50 or 70 mg % ammonium nitrogen stimulates, both the growth of lactococci, and their ability to synthesize bacteriocins. The maximum biomass accumulation by the culture coincided with maximum bacteriocin level in the culture liquid (Figs. 1a–1e). The differences in the bacteriocin-synthesizing activity, recorded in the various settings of this experiment (Figs. 1a–1e), confirm that the assimilation of nitrogen sources is correlated with the concentration of phosphates in the medium.

The results of this experiment demonstrated that changes in the content of organic nitrogen (introduced into the medium as yeast autolysate) play an important role in the regulation of bacteriocin synthesis, confirming earlier observations of augmented (1.5 times) nisin synthesis in the presence of increased amount of nitrogen sources (yeast autolysate or fish flour) in the medium [9, 13].

Bacteriocins are formed in media containing diverse carbon sources. Thus, Japanese researchers demonstrated that the lactococcus *L. lactis* IO-1 synthesizes nisin Z in the presence of glucose, sucrose, or xylose. It is of note that the activity of the culture liquid was higher when the microorganism was grown in a medium with glucose (4000 IU/ml) than with xylose (3000 IU/ml) [16]. On the other hand, sucrose was a more favorable carbon source than glucose for the synthesis of enterocin 1146 [12].

It follows from the results of our studies that *L. lactis* subsp. *lactis* F-116 is capable of fermenting aldohexoses (glucose, galactose, and mannose), disaccharides (sucrose, maltose, and lactose), and one pentose (arabinose); ribose, the trisaccharide raffinose, the polysaccharide dextrin, and sugar alcohols sorbitol and dulcitol are less suitable as substrates. The strain fails to ferment xylose, deoxymannose, rhamnose, the ketohexose fructose, mannitol, and glycerol; no starch hydrolysis is observed (Table 2).

When the bacteria were cultured in media containing glucose, sucrose, or maltose, a decrease in pH was observed, paralleled by and increase in antibiotic activity. Sucrose was the most suitable carbon source for the growth and development of strain F-116 lactococci, which lends further support to the data reported by others [1, 12]. Biomass accumulation in a medium containing 2% KH_2PO_4 and 1% sucrose amounted to 20.6%; the antibiotic activity of the culture liquid was 26.1 and 18.9% higher than in experiments where the bacteria were grown in the presence of the same concentrations of glucose or maltose, respectively. Bacteriocin-synthesizing activity was lower in media with lactose, raffinose, or arabinose (by 6, 51.2, and 53.7%, respectively), which correlated with growth characteristics and bacteriocin-synthesizing activity (Table 2).

The synthesis of bacteriocin increased when the bacteria were grown in the presence of increased

Table 3. Dynamics of growth of *Lactococcus lactis* subsp. *lactis* F-116 and bacteriocin synthesis in the fermentation medium containing 1% KH_2PO_4, yeast autolysate (70 mg % ammonium nitrogen), and 2% glucose (or sucrose)

Duration of culturing, h	Glucose			Sucrose		
	pH	OD_{540}	Activity, IU/ml	pH	OD_{540}	Activity, IU/ml
0	6.9	0.19	0	6.8	0.19	0
3	6.0	0.52	1700	6.6	0.80	2100
6	4.8	0.87	3200	4.4	1.46	4160
9	4.0	1.36	3890	4.0	1.82	5200
12	3.8	1.32	3890	3.6	1.80	5180

Table 4. Effects of amino acids on the synthesis of bacteriocins by the strain *Lactococcus lactis* subsp. *lactis* F-116, grown in the base fermentation medium containing 2% KH_2PO_4 and 1% glucose, for 12 h

Amino acid	Biomass, OD_{540} units	pH	Activity, IU/ml
DL-Threonine	1.00	4.2	3200
DL-Leucine	1.10	4.2	3200
L-Serine	1.10	4.2	4200
L-Lysine	1.20	3.9	2900
DL-Cystine	1.30	3.9	2600
L-Glutamate	1.10	4.2	4500
L-Aspartate	1.10	4.2	2350
L-Isoleucine	1.10	4.2	5250
L-Methionine	1.20	3.9	1200
L-Cysteine	0.82	4.5	3600
DL-Valine	1.10	4.2	4600
Control	1.26	4.2	4100

amounts of carbon and nitrogen sources. Table 3 shows the results of studies of the dynamics of growth and bacteriocin accumulation of strain F-116 grown in low-phosphate (1% KH_2PO_4) media that contained doubled amounts of glucose (or sucrose) and yeast autolysate (70 mg % ammonium nitrogen). Increased concentrations of carbohydrates ensured active fermentation and stimulated bacteriocin-synthesizing activity of the lactococcus: during 9 h of incubation in the presence of 2% glucose, the level of antibiotic activity increased by 18% (compared to the result recorded in the presence of 1% glucose), attaining 3890 IU/ml, which was associated with dramatic acidification (pH 4.0) of the medium (Tables 1 and 2). Substitution of 2% sucrose for glucose further increased the bacteriocin-synthesizing activity (by 33.7%, up to 5200 IU/ml) and biomass accumulation (by 33.8%). Thus, the composition of the medium affects both the growth of the biomass and the dynamics of the bacteriocin-synthesizing activity of the lactococcus: a decrease in the amount of phosphates (KH_2PO_4) from 2 to 0.5% considerably suppressed the level of bacteriocin activity, whereas introduction into the medium of 2% sucrose increased it by 26.8% (compared to the activity of the lactococcus cultured in the base fermentation medium with 1% glucose).

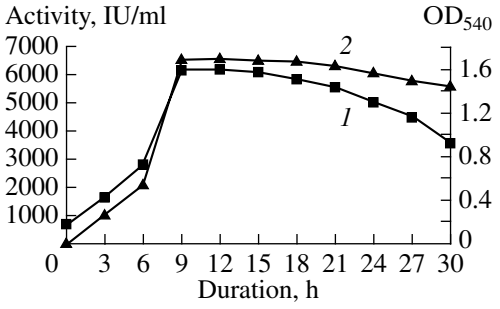

Fig. 2. Dynamics of growth (*1*) and bacteriocin activity (*2*) during culturing of *Lactococcus lactis* subsp. *lactis* F-116 in a fermentation medium containing 1% KH_2PO_4, 70 mg % $[NH_4]^+$ yeast autolysate, 2% sucrose, and 0.1% isoleucine.

In addition to the regulatory effects of the amount and ratio of the main components of the medium, we studied the role of individual amino acids introduced into the medium on the growth of the lactococcus and its ability to synthesize bacteriocins. Our interest in these effects was due to the ability of amino acids to be directly involved in the synthesis of structural proteins, polypeptides, enzymes, bacteriocins, and proteinaceous antibiotics. For example, threonine, serine, cysteine, lysine, and cystine are precursors of lanthionine and β-methyllanthionine and constituents of the nisin molecule [2, 5, 22].

We were able to demonstrate that the addition to the base fermentation medium (2% KH_2PO_4, yeast autolysate equivalent to 35 mg % ammonium nitrogen, and 1% glucose) of serine, glutamate, valine, or isoleucine (0.1% each) increased bacteriocin synthesis by 2.4, 10.4, 12.2, and 28.5%, respectively; in the latter case, the antibiotic activity attained its maximum level of 5250 IU/ml (Table 4).

When the growth dynamics of strain F-116 (in the fermentation medium containing 1% KH_2PO_4, yeast autolysate equivalent to 70 mg % ammonium nitrogen, 2% sucrose, and 0.1% isoleucine) was followed for 30 h, the optical density and the antibiotic activity of the culture liquid increased to 1.7 and 6600 IU/ml within 9 h. Thereafter, the exponential phase was followed by the steady-state phase (Fig. 2). After 15 h of culturing, we observed an insignificant decrease in the antibiotic activity of the culture liquid (to 6530 IU/ml), and, by the end of 30 h of incubation, the activity decreased further (to 5640 IU/ml); the amount of cells also became lower.

Thus, changes in the composition of the low-phosphate (1% KH_2PO_4) fermentation medium—addition of yeast autolysate (70 mg % ammonium nitrogen), 2% sucrose, and 0.1% isoleucine—allowed us to increase the level of bacteriocin-synthesizing activity of the strain *L. lactis* subsp. *lactis* F-116 to 6600 IU/ml, which was 61% higher than bacteriocin level in the base medium with high phosphate content (2% KH_2PO_4), used for culturing nisin-producing *L. lactis* subsp. *lactis* F-116 [1, 7, 9, 17].

In conclusion, we studied the regulation by nutrition sources of the biosynthesis of bacteriocin produced by a new strain, *L. lactis* subsp. *lactis* F-116, which was obtained by the fusion of protoplasts derived from weakly active strains. Bacteriocin synthesis was paralleled by the growth of the culture of the producer strain. We demonstrated that the content of inorganic phosphorus (KH_2PO_4), nitrogen (yeast autolysate), and car-

bohydrates affect both the growth of the producer and the synthesis of the target metabolite. On decreasing the content of KH_2PO_4 from 2 to 0.5%, the biosynthetic activity of the lactococcus attenuated; an increase in the level of yeast autolysate and carbohydrates stimulated the biosynthetic process. Sucrose and isoleucine exemplified the most favorable carbon source and amino acid, respectively, for bacteriocin synthesis.

Based on the results of this work, we recommend the above medium for culturing the bacteriocin producer strain *L. lactis* subsp. *lactis* F-116.

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