The role of aminoacids in nisin and pediocin production by two lactic acid bacteria. A factorial study.

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ABSTRACT

In conventional and repeatedly re-alkalized cultures of two lactic acid bacteria, the effects of five amino acids and their interactions on biomass and bacteriocin (nisin and pediocin) production were studied. Nisin production was stimulated by cysteine and tryptophan, and depressed by proline. Equations describing biomass production in *L. lactis*, and biomass and pediocin productions in *P. acidilactici*, contain numerous terms with three and four variables, indicating that none of the amino acids seems to play a relevant individual role. Stimulation due to the pH-stepwise profiles exceed significantly those of the most effective amino acid combinations and promote much faster consumption of some amino acids. Since a pH-stepwise culture can be considered as a series of successive incubations, each starting up in presence of the metabolic products from the preceding step, these results are more compatible with an auto-stimulatory mechanism rather than a nutritional phenomenon.

INTRODUCTION

Bacteriocins are peptides produced by lactic bacteria with anti-microbial activity. For the cultivation of lactic acid bacteria the use of a complex semisynthetic medium, particularly MRS, is recommended [1-4]. Nonetheless, low efficiency of carbohydrate and

protein utilisation was obtained when the micro-organisms were cultured under uncontrolled pH. When the culture reached the minimum pH and the stationary phase, many essential nutrients remained, indicating that the growth ceases not because limiting effects, but inhibitory ones.

However, when cultures experience a stepwise-pH profile, achieved by a series of successive re-alkalizations followed by spontaneous acidifications [5], the initial protein levels were sufficient to promote the consumption of the initial carbohydrates of the media and the glucose added in fed-bath cultures. However, if the amount of tryptone and peptone added to MRS is calculated in accordance with its consumption in cultures with a conventional time-course of pH, biomass and bacteriocin productions decreased with regard to the maximum levels obtained in the conventional media. Thus, MRS medium seems to be unbalanced with respect to the protein/carbohydrate ratio. Relatively high levels of complex peptidic sources in the media are necessary to supply components for the synthesis of bacteriocins, even when this results in excess concentrations of other components.

That the role of these supplements was initially related to vitamins. Hirsch [6] observed that higher nisin production when calcium pantotenate was present in the medium, although, production did not increase when a mixture of vitamins were added. Later, the contribution of Ca^{2+} to the maturation of prenisin [7] and the increase in nisin production when *Lactococcus lactis* was cultured in presence of CaCl_2 [8] both suggest that this cation is responsible for the observed effects.

The effect of complex protein and yeast extract supplements on bacteriocin production has been studied previously in our laboratory [9], and an empirical model describing the effects of tryptone and yeast extract on nisin production proposed. It was shown that both ingredients stimulated nisin production, although a reduction was observed at high concentrations. The similarity of the results, and the absence of vitamins

in tryptone, led to the presumption that amino acids common to both supplements were responsible of the observed effects. The decreased production at high concentrations can be attributed to the buffering capacity of both supplements, that attenuate the pH fall, or to specific inhibitory effects of some supplement components. These results agree with those obtained in the enterocin production by *Enterococcus faecium* [10] or lactocin production by *Lactobacillus casei* [11]. In the first case, it is possible that an effect of the pH cannot be discarded; in the second, the descent in the lactocin production to high tryptone concentrations seems to be a genuine pH-independent effect.

The stimulation of bacteriocin production by organic nitrogen sources has been investigated by several authors (*e.g.* [3,12,13]), generating diverse explanations that some amino acids (or peptides) are enzymatic inducers, that several amino acids are necessary to synthesise the lanthionine ring (only in lantibiotics), or that there are specific nutritional requirements for normal bacterial growth. Nevertheless, these results are unclear and the specific role of amino acids in bacteriocin production has not still been satisfactorily identified.

Accordingly, the main objective of this work is to study the effects of amino acids on nisin and pediocin productions by two lactic acid bacteria in order to define criteria for the eventual formulation of media with a more appropriate nutrient balance.

MATERIALS AND METHODS

Microorganisms and culture media

Micro-organisms used were *Lactococcus lactis* sp. *lactis* CECT 539 (abbreviated key: Lc 1.04) from the Spanish Standard Culture Collection (Valencia, Spain), and *Pediococcus acidilactidi* NRRL B-5627 (key: Pc 1.02) from the Northern Regional Research Laboratory (Peoria, Illinois, USA). *Carnobacterium piscicola* CECT 4020 was

used as an indicator for bacteriocin bioassay. Stock cultures of both micro-organisms were stored at -50°C in powdered skimmed milk suspension with 25% glycerol.

Culture media for Lc 1.04 were prepared combining two groups of components. A first group constant, or basic medium (Table 1), was formulated according to the criteria of Kozak and Dobrzanski [12], and De Vuyst [3]. Moreover, a group was added of five amino acids on whose relevance in the bacteriocin biosynthesis seems to have general agreement, and whose concentrations were adjusted in order to obtain a complete orthogonal design 2^5 , in the terms that will be described later on. In view of the differences between the amino acid composition of nisin and pediocin (Table 2), tryptophan and serine were added to the basic medium for Pc 1.02, and serine, proline and tryptophan were replaced by asparagine, lysine and glycine in the group of variable amino acids, in order to provide components with a high presence in the pediocin molecule. Although glycine can act as a membrane permeabilizer, its high proportion in pediocin justified its inclusion in the second medium.

Although the lactic acid bacteria are aerotolerant anaerobes, in previous studies [5,14] it was demonstrated that the aeration conditions for the maximum productions of biomass and bacteriocins –not always coincident– can differ with the species considered, often they require high levels of dissolved oxygen and can be provided in a reproducible way by varying the flask volume/medium volume (F/M) ratio. Accordingly, micro-organisms were grown in 250 mL Erlenmeyer flasks with optimal F/M ratios for bacteriocin production (1.56 and 2.5 for Pc 1.02 and Lc 1.04, respectively). All cultures were carried out at 30°C and 200 rpm orbital shaking. For re-alkalizations, pH was adjusted with 2N NaOH. Inocula (1% of the total culture volume) were prepared as cell suspensions in sterile distilled water and adjusted to an optical density (700 nm) of 0.900.

Experimental design

Two complementary type of experiments were accomplished:

1: Effects on bacteriocin production of the amino acids included in the variable group, *i.e.*, threonine (T), cysteine (C), serine (S) (as a part of the lanthionine ring), proline (P) and tryptophan (W) for nisin, and threonine (T), cysteine (C), asparagine (N), lysine (K) and glycine (G) in the case of pediocin. For this study, an orthogonal factorial design [15] was carried out, comprising the 32 combinations of the five variable amino acids at two levels, as well as a sextuple replicate in the centre of the domain for estimation of the experimental error. Range and codification of variables are shown in Table 3. After 12 hours, the culture was re-alkalized to pH=7.0. According to previous findings [5], this step renders higher productions and permits the effects of the variables to be better distinguished.

2: Study of time-course of the culture basic variables and amino acid consumptions in conventional and re-alkalized incubations, on media with the specified basic group of nutrients, and the amino acids of the variable group taken to a concentration half of the maximum used in the factorial design. In re-alkalized cultures, the glucose concentration was maintained at 15 g/L by regular feeding.

Analytical methods

At each sampling interval, each whole experimental unit was split into two aliquots. One aliquot was centrifuged at 15,000g/10 min., the sediment washed twice with distilled water and dried to constant weight at 106°C for biomass estimation. The corresponding supernatant was used for the determination of reducing sugars [16], proteins [17], and characteristic metabolites (lactic and acetic acid, 2,3-buthanediol, ethanol) by means HPLC. Chromatographic analysis was performed using a ION-300 column (Interaction Cromatography USA) with 6mM sulfuric acid as mobile phase (flow = 0.4 mL/min) at 65°C, by means a refractive-index detector. Lactic and acetic acid, ethanol and 2,3-

buthanediol standards were obtained from Sigma. The second aliquot was used for the extraction and quantification of bacteriocins (nisin and pediocin), using *Carnobacterium piscicola* CECT 4020 as an indicator, by methods described in detail by Cabo *et al.* [18] and Murado *et al.* [19].

Amino acids in the supernatant of the first aliquot were determined as OPA (o-phthalaldehyde) derivatives by reverse phase HPLC with a Spherisorb ODS-2 C₁₈ column and fluorimetric detection, according to procedures previously described [20,21]. Standards were obtained from Sigma-Aldrich.

Statistical methods

Results of the factorial designs were used to obtain (orthogonal least-squares calculation) empirical equations which describe the biomass and bacteriocin productions as a function of amino acid levels of the variable group. Statistic significance of the coefficients were verified by means Student t-test ($\alpha=0.05$), and model consistency by means Fisher F test ($\alpha = 0.05$) applied to following means squares ratios:

Model / Total error

(Model + Lack of fitting) / Model

Total error / Experimental error

Lack of fitting / Experimental error.

RESULTS AND DISCUSSION

Effects of amino acids on L. lactis production

Statistical analyses in 48 hours-aged cultures allowed to describe the biomass (*B*) and nisin (*BT*) productions by Lc 1.04 through the following equations:

$$\begin{aligned} B = & 5,73 + 0,56 C + 0,28 S + 0,50 W - 0,50 P + \\ & + 0,39 TS - 0,37 TP + 0,33 CS - 0,36 CP - 0,29 WP + \\ & + 0,32 TCS - 0,30 TCP - 0,66 TSP - 0,61 TCSP \end{aligned} \quad [1]$$

$$\begin{aligned} BT = & 6,00 + 0,62 C + 0,77 W - \\ & - 0,25 TP + 0,26 CW - 0,32 TSP - 0,33 TCSP \end{aligned} \quad [2]$$

The regression coefficients (r^2), corrected according to the number of significant variables were 0.787 and 0.708 for biomass and bacteriocin, respectively. Residual distributions did not show any regularity. The most representative response surfaces (Fig. 1) allowed to establish:

1: Bacteriocin and biomass productions were lower than those obtained with complex nitrogen sources as tryptone or yeast extract [14]. This is in agreement with results obtained by other authors [3,10,13,22].

2: A low effect of the variables was observed, with maximum responses less than two-fold the minimum along the experimental domain. Furthermore, although the Student t-test allows to eliminate many interactions for not being statistically significant, the equation describing biomass production still contains numerous terms with interactions of two and three variables. Thus, it could be argued that none of the amino acids seems to play a relevant individual role and be responsible for the stimulant effects of complex ingredients such as tryptone or yeast extract.

3: Whereas biomass production is dependent on several interactions, bacteriocin production is only stimulated by cysteine and tryptophan. On the other hand, proline is always included in the negative terms of the equation (a first order term is also included in the biomass). This indicates the suppression of its depressor effect when this amino acid is at low concentrations.

Amino acid consumption by L. lactis

Comparison of the results from cultures with and without stepwise pH profiles (Fig. 2) confirmed the low levels of bacteriocin production in synthetic media, as well as the effect of pH-gradient previously described [5]. Additionally, the capacity of the culture to recover the acid pH after each re-alkalization was slower when the producer microorganism was cultured in synthetic media with respect to complex media used by [9].

The realkalized cultures showed two well differentiated phases: one homofermentative and another heterofermentative, the latter with scarce production of lactic acid, but in which the productions of ethanol and acetic acid were detected. No significant consumption of any amino acid studied was detected in the control cultures or in the first phase of the re-alkalized ones. However, a fast decrease in glutamic, serine and arginine content was observed in the second phase of the re-alkalized cultures. Threonine, histidine, alanine, isoleucine and leucine were consumed more slowly and no consumption of valine was detected. Additionally, the variations in amino acid consumption profiles were associated along the time with the difference in biomass and bacteriocin productions between control and pH-stepwise cultures, in which nisin production reached a plateau **simultaneously** to glutamic, serine and arginine consumption completion.

Effects of amino acids on P. acidilactici productions

The results obtained for this assay showed a more poorly defined situation than for *L. Lactis*, with a scarce difference between the extreme responses of the experimental domain (maximum $\sim 1.5 \times$ minimum). The Student t-test eliminated few interactions, and this way the equations, although statistically significant, they contain too many terms and, accordingly, they lack interest to attribute relevant effects to individual amino acids:

$$\begin{aligned} B = & 7.37 - 1.58 C + 1.25 L - 1.10 G - \\ & - 2.20 AT + 1.18 AL - 1.74 AG + 1.03 CT + 0.93 CG - 1.29 TL + 1.35 TG - \\ & - 1.78 LG + 1.64 ACT - 1.68 ACL + 1.45 ACG - 1.28 ATL + 1.12 ATG + \\ & + 1.76 CTL - 1.41 CTG + 1.87 CLG - \\ & - 1.10 ACTG + 2.01 ATLG - \\ & - 1.96 ACTLG \end{aligned}$$

$$\begin{aligned} BT = & 3.52 + 0.07 C + 0.09 T - 0.06 G + \\ & + 0.11 AL - 0.06 AG - 0.14 TL + 0.06 TG - 0.09 LG + \\ & + 0.14 ACT + 0.09 ATG + 0.07 CLG + \\ & + 0.13 ACTL + 0.10 ATLG - \\ & - 0.06 ACTLG \end{aligned}$$

Amino acid consumption by P. acidilactici

Although the kinetics of growth and bacteriocin production were similar to those obtained in Lc 1.04 cultures, several differences can be highlighted (Fig. 3):

1: Synthetic medium depresses the capacity of the culture to recover the acid pH after each re-alkalization less than in Lc 1.04.

2: The stabilization of the bacteriocin production takes place later than in Lc 1.04.

3: Division of the culture into two phases based on amino acid consumptions is not as clearly defined as in Lc 1.04. Additionally, these consumptions were less clearly associated along the time with biomass and bacteriocin production.

Discussion

A global valuation of these results suggests that the application of multivariate designs to groups of amino acids in synthetic media is of scarce utility for the search of individual sources of organic nitrogen as factors affecting the bacteriocin production. Such an experimental approach (similar to those of authors as Parente and Hill [10] or Vignolo *et al.* [11]), seems logical in view of the effects of nutrients as peptone or yeast extract, and, in fact, it allows to attribute stimulating or depressor effects to certain amino acids or combinations of them. On the other hand, the role of the amino acids neither is clear in the realkalized cultures, in spite of its considerably higher productions. This way, it can be concluded that the low productivities are not the principal problem to characterize that role. However, 1) the productions are always much lower in synthetic media than in complex ones, 2) the effects of the amino acids are of scarce magnitude, 3) even in synthetic media, the stimulation due to the pH-stepwise profiles exceeds significantly those of the most effective amino acid combinations (Figs. 2 and 3).

Several authors have proposed, based on metabolic or genetic arguments, that the peptides are more important than amino acids in the bacteriocin synthesis. The first group of arguments has pointed out that the transport systems are more efficient for peptides than for amino acids [23], that amino acids are more protected from catabolism when integrated in peptides [24,25], or that bacterial cells obtain energy through the hydrolysis and subsequent elimination of peptides [13]. The second group of arguments has postulated that peptides are inducers of bacteriocin synthesis by direct activation of transcription once inside the cell, or through a second messenger mechanism (*i.e.* activation of a transmembrane protein generating a cytoplasmic response that activates transcription). In both ways the structure of the inducer could be equal or similar to the bacteriocin produced. Kuipers *et al.* [26] have found auto-induced nisin synthesis by *Lactococcus lactis* through a second messenger mechanism, whilst Eijsink *et al.* [27] have demonstrated that sakacin synthesis by *Lactobacillus sake* is induced by a structurally similar 17 amino acid-peptide.

The stimulating effects due to the pH-stepwise profiles described in this paper seem of interest at this respect, since an incubation process carried out in this way can be considered, to a certain extent, like a series of successive cultures, each starting up in presence of the metabolic products eliminated by the micro-organism during the precedent step. Since it is reasonable to suppose that among these products they are peptides (bacteriocins are, in fact, peptides), the differences between conventional and pH-stepwise cultures on synthetic media are more compatible with an auto-stimulatory mechanism rather than a nutritional phenomenon.

On the other hand, the differences among the consumptions of amino acids in both types of conditions suggest that, at least in the studied cases, the auto-stimulatory effects

could affect the mechanisms responsible for the transport of amino acids, maybe without affecting the transcription processes, since in this case it could be expected differences even more important, as those detected by Kuipers *et al.* [26].

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REFERENCES

- [1] Daba H, Lacroix C, Huang J, Simard RE. Influence of growth conditions on production and activity of mesenterocin 5 by a strain of *Leuconostoc mesenteroides*. Appl Microbiol and Biotechnol 1993;39:166-73.
- [2] Jensen PR, Hammer K. Minimal requirements for exponential growth of *Lactococcus lactis*. Appl Environ Microbiol 1993;59:4363-66.
- [3] De Vuyst L. Nutritional factors affecting nisin production by *Lactococcus lactis* subsp. *lactis* NIZO22186 in a synthetic medium. J Appl Bacteriol 1995;78:28-33.
- [4] De Vuyst L., Vandamme EJ. Nisin, a lanthibiotic produced by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and applications. In: De Vuyst, L., Vandamme, E.J. (eds) Bacteriocins of Lactic Acid Bacteria: Microbiology, genetics and applications. Blackie Academic & Profesional, London, 1994. p. 151-221.
- [5] Cabo ML, Murado MA, González M^aP, Pastoriza L. Nisin production and pH gradient. A mathematical model. Enzyme Microb Technol 2001;29:264-73.
- [6] Hirsch A. Growth and nisin production of a strain of *Streptococcus lactis*. J General Microbiol 1951;5:208-21.

- [7] Siezen RJ, Rollema HS, Kuipers OP, De Vos WM. Homology modelling of the *Lactococcus lactis* leader peptidase NisP and its interaction with the precursor of the lantibiotic nisin. *Prot Engin* 1995;8:117-25.
- [8] Matsusaki H, Endo N, Sonomoto K, Ishizaki A. Lanthibiotic nisin Z fermentative production by *Lactococcus lactis* IO-1: relationship between production of the lantibiotic and lactate and cell growth. *Appl Microbiol Biotechnol* 1996;45:36-40.
- [9] Cabo ML, Murado MA, González M^aP, Vázquez JA, Pastoriza L. An empirical model for describing the effects of nitrogen sources on nisin production. *Lett Appl Microbiol* 2001;33:425-29.
- [10] Parente E, Hill C. A comparison of factors affecting the production of two bacteriocins from lactic acid bacteria. *J Appl Bacteriol* 1992;73:290-98.
- [11] Vignolo GM, De Kairuz MN, De Ruiz AAP, Oliver G. Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. *J Appl Bacteriol* 1995;78:5-10.
- [12] Kozak W, Bardowski J, Dobrzanski WT. Lactostrepcins-A bacteriocin produced by *Streptococcus lactis*. *Bull Acad Poland Scien* 1977;25:217-21.
- [13] Aasen IM, Møretrø T, Katla T, Axelsson L, Storrø I. Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Appl Microbiol and Biotechnol* 2000;53:159-66.
- [14] Vázquez JA. Lactobacterias como probiontes e produtoras de bacteriocinas. Modelos de crecimiento e actividade. Aplicacións á acuicultura. Ph D Thesis, Universidade de Santiago de Compostela, España, 2001. ISBN 84-8121-996-7.
- [15] Box GEP, Hunter WG, Hunter JS. In: Reverté (ed.) Estadística para investigadores. Barcelona, 1989.
- [16] Bernfeld P. (1951) Enzymes of starch degradation and synthesis. In: Advances in Enzymology 1951. p. 379-427.

- [17] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-75.
- [18] Cabo ML, Murado MA, González M^aP, Pastoriza L. (1999) A method for bacteriocin quantification. J Appl Microbiol 1999;87:907-14.
- [19] Murado MA, González MP, Vázquez JA. Dose-response relationships: an overview, a generative model and its application to the verification of descriptive models. Enzyme Microbiol Technol 2002;31:439-55.
- [20] Burbach JPH, Prins A, Leboville JLM, Verhoef J, Witter A. Sensitive and rapid amino acid analysis of peptide hydrolysates by high-performance liquid chromatography of o- phthalaldehyde derivatives. J Chromatog 1982;237:339-43.
- [21] Hancock WS, Sparrow JT. HPLC analysis of biological compounds-a laboratory guide. Marcel Dekker Inc. (eds.), New York, 1984.
- [22] Biswas SR, Ray P, Johnson MC, Ray B. Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. Appl Environ Microbiol 1991;4:1265-67.
- [23] Monnet V, Gripon JC. Métabolisme azoté des bactéries lactiques. In «Bactéries lactiques». Ed. de Roissart H and FM Luquet, Lorica (Uriage), 1994. p. 331-47.
- [24] Law BA, Kolstad J. Proteolytic systems in lactic acid bacteria. Antonie van Leeuwenhoek. 1983;49:225-45.
- [25] Matthews DM, Payne JW. Peptide transport in protein nutrition. Matthews DM & JW Payne (eds). North Holland & American Elsevier, (Amsterdam), 1975.
- [26] Kuipers OP, Beerthuyzen MM, De Ruyter GGAP, Luesink EJ, De Vos WM. Autorregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J Biol Chem 1995;270:27299-27304.
- [27] Eijsink VGH, Brurberg MB, Middelhoven PH, Nes IF. Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. J Bacteriol 1996;178:2232-36.

Table 1: Basal media and concentrations of amino acids used in the experimental plan of *Lactococcus lactis*.

KOZAK & DOBRZANSKI (1977)	g / L	µg / L	Present work	g / L	µg / L
Sucrose	10.0	-	Glucose	20.0	-
MgSO ₄	0.5	-	MgSO ₄	0.5	-
KH ₂ PO ₄	0.7	-	KH ₂ PO ₄	0.7	-
Na ₂ HPO ₄	2.2	-	Na ₂ HPO ₄	2.2	-
NaCl	0.7	-	NaCl	0.7	-
Citrate.Na ₃	10.0	-	Citrate.Na ₃	10.0	-
-	-	-	CaCl ₂ (1)	6.0	-
Biotin	-	0.60	Biotin	-	0.60
Calcium pantothenate	-	70.0	Calcium pantothenate	-	70.0
Nicotinic acid	-	70.0	Nicotinic acid	-	70.0
Pyridoxine	-	70.0	Pyridoxine	-	70.0
Riboflavin	-	70.0	Riboflavin	-	70.0
Aminoacids			Aminoacids		
Alanine	1.3	-	Alanine (2)	1.25	-
Arginine	1.3	-	Arginine	1.25	-
Glutamic acid	1.3	-	Glutamic acid	1.25	-
Histidine	1.3	-	Histidine	1.25	-
Isoleucine	1.3	-	Isoleucine	1.25	-
Leucine	1.3	-	Leucine	1.25	-
Methionine	1.3	-	Methionine	1.25	-
Treonine	1.3	-	-	-	-
Valine	1.3	-	Valine	1.25	-
			Variable aminoacids		
			<i>T</i> : Threonine (Lant)	0.5-1.5-2.5	-
			<i>C</i> : Cysteine (Lant)	0.5-1.5-2.5	-
			<i>S</i> : Serine (Lant)	0.5-1.5-2.5	-
			<i>W</i> : Thryptophan	0.5-1.5-2.5	-
			<i>P</i> : Proline	0.5-1.5-2.5	-

(1): Kozak and Dobszanski didn't use this component; De Vuyst (1995) used 55 µg / L.

(2): De Vuyst (1995) changed alanine by cysteine in the basal medium (in this work both are included as variable aminoacids).

(Lant): Aminoacids that form the ring of lanthionine.

Table 2: Aminoacids of nisin A and pediocin (mature peptides).

Aminoacid	Nisin A	Pediocin
G: glycine	3	8
A: alanine	12 (1)	4
K: lysine	3	4
Y: tyrosine	0	2
N: asparagine	1	4
V: valine	1	2
T: threonine	0 (2)	4
C: cysteine	0 (1)	4
H: histidine	2	3
S: serine	1	2
D: aspartic acid	0	1
W: thryptophan	0	2
I: isoleucine	3	2
M: methionine	2	1
Q: glutamine	0	1
Dha: dehydroalanine	2	0
Dhb: dehydrobutyrine	2	0
Abu: butyrine	4 (2)	0

(1) 5 cysteine and 1 serine found in the mature peptide as members of disulfure bridges come from alanines.

(2) Result of a disulfure bridge formed between a threonine and a non-adjacent cystein.

Table 3: Variables, experimental domain and codification criteria used in the experimental plans.

Codified values	Natural values (g / L)				
	T: threonine ¹ T: threonine ²	C: cysteine ¹ C: cysteine ²	K: lysine ¹ S: serine ²	N: asparagine ¹ W: thryptophan ²	G: glycine ¹ P: proline ²
-1	0.5	0.5	0.5	0.5	0.5
0	1.5	1.5	1.5	1.5	1.5
1	2.5	2.5	2.5	2.5	2.5

Codification: $V_c = (V_n - V_o) / \Delta V_n$; decodification: $V_n = V_o + (\Delta V_n \times V_c)$. V_c : coded value; V_n : natural value; V_o : natural value in the center of the experimental domain; ΔV_n : increment of V_n corresponding to one unit of V_c .

(1): *Pediococcus acidilactici*; (2): *Lactococcus lactis*.

FIGURE CAPTIONS

Figure 1: Response surfaces of the effects of the most relevant amino acids on the biomass and bacteriocin productions by *Lactococcus lactis* (Lc 1.04) in synthetic media.

Figure 2: *Lactococcus lactis* cultures on synthetic medium with spontaneous (left) and stepwise pH profile (right). B: biomass; BT: bacteriocin; RS: reducing sugars; La: lactic acid ($\square$); Et: ethanol ($\circ$); Aa: acetic acid ($\diamond$); aa1: aminoacids of moderate-high consumption ($\diamond$: glutamic, $\circ$: serine, $\square$: arginine); aa2: aminoacids of moderate-low consumption (∇ : histidine, $\triangle$: threonine, $\diamond$: alanine, $\circ$: isoleucine, $\square$: leucine, $\odot$: valine). Initial values of aminoacids are showed in table 1. The vertical dotted line separates the two phases of the culture (see text).

Figure 3: *Pediococcus acidilactici* cultures on synthetic medium with spontaneous (left) and stepwise pH profile (right). B: biomass; BT: bacteriocin; RS: reducing sugars; La: lactic acid ($\triangle$); Bd: buthanediol ($\circ$); Aa: acetic acid ($\diamond$); aa1: aminoacids of moderate-high consumption (∇ : glutamic acid, $\triangle$: serine, $\diamond$: histidine, $\circ$: glycine, $\square$: alanine, $\odot$: asparagine); aa2: aminoacids of moderate-low consumption (∇ : threonine, $\triangle$: tyrosine, $\diamond$: isoleucine, $\circ$: lysine, $\square$: valine). Initial values of aminoacids are showed in table 1. The vertical dotted line separates the two phases of the culture (see text).

FIGURE 1

FIGURE 2

FIGURE 3