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Prevention of Histamine Formation in Cheese by Bacteriocin-Producing Lactic Acid Bacteria

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The susceptibility of 13 amine-forming lactobacilli to several bacteriocins was investigated by an agar diffusion assay. All strains were susceptible to nisin and to five bacteriocins of enterococcal origin. Pediocin PA-1, bavaricin A, lactococcin A, and a bacteriocin from *Enterococcus faecalis* 1061 did not show inhibitory activity. Two bacteriocin-producing enterococci and a nisin-producing *Lactococcus lactis* strain were employed as starters in separate cheese-making experiments. Outgrowth of histamine producer *Lactobacillus buchneri* St2A, which was added to the milk at levels of up to 190 CFU/ml, was almost completely inhibited. No histamine formation was detected in the cheeses made with bacteriocin-producing starters. In the control cheese without bacteriocin, St2A reached levels of 1.1×10^8 CFU/g, and 200 mg of histamine per kg was found after 4 months of ripening. To our knowledge, this is the first report of bacteriocin-mediated inhibition of histamine formation in foods.

One of the biochemical processes which take place during cheese ripening is the degradation of proteins, leading to the accumulation of free amino acids. Because of the activity of bacterial decarboxylases, some amino acids can be converted into amines. Most of these so-called biogenic amines are pharmacologically active, but oral administration generally does not provoke adverse reactions because amine oxidases in the intestine rapidly detoxify these compounds (3). However, if the amine-metabolizing capacity of the human body is saturated by ingestion of a high dose, or when it is impaired in its activity by specific inhibitors (25), food intoxication may occur. Several cases of cheese-related outbreaks of amine poisoning have been reported, and histamine was implicated in most of them (10, 22, 27). Commonly observed symptoms of histamine poisoning include flushing, sweating, nausea, vomiting, diarrhea, headache, palpitations, and rash (17).

Because most cheeses do not contain histidine-decarboxylating bacteria and because the free histidine concentration in cheese is usually low, histamine levels mostly do not exceed 100 mg/kg (10). Since histamine poisoning probably often depends on the presence of potentiators (such as other biogenic amines like cadaverine and putrescine), it is difficult to establish a threshold value. However, data obtained from the reported cases of food poisoning indicate that a level of 500 to 1,000 mg/kg is potentially dangerous.

Histamine formation can be controlled by reduction of the length of the maturation period, but it is clear that this would be detrimental for flavor development. Suppression of histidine-decarboxylating bacteria is a more appropriate approach to avoid histamine production. Histidine-decarboxylating bacteria are found among different gram-positive and gram-negative genera (21, 22, 26). For histamine buildup in cheese, probably only some heterofermentative mesophilic lactobacilli are important since they combine high decarboxylase activity with the potential to grow in the aging cheese (12). Besides, after reaching their maximum density, which may be as high as 2×10^8 CFU/g, they remain viable for up to 1 year, meanwhile not losing their decarboxylase activity (15). This is crucial since histidine is liberated throughout the whole ripening period and it can be transformed into histamine only if the corresponding decarboxylase remains active.

Pasteurization of the cheese milk and subsequent hygienic processing reduce the threat of heterofermentative lactobacilli, but cheese-making experiments with *Lactobacillus buchneri* St2A showed that contamination of the milk with only a few CFU per milliliter may cause significant amine formation (13). In cheese with accelerated ripening, this strain produced more than 1,000 mg/kg within 3 months. An extra hurdle would therefore be desirable. This hurdle may be found in the application of bacteriocin-producing starter cultures. Nisin-producing cultures have been employed successfully to control various gram-positive spoilage bacteria and pathogens in cheese. There are no specific reports on the susceptibility of histamine-producing lactobacilli to bacteriocins, but the inhibitory spectra of various bacteriocins include heterofermentative lactobacilli (8).

The objective of this work was to compare the activities of various bacteriocins against a panel of 13 different histidine- and tyrosine-decarboxylating lactobacilli in agar media. In addition, selected bacteriocin-producing lactic acid bacteria were used in a cheese-making trial to determine whether they can prevent histamine formation in cheese.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are given in Table 1. All strains were propagated at 30°C in APT broth (Difco catalog no. 0655-01-7).

Cell-free supernatant preparation. Because the bacteriocin-producing bacteria were intended for use as a starter or adjunct starter in cheese-making, most strains were cultivated in milk, supplemented with 0.2% Bacto Tryptone (Difco catalog no. 0123-01-1). The latter was added to promote the growth of possibly proteinase-negative strains. Since *Pediococcus acidilactici* PAC 1.0 and *Lactobacillus bavaricus* MI 401 showed very poor growth in this medium, they were cultivated in APT broth. Bacteriocins were harvested after 16 h of incubation at 30°C as described previously (11). To this end, the pH of the cultures was adjusted to 4.3 with 1 N HCl. After centrifugation (10,000 × *g*, 10 min) the supernatant was filtered through a 0.22-μm-pore-size low-protein-binding filter (Millex GV; Millipore). Finally, the pH was raised to 6.0 with 2 N NaOH.

Susceptibility testing of lactobacilli. The sensitivities of the lactobacilli to the bacteriocins were determined by an agar diffusion assay as described previously (14). Fifty microliters of fresh indicator culture (A_{600} = 1.0) was mixed with 20 ml of molten APT agar (45°C) and poured in a petri dish. The wells (5-mm

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TABLE 1. Bacterial strains used in this study

Species and strain	Source[a]	Bacteriocin produced	Reference
Bacteriocin producers			
Enterococcus faecalis			
EFS 2	INRA	Enterococcin EFS 2	19
INIA 4-07	INIA	Enterocin 4	11
INIA 4-071	INIA	−[b]	11
Enterococcus faecium			
JBL 1061	FRI	+[c]	2
7C5	ILC	+	28
INIA 66	INIA	+	
INIA 70	INIA	+	
Pediococcus acidilactici PAC1.0	URL	Pediocin PA-1	6
Lactobacillus bavaricus MI 401	RVAU	Bavaricin A	16
Lactococcus cremoris LMG 2130	LMGT	Lactococcin A	7
Lactococcus lactis ESI 561	INIA	Nisin	
Heterofermentative lactobacilli			
Lactobacillus buchneri[d]			
NZHD1	NIZO		13
NZHD2	NIZO		13
NZHD3	NIZO		13
NZHD4	NIZO		13
NZHD5	NIZO		13
St2A	UNL		24
Lactobacillus brevis[e]			
2B5B	NIZO		12
Hem3	NIZO		12
NZTD1	NIZO		13
38	INIA		
113	INIA		
115	INIA		
357	INIA		

[a] Abbreviations: INRA, J. Richard, Station de Recherches Laitières, Jouy-en-Josas, France; FRI, J. Luchansky, Food Research Institute, Madison, Wis.; ILC, G. Giraffa, Istituto Sperimentale Lattiero-Caseario, Lodi, Italy; URL, A. Ledeboer, Unilever Research Laboratorium, Vlaardingen, The Netherlands; RVAU, A. Larsen, RVAU Centre for Food Research, Frederiksberg, Denmark; LMGT, H. Holo, Laboratory of Microbial Gene Technology, Agricultural University, As, Norway; INIA, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain; NIZO, Z. Kruiswijk, Netherlands Institute for Dairy Research, Ede, The Netherlands; UNL, S. L. Taylor, University of Nebraska, Lincoln.

[b] *E. faecalis* INIA 4-071 is a bacteriocin-negative variant of INIA 4-07.

[c] +, nameless bacteriocin.

[d] All *Lactobacillus brevis* strains listed produce tyramine.

[e] All *Lactobacillus buchneri* strains listed produce histamine.

diameter) in the agar were filled with 30 μl of the bacteriocin-containing cell-free supernatants and serial twofold dilutions thereof in 0.1 M acetate buffer (pH 5.6) containing 0.1% Tween 80. The diameter of the inhibition zone was plotted against the logarithm of the dilution factor. By use of the calculated regression equation, the sensitivity of the indicator strain was determined; it is expressed as the theoretical dilution for which the diameter of the halo was 0 mm.

Cheese-making. Twelve cheeses were manufactured in 16-liter vats from pasteurized cow's milk by standard procedures for Manchego cheese production (5). Three bacteriocin producers were employed: *Lactococcus lactis* ESI 561, *Enterococcus faecalis* INIA 4-07, and *E. faecalis* EFS 2. *Lactococcus lactis* ESI 561 was grown in milk, while both enterococcal strains were grown in milk supplemented with 0.5% yeast extract. All strains were added to the vats at a 1% inoculation level. The commercial starter MAO11 (Texel; Rhône-Poulenc, Dangé Saint Romain, France) served as a bacteriocin-free control (1% inoculation), but it was also added (1%) to the vats containing strain INIA 4-07 or EFS 2 because these enterococci are not able to acidify the milk sufficiently. Three vats were prepared with each starter or starter combination, to which *Lactobacillus buchneri* St2A was added at levels of 1.9, 19, and 190 CFU/ml. All cheeses were ripened at 14°C.

Chemical analysis. The histidine and histamine contents of the cheeses were determined after 4 months of ripening by the high-performance liquid chromatography (HPLC) method of van Boekel and Arentsen-Stasse (29). A 5-μm-particle-size column was used (Beckman ultrasphere, 250 by 4.6 mm [inside diameter]), and the methanol concentration of the mobile phase was increased to 24%. Under these conditions, the retention times of histidine and histamine were 5.1 and 37.0 min, respectively.

Bacteriological analysis. The number of *Lactobacillus buchneri* St2A organisms in cheese was determined after 1, 4, 8 and 17 weeks of ripening. To this end, Rogosa agar in which glucose was replaced by arabinose was used. Only a few lactobacilli (of which St2A is one) can ferment arabinose, and preliminary experiments had shown that strain St2A grew well in this medium, forming normal-sized colonies (about 3 mm in diameter), whereas most other lactobacilli produced only small colonies. The starter bacteria and bacteriocin-producing lactococci and enterococci did not grow on this agar. Plates were incubated for 96 h at 37°C under anaerobic conditions (AnaeroGen; Oxoid), after which for each cheese 10 small and 10 normal-sized colonies (if present) were streaked onto histidine decarboxylase agar (13). Colonies producing a positive reaction on this agar after 2 days at 37°C were assumed to be *L. buchneri* St2A, and counts on modified Rogosa agar were adjusted for the percentage of positive reactions.

Bacteriocin detection in cheese extracts. Ten grams of cheese was suspended in 40 ml of saline containing 0.1% Tween 80 at 45°C. From this suspension, a cell extract was prepared as described above, and bacteriocin activity was determined with an agar diffusion assay, with *L. buchneri* St2A as an indicator organism (14).

TABLE 2. Sensitivity of amine-producing lactobacilli to various bacteriocins[a]

Indicator species and strain	Sensitivity to[a]: Enterococcal strain					*L. lactis* ESI 561
	INIA 4-07	7C5	EFS 2	INIA 66	INIA 70	
Lactobacillus buchneri						
NZHD1	100	144	240	45	40	60
NZHD2	44	64	100	20	22	56
NZHD3	38	34	55	13	12	46
NZHD4	39	51	72	22	24	56
NZHD5	290	417	724	50	54	102
St2A	540	704	1,770	240	210	110
Lactobacillus brevis						
38	55	115	230	35	31	69
113	210	540	724	60	55	32
115	50	85	129	26	27	102
357	35	106	180	24	21	64
2B5B	125	256	410	51	54	23
Hem3	160	390	510	43	42	26
NZTD1	56	95	178	23	30	27

[a] The sensitivity is expressed as the theoretical dilution for which the diameter of the inhibition zone in the agar diffusion assay was 0 mm. All tested lactobacilli were resistant to pediocin PA-1, bavaricin A, lactococcin A, and the bacteriocin produced by *E. faecium* JBL 1061.

RESULTS AND DISCUSSION

Sensitivity of lactobacilli to bacteriocins. Six strains of histamine-producing lactobacilli and seven tyramine-producing lactobacilli, all belonging to the species *Lactobacillus buchneri* or *Lactobacillus brevis*, were tested for their susceptibility to various bacteriocins in an agar diffusion assay (Table 2). All strains were resistant to pediocin PA-1, bavaricin A, lactococcin A, and the bacteriocin produced by *Enterococcus faecium* JBL 1061. The other five enterococcal bacteriocins and nisin clearly inhibited the lactobacilli. Strains that were very susceptible to nisin were also more sensitive to the enterococcal bacteriocins.

Inhibition of histamine formation in cheese. Two enterococcal strains (INIA 4-07 and EFS 2) and nisin-producing *Lactococcus lactis* ESI 561 were used for cheese-making. *Lactobacillus buchneri* St2A was added at 1.9, 19, and 190 CFU/ml to the cheese milk. As bacteria are entrapped in the curd, theoretical concentrations of 19, 190, and 1,900 CFU/g can be expected after whey drainage. In the control cheese, St2A reached levels of 1.5×10^6, 3×10^7, and 1.1×10^8 CFU/g after

TABLE 3. Inhibition of histamine formation in cheese by bacteriocin-producing starters

Bacteriocin-producing starter	*L. buchneri* St2A		Histamine (mg/kg)[a]	Histidine (mg/kg)[a]
	Inoculum[b] (CFU/ml)	Maximum density[c] (CFU/g)		
E. faecalis INIA 4-07	1.9	$<10^2$	<10	273
	19	$<10^2$	<10	234
	190	2×10^3	<10	250
E. faecalis EFS 2	1.9	$<10^2$	<10	475
	19	$<10^2$	<10	470
	190	$<10^2$	<10	443
L. lactis ESI 561	1.9	$<10^2$	<10	167
	19	$<10^2$	<10	173
	190	$<10^2$	<10	213
None[d]	1.9	1.5×10^6	177	63
	19	3×10^7	194	21
	190	1.1×10^8	214	<10

[a] Histamine and histidine concentrations were determined after 4 months of ripening.
[b] In milk.
[c] In cheese.
[d] The control cheese was made with the regular starter, MAO11.

4 weeks of ripening (Table 3). In the cheeses made with the bacteriocin-producing starters, St2A remained below the detection limit of 100 CFU/g, with the exception of the cheese made with INIA 4-07 at an inoculation level of 190 CFU/ml. In this cheese, fewer than 100 CFU/g were found after 1 week, but 2,000 CFU/g were detected after 1 month of ripening. After 2 and 4 months of ripening, fewer than 100 and 300 CFU/g were found, respectively.

Although the existence of other inhibitory mechanisms cannot be ruled out, it seems very probable that the observed suppression is caused by the activity of the bacteriocins. This hypothesis was further supported by the finding that the growth of *L. buchneri* St2A in milk was not suppressed when it was cocultured with a bacteriocin-negative variant of *E. faecalis* INIA 4-07 and starter MAO11 (data not shown). Besides, extracts from the cheeses made with the bacteriocin-producing starters were active in the agar diffusion assay, whereas extracts from the control cheese did not produce a halo.

It is not likely that the inhibitory effect is related to the pH: after 1 week of ripening, the pH values of all cheeses were in the range of 5.03 to 5.16. Previous experiments with *L. buchneri* St2A in cheddar cheese revealed that this strain develops in cheese with a pH even as low as 4.8 (23).

Histamine was found only in the control cheese; after 4 months of ripening, it contained 177, 194, and 214 mg/kg, depending on the inoculation level (Table 3). In the cheeses made with bacteriocin-producing starters, no histamine was detectable (<10 mg/kg), most probably as a consequence of the strong inhibition of *Lactobacillus buchneri* St2A. The residual histidine concentration was low in the control cheese (63, 21, and <10 mg/kg). Cheese made with *E. faecalis* INIA 4-07 or *Lactococcus lactis* ESI 561 contained 167 to 273 mg of histidine per kg, but the histidine concentration in the cheese made with *E. faecalis* EFS 2 was much higher (443 to 475 mg/kg). Levels of other aromatic amino acids were also higher in this cheese (data not shown), a condition which is probably related to the proteolytic activity of *E. faecalis* EFS 2. The proteolytic activity of this strain was also apparent when it was grown in litmus milk: after prolonged incubation, it had digested the coagulated caseins, whereas *E. faecalis* INIA 4-07 lacked this capacity. An increased rate of proteolysis was also found for Gouda cheese made with a combination of a regular starter and *E. faecalis* H1 (9). However, because strain H1 apparently does not inhibit *Lactobacillus buchneri* St2A, the latter developed prosperously and produced more than 1,500 mg of histamine per kg after 1 year of ripening (9).

With respect to ripening parameters that are critical for histamine formation, Manchego cheese is comparable to Gouda cheese (4), and therefore it seems likely that the kinetics of histamine formation are also similar (15). This would suggest that within 1 year, which is not an uncommon ripening period for Manchego cheese, toxic amounts of histamine will have accumulated in the control cheeses.

These results demonstrate that it is possible to prevent the formation of histamine in cheese by use of bacteriocin-producing starters. It is recognized that the strain used for these experiments, *Lactobacillus buchneri* St2A, was the most susceptible strain of the panel tested in the agar diffusion assay. On the other hand, the inoculum levels employed in this study can be considered very high. As reported by Perry and Sharpe (18), raw milk generally contains fewer than 10 lactobacilli per ml. Stadhouders et al. (20) found a maximum of 29 lactobacilli per ml in milk from nine different Dutch cheese factories, among which homofermentative species were clearly dominant. Nevertheless, more trials to investigate the effectiveness against less susceptible strains are needed. Besides, the technological consequences of the application of enterococci for cheese-making require further research. The organoleptic properties of the cheese may be affected by enzymatic activity of the enterococci and by bacteriocin-mediated inhibition of lactic acid bacteria involved in the ripening process. Safety consequences of the application of enterococci should also be considered. Many enterococci produce the toxic substance tyramine, but this problem can be avoided by selection of strains lacking this capacity (10). Of greater concern is the involvement of some enterococci in clinical infections (1) and as indicators of fecal contamination, which could impede the use of these bacteria in starter cultures.

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