Antibacterial activity produced by *Lactococcus lactis* ssp. *lactis* CECT 539 in different culture media

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In this work, the antagonistic activity of a cell-free supernatant (CFS) from a culture of *Lactococcus lactis* subsp. *lactis* CECT 539 was firstly characterised by determining its antibacterial spectrum and potency against related lactic acid and pathogenic bacteria by employing the agar well-diffusion method. The antagonistic activity produced by the nisin-producing strain was similar to those obtained with a solution of 1 g/L of Nisaplin and a CFS from a culture of *Pediococcus acidilactici* NRRL B-5624, a pediocin producing-strain. Subsequently, a photometric bioassay in culture tubes was used to quantify the individual antibacterial activity against *Carnobacterium piscicola* CECT 4020, of different metabolites (nisin, lactic acid, acetic acid, butane-2,3-diol and ethanol) present in CFS obtained from three different realkalised fed-batch cultures of *L. lactis* CECT 539. In this study, nisin was found to be the most potent antibacterial product while butane-2,3-diol did not exhibited a significant inhibitory effect.

Keywords Nisin; *Lactoccoccus lactis*; *Pediococcus acidilactici*; antibacterial products; cell-free supernatant; inhibition.

1. Introduction

Chemical preservatives (*e.g.* nitrite, nitrate and sodium benzoate, SO_2) and heat treatment have been usually used to control the growth of undesirable microorganisms in foods. However, the serious risk that some of these preservatives have to the health and safety of consumers, as well as the growing consumer rejection of heat-treated foods have focused the attention on the production of natural (less-processed) foods. This approach includes the vacuum packaging and storage at low temperatures, among other alternatives. However, these foods may contain pathogenic microorganisms capable of growing even under these conditions [1–3].

The use of ionizing radiation can destroy these organisms, but this method often causes some flavour changes in foods and, in addition, radiation does not protect food from contamination after treatment [4]. One solution to this dilemma could be the use of antimicrobial metabolites (*e.g.* bacteriocins) produced by lactic acid bacteria (LAB). This procedure may reduce the addition of chemical preservatives, as well as the thermal processing of low-acid foods with high water activity, resulting in less-processed food products that preserve their initial characteristics, especially those related with the texture and flavour [5, 6].

Bacteriocins are ribosomally synthesised antibacterial peptides with a narrow (against related species) or a broad (against unrelated species) antibacterial activity spectrum [7, 8]. Combination of bacteriocins with other stress-inducing processes, including freezing, acid treatment, chelating agents, high hydrostatic pressure and electroporation, has found to be a way to control the growth of Gram negative or resistant Gram-positive bacteria [9, 10].

Nisin, produced by *Lactococcus lactis*, has been the most studied bacteriocin to date and has been authorised for food preservation in different countries [11, 12]. This bacteriocin exhibits antibacterial activity towards different important spoilage and pathogenic microorganisms such as *Listeria monocytogenes* and *Bacillus cereus* and also the outgrowth of spores of bacilli and clostridia [13–15].

During the fermentation process, nisin and other antimicrobial substances such as lactic, acetic and propionic acids accumulate in the culture media. Therefore, the antibacterial activity of cell-free supernatants (CFS) from these culture media towards any indicator strain could be due to the synergistic effect of nisin and the other antimicrobial compounds [16]. For this reason, to quantify the individual antibacterial activity and the concentration of nisin in the CFS is necessary to determine the individual activity of each antimicrobial compound.

In the present work, the antibacterial spectrum and potency of a cell-free supernatant obtained from a *Lactococcus lactis* CECT 539 batch culture in MRS broth was compared to those of the cell-free supernatant from a *Pediocccus acidilactici* NRRL B-5624 (a pediocin-producing strain) culture in MRS broth and a solution of 1 g/L of Nisaplin. Subsequently, the contribution to the overall antibacterial activity of nisin and other antimicrobial products (lactic acid, acetic acid, ethanol and butane-2,3-diol) produced in three different realkalised fed-batch cultures of strain CECT 539 [16], was estimated by using response surface methodology and empirical modelling.

2. Materials and methods

2.1. Microorganisms and culture media

L. lactis subsp. *lactis* CECT 539, the nisin-producing strain and *Ped. acidilactici* NRRL B-5624, the pediocin-producing strain, were respectively acquired from the Spanish Type Culture Collection (CECT) and the Northern Regional Research Laboratory (NRRL, Peoria, IL, USA). The strains used as indicators in the agar well-diffusion bioassay are listed in Table 1. All the cultures were maintained as frozen stocks at −40 °C in Nutrient Broth containing 15% (v/v) of glycerol and were propagated twice in appropriate media before use. All *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Pediococcus* and *Leuconostoc* strains were grown on MRS broth. Strains of *Enterococcus* and *Listeria* were respectively grown on Rothe and Mc Bride broth.

Table 1 Indicator bacterial strains used in this work.

Indicators	*Strain*	*Designations*
Carnobacterium piscicola	CECT 4020	Cb 1.01
Lactobacillus casei ssp. *casei*	CECT 277	Lb 3.01
Lactobacillus delbrueckii ssp. *lactis*	JCM 1106	Lb 4.05
Lactobacillus fermentum	CECT 285	Lb 5.01
Lactobacillus fermentum	CECT 4007	Lb 5.02
Lactobacillus helveticus	CECT 404	Lb 6.02
Lactobacillus helveticus	CECT 541	Lb 6.04
Lactobacillus helveticus	CECT 800	Lb 6.05
Lactobacillus helveticus	CECT 402	Lb 6.07
Lactobacillus curvatus	CECT 904	Lb 7.01
Lactobacillus plantarum	CECT 229	Lb 8.04
Lactobacillus plantarum	CECT 4044	Lb 8.05
Lactococcus lactis ssp. *lactis*	CECT 540	Lc 1.05
Lactococcus lactis ssp. *lactis*	CECT 916	Lc 1.06
Leuconostoc mesenteroides ssp. *cremoris*	CECT 827	Ln 3.01
Leuconostoc mesenteroides ssp. *mesenteroides*	CECT 4046	Ln 3.07
Enterococcus faecium	CECT 410	Ec 1.01
Enterococcus faecium	CECT 964	Ec 1.02
Enterococcus hirae	CECT 279	Ec 2.01
Listeria monocytogenes	ES	Lm1.01
Listeria monocytogenes	ES	Lm1.02

Abbreviations: CECT: Spanish Type Culture Collection (Valencia, Spain); JCM: Japan Collection of Microorganisms; ES: Provincial Delegation of Health (La Coruña, Spain).

2.2. Fermentation conditions

Batch cultures of strains CECT 539 and NRRL B-5624 were carried out in 250 mL Erlenmeyer flasks containing 50 mL of MRS broth, on a rotary shaker (200 rpm) at 30 °C, until the early stationary phase. The two cultures were started with a 2% (v/v) inoculum of a 12-h culture in the same medium. Samples were taken at the end of the cultures for determination of antibacterial activity by means of an agar well-diffusion bioassay [15].

Constant volume fed-batch fermentations with *L. lactis* CECT 539 were carried out in culture media prepared with mussel processing wastes (MPW) and diluted whey (DW), in a 6-L bench-top fermentor (New Brunswick Scientific, Edison, NJ, USA) with 4 L working volume of medium, at a constant temperature, agitation and aeration levels of 30 ºC, 200 rpm and 0.5 L/h, respectively [16]. The cultures were repeatedly re-alkalised (up to initial pH 7.0) and fed with

the feeding substrates to restore the initial total sugars concentration in the fermentation media, after 8 h for MPW cultures and 12 h for DW culture. The feeding substrates used in the two MPW cultures were a 240 g/L concentrated glucose (fermentation 1) and a concentrated MPW (CMPW; ~ 100 g of glucose/L) medium (fermentation 2). In whey culture (fermentation 3), the feeding substrate consisted of a mixture of concentrated whey (CW; ~ 50 g of lactose/L) and a 400 g/L concentrated lactose. The inoculum in the three fed-batch cultures consisted in 2% (v/v) of a 12-h culture in the corresponding MPW and DW medium. The samples taken at the end of the cultures were used to quantify the individual antibacterial activity against *C. piscicola* CECT 4020, of the different metabolites (nisin, lactic acid, acetic acid, butane-2,3-diol and ethanol) present in cell-free supernatants obtained in each realkalised fed-batch culture. For this purpose, a photometric bioassay in culture tubes was used [16].

In all cases, culture samples were adjusted to pH 3.5 and then heated for 3 min to kill the cells. Subsequently they were centrifuged at 27,200 × *g* for 15 min at 4 ºC to obtain the cell-free supernatants containing the total antibacterial activity.

2.3. Bacteriocin activity assays

Two methods were employed. Firstly, an agar well-diffusion method [17] was used to compare the antibacterial spectrum of cell-free supernatants obtained from batch cultures of strains CECT 539 and NRRL B-5624, and a solution of 1 g/L of Nisaplin. In this assay, an overnight culture of the corresponding indicator bacterium (Table 1) was spread on Rothe agar (in case of *Enterococcus* strains), Mc Bride agar (in case of *Listeria* strains) or MRS agar (in case of *Lactococcus*, *Lactobacillus*, *Carnobacterium* and *Leuconostoc* strains). Wells (5 mm in diameter) were cut into plates and 50 µL of culture supernatant fluid of the producing-strains adjusted to pH 6.0 were placed into each well. Plates were kept for 4 h at 4 °C to allow the diffusion of the cell-free supernatants and later they were incubated at 30 °C for 24 h, after which the widths of the clear inhibition zones around the well were measured. A solution of 1 g/L of Nisaplin™ adjusted to pH 6.0, was used as bacteriocin standard. The antibacterial activity was quantified as the mean diameters, respectively (obtained from triplicate experiments) of the inhibition zones produced by the cell-free supernatants from the cultures of strains CECT 539 and NRRL B-5624, and the Nisaplin solution.

Secondly, a photometric bioassay in culture tubes [18] was used to quantify the individual and combined antibacterial activities against *C. piscicola* CECT 4020 of the antimicrobial products (nisin, lactic acid, acetic acid, butane-2,3-diol and ethanol) at the concentrations obtained at the end of the realkalised fed-batch cultures of *L. lactis* CECT 539 [16]. Stock solutions of each compound were prepared in distilled water and conveniently mixed to obtain the concentrations indicated in the experimental design described below (Table 2).

Diluted samples (2.5 mL) containing the antibacterial compounds were added in sterile culture tubes. Each tube was inoculated with 2.5 mL of a culture of *C. piscicola* CECT 4020 (diluted to an absorbance of 0.2 at 700 nm with sterile buffered MRS broth at pH 6.3) and incubated for 6 h at 30 ºC. Controls consisted in three culture tubes in which the diluted sample was substituted by distilled sterile water. Growth inhibition was measured spectrophotometrically at 700 nm. Dose/response curves were obtained from these data. One antibacterial activity (AU) was defined as the reciprocal of the dilution causing 50% growth inhibition (inhibitory dose 50: ID_{50} obtained from duplicate samples) compared with control tubes. The titres of antibacterial activity (AbA) were expressed in AU/mL [19].

Firstly, the individual antibacterial activities of solutions of lactic acid, acetic acid, butane- 2,3-diol and ethanol, containing each compound at the final concentrations produced in each realkalised fed-batch culture, were quantified. The contribution of nisin in terms of antibacterial activity, considering the absence of synergistic effects, was estimated from the difference between the corresponding total antibacterial activity obtained at the end of each realkalised fed-batch culture and that of each compound. Then, the individual concentration of nisin (g/L) to assay was determined from a standard curve (nisin concentration versus antibacterial activity).

Nisin used in these assays was the commercial preparation Nisaplin™ (Aplin & Barrett Ltd, Beaminster, Dorset, UK) containing 25 mg of nisin/g of Nisaplin. Stock solution of nisin was prepared by dissolving Nisaplin™ in 0.02 M HCl (pH 2) and stored at 20ºC. All chemicals (lactic acid, acetic acid, butane- 2,3-diol and ethanol) used were of analytical grade and were obtained from Cultimed Panreac Química S.A. (Barcelona, Spain).

2.4. Experimental design

A complete factorial design [20, 21] based on two levels and four variables was used to quantify the individual and combined antibacterial activities of nisin, lactic acid, acetic acid, butane-2,3-diol and ethanol. The design consisted of 20 experiments with 16 (2^4) factorial points and four replicates of the central treatment (Table 2). Those concentrations obtained for each metabolite at the end of each realkalised fed-batch culture of *L. lactis* CECT 539 [16] were taken as the higher level (+ 1) in the experimental designs.

Results were analyzed by Experimental Design Module of the Statistica software package (Statistica 5.1 for Windows computer program manual; StatSoft Inc. Tulsa, OK, USA). The Student's *t*-test was employed to check the statistical significance of the regression coefficients. The Fisher's *F*-test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model. The coefficients of the models with *P* values lower than 0.05 were considered statistically significant.

Table 2 Experimental domain and codification of the variables in the three experimental designs used to quantify the individual and combined antibacterial activities of nisin (Nis), lactic acid (LA), acetic acid (AA), butane-2,3-diol (B) and ethanol (Et) produced at the end of the three realkalised fed-batch cultures of *L. lactis* CECT 539.

	Actual values (g/L)											
	Fed-batch fermentation (1)				Fed-batch fermentation (2)				Fed-batch fermentation (3)			
Coded values	Nis	LA	AA	B	Nis	LA	AA	Et	Nis	LA	AA	B
–1	0	0	0	0	0	0	0	0	0	0	0	0
0	0.32	9.60	0.25	1.05	0.63	31.05	1.36	7.35	0.88	9.65	0.85	2.90
1	0.64	19.20	0.50	2.10	1.25	62.10	2.72	14.70	1.75	19.30	1.70	5.80

3. Results and discussion

3.1. Antibacterial activity of a cell-free supernatant from a batch culture of *L. lactis* CECT 539 in MRS broth

Figure 1 shows the antibacterial activities of cell-free supernatants obtained from batch cultures of *L. lactis* CECT 539 and *Ped. acidilactici* NRRL B-5627 in MRS broth and a solution of 1 g/L of Nisaplin against 21 indicator strains (Table 1). The Nisaplin solution inhibited the growth of 19 strains while the *L. lactis* CECT 539 and *Ped. acidilactici* NRRL B-5627 cell-free supernatants were respectively inhibitory to 18 and 16 indicator strains, including the two *Listeria* and three *Enterococcus* strains.

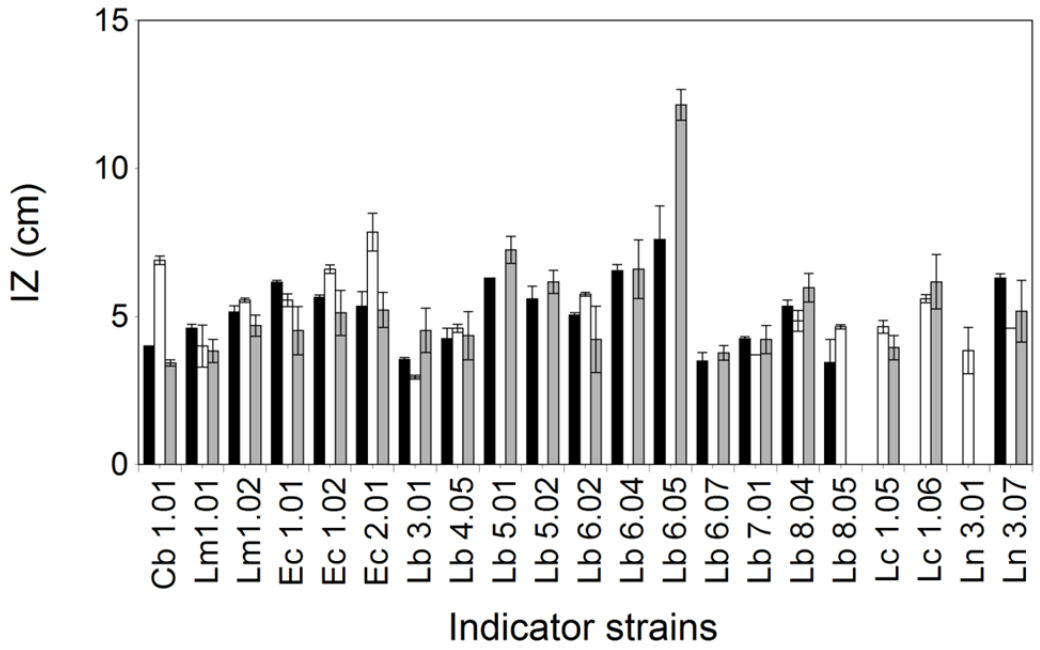

Fig. 1 Mean diameter (obtained from triplicate experiments) of the inhibition zones (IZ) produced by the cell-free supernatants obtained from cultures of *L. lactis* CECT 539 (black bars) and *Ped. acidilactici* NRRL B-5624 (white bars) in MRS broth, and by a solution of 1 g/L of Nisaplin (gray bars). The error bars indicate the range of standard deviation of the experimental data.

The potency of each antibacterial sample (IZ value) exhibited a high variability, indicating that the inhibitory effect was dependent on the indicator strain used. In this way, the IZ values for the Nisaplin solution and the cell-free supernatants from *L. lactis* and *Ped. acidilactici* cultures varied respectively from 0 (in case of strains Lb 8.05 and Ln 3.01) to 12.2 cm (in case of strain Lb 6.05), from 0 (in case of strains Lc 1.05, Lc 1.06 and Ln 3.01) to 7.6 cm (in case of strain Lb 6.05), and from 0 (in case of strains Lb 5.01, Lb 5.02, Lb 6.04, Lb 6.05 and Lb 6.07) to 7.9 cm (in case of strain Ec 2.01). The different sensitivity observed between the indicator strains to the three antibacterial samples, has been observed before for other strains [22]. This behaviour could be attributed to both the differences in the specificity of adsorption of peptides to the cell surface of the indicator strains and the existence of specific receptors for each bacteriocin [23].

3.2. Antibacterial activity of the end products synthesised by *L. lactis* CECT 539 in realkalised fed-batch cultures

The contributions to the total antibacterial activity (AbA) of nisin, lactic acid, acetic acid, butane-2,3-diol and ethanol produced by *L. lactis* CECT 539 at the end of the first, second and third realkalised fed-batch fermentations were studied by using a complete factorial design based on two levels and four variables. The empirical models obtained with their significant parameters ($P < 0.05$) in each case were:

$$\text{AbA (AU/mL)} = 24.3 + 21.0\times[\text{Nis}] + 2.9\times[\text{LA}] + 1.1\times[\text{AA}] \quad (1)$$

$$\text{AbA (AU/mL)} = 55.2 + 41.8\times[\text{Nis}] + 9.0\times[\text{LA}] + 1.8\times[\text{AA}] + 1.2\times[\text{Et}] \quad (2)$$

$$\text{AbA (AU/mL) } 63.0 + 59.5\times[\text{Nis}] + 2.7\times[\text{LA}] \quad (3)$$

Where [Nis], [LA], [AA] and [Et] are the concentrations of nisin, lactic acid, acetic acid and ethanol, respectively.

The coefficients obtained were significant according to the Student's *t*-test ($\alpha < 0.05$) and the equations obtained were significant in Fisher's *F*-tests ($\alpha < 0.05$) applied to both quotients total error/experimental error and lack of fitting/experimental error. In addition, the high values of the regression coefficients (R^2) obtained (> 0.998, in all cases) have strengthened the usefulness of these models for predicting the individual and combined antibacterial activities of nisin, lactic acid, acetic acid, ethanol and butane-2,3-diol against *C. piscicola* CECT 4020.

The response surfaces generated from the three empirical models are shown in Figures 2, 3 and 4. From the detailed observation of the results obtained, it can be noted that nisin showed the highest coefficient values in the three models, indicating that this bacteriocin produced in all cases, the greatest contribution to the total antibacterial activity against *C. piscicola* CECT 4020 and therefore, nisin had the main effect on the responses. Probably due to this fact, the coefficients for the interaction between the independent variables were not significant ($P > 0.05$).

Lactic acid was also present in the three models, but with coefficient values lower that those of the nisin. Taking into account the coefficients obtained for the other compounds, it can be concluded that acetic acid and ethanol contributed only slightly to the total antibacterial activity, while butane-2,3-diol did not produce a significant antibacterial effect.

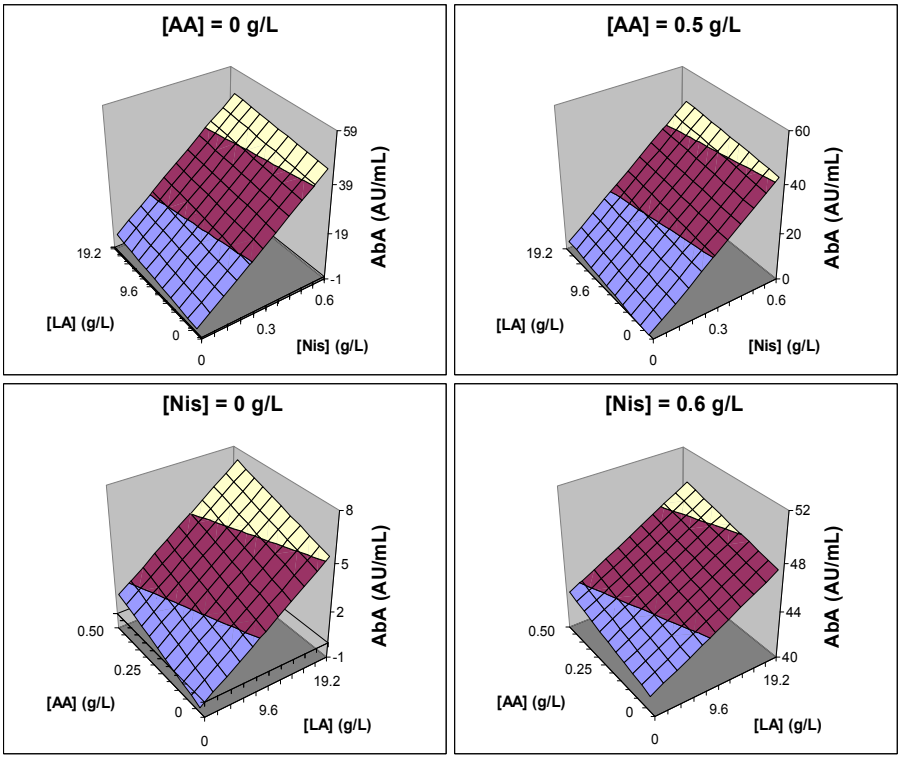

Fig. 2 Response surfaces showing the total antibacterial activities (AbA) against *C. piscicola* CECT 4020 predicted by model (1) for nisin (Nis), lactic acid (LA) and acetic acid (AA).

In the first assay, that was carried out taking as reference the concentration of the products obtained at the end of fermentation 1, the increase in nisin concentration led to an increase in the total antibacterial activity for low and high concentrations of acetic acid (left and right higher parts of Figure 2) with independence of the concentration of butane-2,3-diol (the effect of this compound was not significant). Thus, the individual antibacterial activity predicted by model (1) for nisin was 41.36 AU/mL, which represents the 83.8 % of the maximum antibacterial activity (49.34 AU/mL)

obtained according to the same model for the highest concentrations of nisin (0.64 g/L), lactic acid (19.20 g/L) and acetic acid (0.50 g/L).

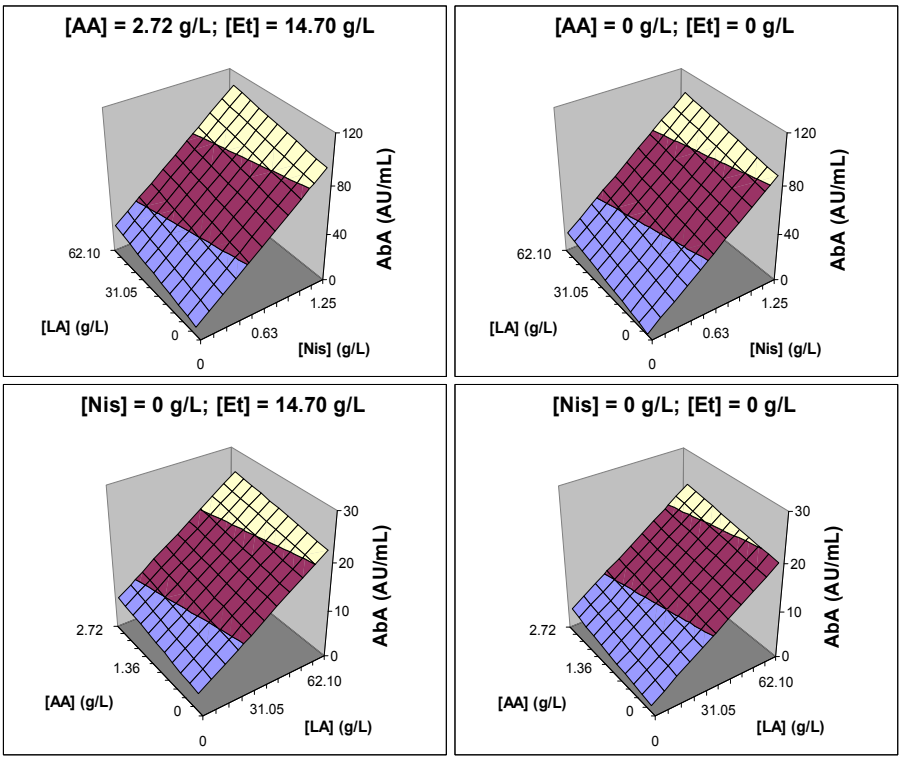

Fig. 3 Response surfaces showing the total antibacterial activities (AbA) against *C. piscicola* CECT 4020 predicted by model (2) for nisin (Nis), lactic acid (LA), acetic acid (AA) and ethanol (Et).

In the same way, the joint contribution of the other metabolites (lactic acid and acetic acid) was 7.98 AU/mL in absence of nisin (left lower part of Figure 2), which represents 16.2 % of the maximum antibacterial activity predicted by model (1).

In the second assay (Figure 3), that was carried out considering the concentration of the products obtained at the end of fermentation 2, the maximum AbA value predicted by model (2) for nisin was 90.99 AU/mL, which represents the 83.4 % of the maximum AbA level (109.04 AU/mL) obtained according to the same model for the highest concentrations of nisin (1.25 g/L), lactic acid (62.10 g/L), acetic acid (2.72 g/L) and ethanol (14.70 g/L).

From the comparisons between the results obtained in the two assays, it can be concluded that the higher total AbA value (109.04 AU/mL) obtained in the second experiment (Figure 2) in comparison to that (49.34 AU/mL) of the first experiment (Figure 1), is due to the highest concentrations of nisin produced in the second realkalised fed-batch culture, since the significant contributions of lactic acid, acetic acid and ethanol in terms of antibacterial activity were very low.

In the third experiment, corresponding to the third realkalised fed-batch experiment, only nisin and lactic acid produced a significant antibacterial activity against *C. piscicola* CECT 4020, because the effect of acetic acid and butane-2,3-diol was not significant. In this case, the effect of nisin (119.79 AU/mL) accounted for almost 96.0 % of the total antibacterial activity (125.21 AU/mL) predicted by model (3), while the activity of the lactic acid (5.41 AU/mL) accounted only for a 4 %.

Although the concentration of acetic acid in the first experiment (0.50 g/L) was three times lower than that of the third experiment (1.70 g/L), the antibacterial effect of this compound in the latter assay was not significant. This was probably due to a stronger inhibitory effect against *C. piscicola* CECT 4020 produced by the higher nisin concentration (1.75 g/L) used in the third experiment in comparison to that (0.64 g/L) of the first assay, which probably masked the antibacterial effect of acetic acid.

All these observations corroborate that the individual contribution of nisin to the total antibacterial activity was considerably higher than those of the other antibacterial compounds in the three experiments. Therefore, the highest total antibacterial activity was obtained in the third assay mainly because the nisin concentration in this experiment (1.75 g/L) was higher than those of the two previous assays (0.64 and 1.25 g/L, respectively).

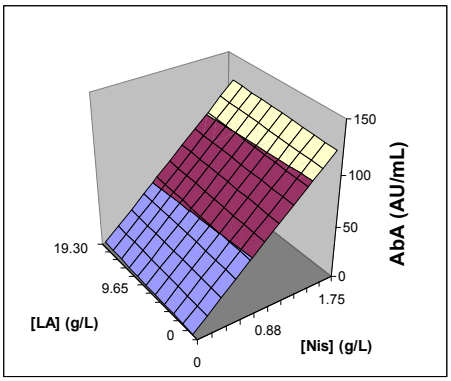

Fig. 4 Response surface showing the total antibacterial activities (AbA) against *C. piscicola* CECT 4020 predicted by model (3) for nisin (Nis) and lactic acid (LA).

From a practical point of view, the results obtained in the present work could be very important. On the one hand, the cell-free supernatants of *L. lactis* CECT 539 could be used to treat food contact surfaces to prevent the initial adhesion of unwanted bacteria, such as *L. monocytogenes* [2]. This could avoid the use of high levels of disinfectants (chlorine, iodine, anionic acid and quaternary ammonium sanitisers) that are needed to eliminate the bacteria attached to the surfaces. These compounds at high concentrations may pose health risks to personnel using these agents, probably result in unacceptable chemical residuals in foods and increased production costs [24].

On the other hand, the cell-free supernatants could be used to produce antibacterial food packaging materials to control the outgrowth of nisin-sensitive strains, including species of *Listeria*, *Clostridium*, *Micrococcus* and *Staphylococcus* that result from postpasteurization contamination. This approach could contribute to the extension of the shelf life of foods [2, 25–29].

4. Conclusions

Lactococcus lactis subsp. *lactis* CECT 539 and *Pediococcus acidilactici* NRRL B-5624 cell-free supernatants exhibited a broad antibacterial activity spectrum with a high potency, which was comparable to that of the commercial nisin preparation (Nisaplin).

Since the antibacterial activity of a compound depends on the indicator strain used, it can be concluded that *C. piscicola* CECT 4020 was highly sensible to nisin, but this bacterium has a great tolerance to the concentrations of lactic acid, acetic acid, ethanol and butane-2,3-diol produced at the end of the three realkalised fed-batch cultures of *L. lactis* CECT 539 in culture media prepared with mussel processing wastes and whey. Thus, *C. piscicola* CECT 4020 could be used as an appropriate indicator strain to quantify the individual antibacterial activity of nisin present in cell-free supernatants of nisin-producing strains.

The results obtained in this work indicate that the cell-free supernatants obtained from the three realkalised fed-batch cultures of *L. lactis* CECT 539 could be used in the food industry to control the growth of spoilage and pathogenic microorganisms.

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