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Inhibitory substances produced by *Lactobacilli* isolated from sourdoughs—a review

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Abstract

Several sourdough lactic acid bacteria (LAB) produce inhibitory substances other than organic acids. Bacteriocins (bavaricin A, and plantaricin ST31), a bacteriocin-like inhibitory substance (BLIS C57), and a new antibiotic (reutericyclin) have been discovered. Maximum antimicrobial production was found in the pH range 4.0–6.0. Temperature optima vary strongly. The substances are resistant to heat and acidity, and inactivated by proteolytic enzymes, except for reutericyclin. Bavaricin A and plantaricin ST31 have been purified to homogeneity. Bavaricin A is classified as a class IIa bacteriocin. Reutericyclin is a new tetramic acid. The mode of action of bavaricin A, BLIS C57, and reutericyclin is bactericidal. Some of these substances are active towards some *Bacilli*, *Staphylococci* and *Listeria* strains. Up to now, only the application potential of purified bavaricin A has been examined. More research should be done to study the production, the activity, and the stability of these inhibitory substances in food systems as these often differ from the broths mostly used in this kind of studies. Furthermore, an extensive screening of the sourdough microflora must be performed, in particular towards *Bacilli* and fungi. This could lead to the discovery of additional inhibitory substances, although it seems that the frequency of isolating bacteriocin-producing sourdough LAB is low. However, potent antimicrobials towards *Bacilli* as well as antifungal substances will have to be found using rational screening strategies and novel purification and analytical techniques.

Keywords: Sourdough; Lactic acid bacteria; Antimicrobial substance; Bacteriocin; Antibiotic

1. Introduction

Lactic acid bacteria (LAB) are an important group of industrial starter cultures, applied in the production of fermented foods like yoghurt, cheese, dry sausage, sauerkraut, and sourdough. They contribute to the enhancement of the organoleptic attributes of these foods, as well as to their preservation and microbial safety (Caplice and Fitzgerald, 1999). Their antimicrobial activity is due to the production of organic acids (in particular, lactic acid and acetic acid), carbon dioxide, ethanol, hydrogen peroxide, and diacetyl (De Vuyst and Vandamme, 1994a). The inhibition, however, can also be caused by bacteriocins that are low-molecular-mass peptides, or proteins, with a bactericidal or bacteriostatic mode of action, in particular against closely related species (De Vuyst and Vandamme, 1994b). The inhibitory spectrum of some bacteriocins also includes food spoilage and/or foodborne pathogenic microorganisms. Those bacteriocins may thus contribute to the competitiveness of the

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producing strain in the fermented food ecosystem (Caplice and Fitzgerald, 1999). In most fermented food processes, the raw material (milk, meat, cereals, etc.) is not sterile, except for milk fermentations where a heat treatment can be and is mostly performed before the fermentation starts. Examples of the in situ bacteriocin production and improved competitiveness of the producing LAB strain are known for the manufacture of cheeses (Sulzer and Busse, 1991), fermented sausages (Vogel et al., 1993), and fermented olives (Leal et al., 1998). The use of bacteriocin producing LAB starter cultures or co-cultures in the fermentation of cereals is currently under investigation.

Bacteriocins from LAB may be subdivided into three classes (Nes et al., 1996). One class of bacteriocins is formed by the lantibiotics. These are small and heat-stable peptides that contain thioether amino acids like lanthionine. The next class of LAB bacteriocins consists of small, heat-stable and hydrophobic peptides with an antilisterial activity. The bacteriocins are either composed out of one (class IIa) or two (class IIb) polypeptide chains. Another class of bacteriocins consists of large, heat-labile and hydrophilic proteins. A fourth class of complex bacteriocins that also need a carbohydrate or lipid moiety for full activity, as previously proposed by Klaenhammer (1993), is omitted here, because up to now no purified bacteriocins have been shown to belong to this class (Nes et al., 1996). However, antimicrobial molecules not purified to homogeneity but displaying characteristics similar to bacteriocins, independent of the class they belong to, may be indicated as bacteriocin-like inhibitory substances (BLIS) (Tagg, 1991).

Even under stringent conditions of production, bread can become contaminated with moulds or bacteria such as *Bacillus subtilis* and clostridia that subsequently grow and spoil the product. To avoid outgrowth of these contaminating microorganisms, addition of organic acids or approximately 15% sourdough to the common dough recipe is performed (Voysey and Hammond, 1993). LAB are the predominant microorganisms in sourdough, and in many cases yeasts are present in significant numbers (Vogel et al., 1999). The fungistatic effect of sourdough addition is attributed to lactic acid, and especially acetic acid, produced by the LAB strains. Sourdough addition is the most promising procedure to preserve bread from spoilage, since it is in agreement with the consumer demand for natural and additive-free food products (Rosenquist and Hansen, 1998). Moreover, it has been reported to improve dough properties, bread texture and flavour, to retard the staling process, and to extend the mould-free shelf life (Corsetti et al., 1998a; Hammes and Gänzle, 1998). Whereas sourdough is an essential ingredient for ensuring baking properties of doughs containing more than 20% rye flour, its addition to wheat dough is optional. The advantages associated with the use of sourdoughs for rye breads have led to their recent use in the production of wheat bread too (Röcken and Voysey, 1995).

Based on the technology applied for their production, sourdoughs have been grouped into three types. Type I sourdoughs are traditional doughs sustained by continuous propagation at ambient temperature (20–30 °C). Mostly, traditional three-stage fermentation processes are used (Hammes and Gänzle, 1998). *Lactobacillus sanfranciscensis* and *Lb. pontis* are the predominant LAB in these doughs (Vogel et al., 1999). Also, *Lb. fructivorans*, *Lb. fermentum* and *Lb. brevis* are found in some doughs. In contrast to type I doughs, leavening of type II sourdoughs is achieved by addition of bakers' yeast to the dough. This is essential since type II doughs employ a less time-consuming, one-stage fermentation process at temperatures exceeding 30 °C. Type II doughs are mostly used in industrial processes. Dominant strains in these doughs are *Lb. panis*, *Lb. pontis*, *Lb. reuteri* (Hammes and Gänzle, 1998; Vogel et al., 1999), *Lb. johnsonii*, *Lb. sanfranciscensis* (Hammes and Gänzle, 1998), *Lb. fermentum*, *Lb. delbrueckii*, *Lb. acidophilus*, *Lactococcus lactis*, *Lb. brevis* and *Lb. amylovorus* (Vogel et al., 1999). Type III doughs are dried preparations of doughs (Hammes and Gänzle, 1998). They are made by (traditional) sourdough fermentation with subsequent water evaporation by freeze-drying, roller spray drying or drying in a fluidised bed reactor (Corsetti et al., 1998b).

It has been shown that the antifungal activity of sourdough LAB varies. It is mainly associated with obligatory heterofermentative *Lactobacillus* spp. Within this group, *Lb. sanfranciscensis* CB1 displays the largest spectrum of antifungal activity due to the production of a mixture of organic acids. Caproic acid plays a key role in inhibiting mould growth (Corsetti

et al., 1998b). Recently, novel antifungal compounds such as phenyllactic and 4-hydroxy-phenyllactic acids are isolated from *Lb. plantarum* 21B, previously derived from sourdough (Lavermicocca et al., 2000).

The screening of sourdough LAB for antimicrobial activity has shown that, besides acidification of the dough, some sourdough LAB produce inhibitory substances other than organic acids. Bacteriocins, a bacteriocin-like inhibitory substance (BLIS), and a new antibiotic have been discovered. Larsen et al. (1993) were the first to screen LAB, isolated from different sourdoughs, for antimicrobial activity. This screening of 335 LAB strains resulted in 18 isolates of which the antimicrobial activity is due to a proteinaceous compound. Those 18 isolates belong to three different *Lactobacillus* species: *Lb. sakei* (formerly *Lb. bavaricus*), *Lb. curvatus*, and *Lb. plantarum*. *Lb. sakei* MI401 that produces the bacteriocin **bavaricin A** was chosen for further study. The strain has been isolated from freshly prepared spontaneous sourdoughs. Corsetti et al. (1996) screened 232 *Lactobacilli* isolates from wheat sourdoughs for antimicrobial activity. Fifty-two strains belonging to one of the species *Lb. sanfranciscensis*, *Lb. brevis*, *Lb. fructivorans*, *Lb. fermentum*, *Lb. plantarum*, *Lb. farciminis*, *Lb. acidophilus*, *Lb. alimentarius*, and *Lb. hilgardii* have been found to exhibit antimicrobial activity. Because *Lb. sanfranciscensis* is the key sourdough starter for several baked products and because strain C57 exhibited a large spectrum of activity against sourdough-related lactobacilli, the antimicrobial compound, designated as **BLIS C57**, produced by *Lb. sanfranciscensis* C57, was characterised. Gänzle (1998) screened 65 strains of *Lactobacilli* previously isolated from wheat and rye sourdoughs. Three of these 65 strains exhibit antimicrobial activity, namely two strains of *Lb. reuteri* (*Lb. reuteri* LTH2584 and *Lb. reuteri* LTH3566), and *Lb. sanfranciscensis* LTH2594. Since the antimicrobial compound produced by *Lb. reuteri* LTH2584 displays the broadest inhibitory spectrum, this strain was selected for further characterisation. It has been isolated from a type II rye-based sourdough. The inhibitory compound was first referred to as sourdough inhibitory compound 64 (SIC64) (Gänzle, 1998). Recently, it has been fully characterised and it is found to be a new antibiotic, called **reutericyclin** (Gänzle et al., 2000; Holtzel et al., 2000). It is worthwhile to mention that certain strains of *Lb. reuteri* can also produce reuterin from glycerol under anaerobic conditions. Its antimicrobial activity against a broad range of microorganisms is attributed to monomers, hydrated monomers, and cyclic dimers of β-hydroxypropionic aldehyde (El-Ziney et al., 2000). The screening of nearly 100 strains of *Lb. plantarum*, isolated from sourdough by Todorov et al. (1999), led to the characterisation of the bacteriocin **plantaricin ST31** produced by *Lb. plantarum* ST31.

Interestingly, when the antimicrobial substances mentioned above are resistant to baking conditions and active at the physical characteristics of bread, they can help to control the growth of spoilage organisms by microbial interactions (Rosenkvist and Hansen, 1995). Also, bacteriocin production may be interesting to explain competition among the bacterial sourdough flora. It could be a criterion for selection of more competitive starters for implantation and stability of sourdough (Todorov et al., 1999).

In this paper, the production, molecular properties, antimicrobial activity, biological advantages, and applications of the inhibitory compounds bavaricin A, BLIS C57, plantaricin ST31, and reutericyclin, produced by LAB isolated from sourdough, are reviewed.

2. Production

The production of bavaricin A (Larsen et al., 1993) and plantaricin ST31 (Todorov et al., 1999) was studied in de Man, Rogosa, Sharpe (MRS) broth (De Man et al., 1960), a common LAB laboratory medium with glucose as sole carbohydrate source. Reutericyclin has been produced in modified MRS (mMRS) broth containing 10 g l^{-1} of maltose and 5 g l^{-1} each of glucose and fructose as carbohydrate sources (Gänzle, 1998). BLIS C57 (Corsetti et al., 1996) has been produced in sourdough bacteria (SDB) broth containing 20 g l^{-1} of maltose as sole carbohydrate source (Kline and Sugihara, 1971).

Temperature strongly influences the antimicrobial production, as shown for bavaricin A (Larsen et al., 1993), reutericyclin (Gänzle, 1998), and plantaricin ST31 (Todorov et al., 1999). Bavaricin A production was studied at 4, 10, and 30 °C (Larsen et al., 1993). At higher temperatures, cells grow faster but bavaricin

A activities are lower. At 30 °C, the maximum bacteriocin activity (10,000 AU ml^{-1}) is detected in the late logarithmic growth phase. At 10 and 4 °C, the maximum bacteriocin activity is 10,000–15,000 AU ml^{-1} after 3 days of growth and 25,000 AU ml^{-1} after 8 days of growth, respectively (Larsen et al., 1993). Both the growth rate of *Lb. reuteri* LTH2584 and the production of reutericyclin increase when the temperature increases from 25 to 42 °C. Appreciable production occurs only at temperatures above 30 °C (Gänzle, 1998). Maximum production of plantaricin ST31 has been observed at a controlled pH of 6.0 and at 30 °C. Under these conditions, detectable amounts of plantaricin ST31 are produced during the exponential growth phase, while maximum activities (3200 AU ml^{-1}) are observed in the stationary phase (Todorov et al., 1999). BLIS C57 production was only studied at 28 °C. From 6 h of incubation onwards, BLIS C57 activity has been detected. A drastic increase of activity was observed in the stationary growth phase, i.e. from 8 to 36 h, while a loss of activity was observed after 72 h of fermentation (Corsetti et al., 1996). Thus, bavaricin A production better fits the temperature conditions used for type I sourdough fermentations. Reutericyclin and plantaricin ST31 production on the other hand is optimal at 30 °C or above, hence matching the conditions used in type II sourdough fermentations.

Acidity also influences the antimicrobial production, as shown for BLIS C57 (Corsetti et al., 1996), and reutericyclin (Gänzle, 1998). The optimum pH for the production of BLIS C57 and reutericyclin is between pH 4.0 and 5.0, and differs from the optimum growth conditions of the producing bacteria (Corsetti et al., 1996; Gänzle, 1998). The pH required for optimum production of BLIS C57 and reutericyclin thus match the conditions encountered in sourdough fermentations.

When considering the in situ food applications of bacteriocin-producing strains, the interference of mineral compounds, such as salt and nitrite, present in the cell environment, with bacteriocin production is important (Leroy and De Vuyst, 2000). Sourdoughs usually are prepared without the addition of salt; nevertheless, several processes that make use of the incorporation of 2–5% NaCl have been developed (Gänzle et al., 1998). Addition of 1% NaCl does not influence the bavaricin A production at 4 and 10 °C. When the NaCl concentration is increased to 3%, the production of active bavaricin A is lost at 4 °C, and lowered (10,000–15,000 AU ml^{-1}) at 10 °C. At 5% NaCl, bavaricin A activity is no longer detected at 10 °C, although the bacteria are still growing. Nitrite (up to 0.01%) does not affect the growth of *Lb. sakei* MI401 or the production of bavaricin A at 4 and 10 °C (Larsen et al., 1993).

The source of fatty acids present in the growth medium affects the production of reutericyclin by *Lb. reuteri* LTH2584. No growth has been observed when fatty acids were not present in the growth medium. Wheat germ oil, the natural source of unsaturated fatty acids in wheat sourdoughs, favours reutericyclin production compared to other sources of fatty acids like Tween 80, oleic acid, and linoleic acid (Gänzle et al., 2000). This is an advantage for the use of the reutericyclin-producing strain as a co culture in sourdough fermentations. When analysing the neutralised cell-free culture supernatant, however, a high inhibitory activity is only observed in media containing Tween 80. This is possibly to be explained by the fact that Tween 80 affects the solubility and activity of hydrophobic molecules like those antimicrobials, due to its emulsifying properties. Consequently, reutericyclin present in foods may be removed from the aqueous phase in foods with high fat content, due to adsorption (Gänzle et al., 2000).

3. Molecular properties

3.1. Isolation and purification

Bavaricin A produced by *Lb. sakei* MI401, plantaricin ST31 produced by *Lb. plantarum* ST31, and reutericyclin produced by *Lb. reuteri* LTH2584 have been purified to homogeneity (Corsetti et al., 1996; Gänzle, 1998; Todorov et al., 1999).

3.2. Physico-chemistry

The antimicrobials mentioned above, all isolated from sourdough, are generally resistant to heat and acidity. Heat stability has been demonstrated for bavaricin A and plantaricin ST31: no activity is lost after boiling (100 °C) of bavaricin A samples for 60

min (Larsen et al., 1993), and of plantaricin ST31 samples for 40 min (Todorov et al., 1999). By contrast, the activity of bavaricin MN, another bacteriocin produced by a *Lb. bavaricus* strain isolated from meat, is completely lost after heating at 60 °C for 15 min and at 100 °C for 10 min (Lewus and Montville, 1992). Reutericyclin is stable to exposure to 60 °C for 30 min. Heat treatment at 90 and 121 °C for 30 min reduces the activity by 50% and >90%, respectively (Gänzle, 1998).

Stability in a wide pH range, including the pH values prevailing in sourdoughs, has been reported for bavaricin A (pH 1.3–9.7) (Larsen et al., 1993) and plantaricin ST31 (pH 3.0–8.0) (Todorov et al., 1999). However, at more alkaline pH values, a significant reduction of activity is observed. At pH 10.4, bavaricin A activity is lowered, while at pH 12.5, no activity can be found (Larsen et al., 1993). At pH 9.0 and above, a significant loss of activity of plantaricin ST31 is found (Todorov et al., 1999). Thus, at the pH conditions prevailing in most food applications, the bacteriocins remain stable.

The proteinaceous nature of bacteriocins makes them very sensitive to the action of certain proteases. Trypsin and pronase E completely inhibit the activity of bavaricin A (Larsen et al., 1993) and plantaricin ST31 (Todorov et al., 1999). Bavaricin A is also inhibited by proteinase K, pepsin and chymotrypsin A_4 (Larsen et al., 1993), while plantaricin ST31 is inhibited by protease IV and protease VIII (Todorov et al., 1999). BLIS C57 is sensitive to proteinase K and pepsin but insensitive to a proteinase from *Streptomyces griseus* and to ficine (Corsetti et al., 1996). The inhibitory activity of reutericyclin is not destroyed upon incubation with proteinase K or trypsin, excluding the possibility that bacteriocins are the antimicrobially active components in the neutralised cell-free culture supernatant of *Lb. reuteri* LTH2584 (Gänzle et al., 2000). Carbohydrate and lipid moieties that are required for the inhibitory activity of any of these antimicrobial substances have not been found.

Addition of divalent ions (Ca^{2+}, Mg^{2+}, Mn^{2+}), emulsifiers (lecithin, Tween 20, Tween 80), and proteins (bovine serum albumin, casein) to the culture supernatant of *Lb. reuteri* LTH2584 in concentrations prevailing in food significantly reduces the antimicrobial activity of reutericyclin. No effect is observed upon the addition of monosaccharides, i.e. galactose, rhamnose, or polysaccharides, i.e. starch, inulin (Gänzle, 1998). Addition of lactic acid, resulting in a pH decrease from 5.5 to 4.5, or addition of 2% NaCl enhances the inhibitory activity (Gänzle et al., 2000). Bavaricin A is not inactivated by addition of 1–10% NaCl to the culture supernatant, although higher amounts (20%) result in a considerable (30%) loss of activity (Larsen et al., 1993). The surface-active agents SDS, Tween 20, and Tween 80 at a final concentration of 1% in the culture supernatant, do not affect the activity of plantaricin ST31. Plantaricin ST31 is sensitive to urea, indicating that this bacteriocin may exist in a multimeric form (Todorov et al., 1999). For food applications, the influence of all these substances on the activity of the antimicrobial needs to be taken into account.

3.3. Structure

The complete amino acid sequence of the bacteriocins bavaricin A (Larsen et al., 1993) and plantaricin ST31 (Todorov et al., 1999) has been determined (Table 1). Both antibacterial molecules are low-molecular-mass peptides. Bavaricin A consists of 41 amino acid residues and has a calculated molecular mass above 4300 Da; SDS-PAGE reveals a molecular mass of 3500–4000 Da (Larsen et al., 1993). Plantaricin ST31 consists of 20 amino acid residues and has a calculated molecular mass above 2700 Da; a molecular mass of 2755.6 Da has been determined by electrospray mass spectrometry (Todorov et al., 1999). Reutericyclin does not contain amino acids, and has a molecular mass of 349 Da as estimated by electrospray mass spectrometry. The results of the molecular mass estimation by SDS-PAGE and gel filtration are 3100 and 2150 Da, respectively, indicating that reutericyclin forms stable multimers in aqueous solutions, even in the presence of denaturing agents or organic solvents (Gänzle, 1998).

The N-terminal region of bavaricin A contains the Lys-Tyr-Tyr-Gly-Asn-Gly-Val consensus motif common to several class IIa bacteriocins, including bavaricin MN (Kaiser and Montville, 1996), leucocin A-UAL 187 (Hastings et al., 1991), pediocin PA-1 (Marugg et al., 1992), sakacin A (Holck et al., 1992), sakacin P (Tichaczek et al., 1994), mesenter-

Table 1
Amino acids (AA) and molecular mass of bacteriocins of some lactic acid bacteria isolated from sourdough and comparison with other bacteriocins

Bacteriocin	Producer	Isolated from	AA residues	Molecular mass (Da)	Reference
Bavaricin A	*Lb. sakei* MI401	sourdough	41	>4300[a] 3500–4000[b]	Larsen et al. (1993)
Sequence KYYGNGVHx[c] GKHSx[c]TVDWG TAIGNIGNNA AANx[c]ATGx[c]NA GG[d]					
Bavaricin MN	*Lb. sakei* MN	retail beef	42	4769[a]	Kaiser and Montville (1996)
Sequence TKYYGNGVYx[c] NSKKx[c]WVDWG QAAGGIGQTV Vx[c]GWLGGAIP GK[d]					
Plantaricin ST31	*Lb. plantarum* ST31	sourdough	20	>2700[a] 2755.6[e]	Todorov et al. (1999)
Sequence KRKKHRX[c]QVYNNGMPTGMYR					

[a] Calculated from known amino acid residues.
[b] Estimated by gel electrophoresis.
[c] x, amino acid residue not determined.
[d] Significant regions of homology are underlined.
[e] Determined by electron spray mass spectrometry.

ocin Y105 (Héchard et al., 1992), and carnobacteriocin B2 (Quadri et al., 1994). Other regions with some homology include the following amino acid residues: valine at position 17, tryptophan at position 19, glycine at position 20, and alanine at position 22. The amino acids at positions 10 and 15 in both bavaricin A and bavaricin MN may be cysteine, given the high degree of homology to cysteines at the same position in other class IIa bacteriocins (Kaiser and Montville, 1996). These cysteine residues may contribute to the range of the antimicrobial spectrum, since it has been postulated that the higher their number is the more extended the corresponding antimicrobial spectrum is (Ennahar et al., 2000). On the basis of its strong amino acid sequence similarity, in particular its distinctive N-terminal part, with other class IIa bacteriocins, and its antilisterial activity (see below), bavaricin A has been classified as a class IIa bacteriocin (Ennahar et al., 1999). Plantaricin ST31 shares no homology with other bacteriocins characterised up to now (Todorov et al., 1999).

Bavaricin A has a significant region of hydrophobicity between amino acids 17 and 40. This was also observed for bavaricin MN between amino acids 22 and 39 (Kaiser and Montville, 1996). Also plantaricin ST31 has a hydrophobic region situated between amino acids 8 and 18. Hydrophobicity is a common feature of class II bacteriocins, in particular those isolated from *Lb. plantarum* (Todorov et al., 1999). Also reutericyclin is a highly hydrophobic molecule that is negatively charged (Gänzle et al., 2000). Recently, structural characterisation revealed that reutericyclin is a novel tetramic acid derivative, structurally related to tenuazonic acid (Fig. 1). It has a 5*S*-conformation. This characterisation allowed the preparation of synthetic reutericyclin as a racemic mixture of (5*R*,5*S*)-reutericyclin (Holtzel et al., 2000). This racemic mixture inhibits the growth of indicator strains at a higher concentration than the (5*S*)-reutericyclin. This points out that the stereochemistry of reutericyclin is important for its antimicrobial activity (Gänzle et al., 2000).

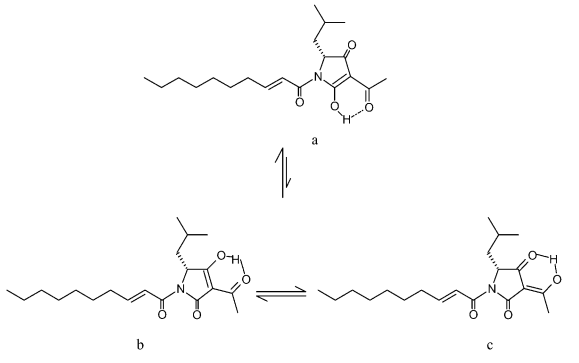

Fig. 1. Chemical structure of reutericyclin showing keto-enol-tautomery. About 60% of form (a) and 20% of forms (b) and (c), respectively, are present in [D_3] acetonitril at 298 K (after Gänzle et al., 2000).

4. Antimicrobial activity

4.1. Mechanism of action

Bavaricin A is bactericidal towards *Lb. sakei* LMG 9468 as indicator organism (Larsen et al., 1993). This could be expected since class IIa bacteriocins are antibacterial peptides that act primarily by permeabilizing the membranes of susceptible bacteria, causing leakage of intracellular compounds. Membrane permeabilization follows bacteriocin–membrane interaction, which is thought to involve the formation of water-filled membrane channels through a multistep process of binding, insertion and aggregation of bacteriocin monomers in the membrane leading to formation of poration complexes (Ennahar et al., 1999). Also the action of BLIS C57 towards *Lb. farciminis* CC1 as target strain is bactericidal (Corsetti et al., 1996). Reutericyclin present in the neutralised culture supernatant (NCS) of *Lb. reuteri* LTH2584 exhibits a bactericidal mode of action against *Lb. sanfranciscensis* ATCC 27651, *Staphylococcus aureus* LTH1493 and *B. subtilis* FAD109 in mMRS medium. Germination of spores of *B. subtilis* is also inhibited by reutericyclin, but spores remain unaffected under conditions that do not permit germination. Cells of *Lb. sanfranciscensis* ATCC 27651, incubated with NCS in mMRS containing 4% NaCl, are lysed in a dose-dependent manner. Lysis of *Lb. sanfranciscensis* is not observed when incubated with NCS in phosphate buffer, indicating that NCS triggers the cell lysis, but is not the lytic principle itself (Gänzle et al., 2000).

Finally, it has to be mentioned that the antibacterial mode of action of bacteriocins seems to be dependent on several factors such as the concentration and purity of the bacteriocin preparation, the type of buffer or broth used, the sensitivity of the indicator strain tested, and the density of the cell suspension applied (Leroy and De Vuyst, 2000).

4.2. Influencing factors

The bioavailability and efficiency of antibacterial action, and hence the stability of bacteriocin activity is affected by environmental conditions such as pH, temperature, and the presence of chemicals. Temperature will influence the fluidity of the membrane of the indicator organism and can thus result in a decreased efficiency of pore formation. Many bacteriocins display greater antibacterial activity at lower pH values (pH 5 and below) than at physiological pH, because a higher amount of bacteriocin molecules are available at lower pH values. At lower pH values, the solubility is often increased, less aggregation of hydrophobic peptides occurs, and less bounding of bacteriocins to the cell surface takes place. Also, hydrophilic bacteriocins may have an enhanced capacity to pass through hydrophilic regions of the cell surface of the sensitive target bacteria (Jack et al., 1995). For instance, pore formation by bavaricin MN is optimal at pH 6.0 (Kaiser and Montville, 1996).

The effect of pH (4.5, 5.0, and 5.5) and NaCl concentrations (0%, 1%, and 2%) on the inhibitory activity of reutericyclin has been evaluated using *Lb. sanfranciscensis* ATCC 27651 as indicator organism. Synergistic effects are observed both at low pH and high NaCl concentrations (Gänzle, 1998). These data indicate that the inhibitory activity of *Lb. reuteri* LTH2584 should increase in fermented foods, because the latter are often characterised by a low pH and high salt content (Gänzle et al., 2000).

4.3. Inhibitory spectrum

In general, LAB bacteriocins tend to be active against a wide range of mostly closely related Gram-positive bacteria (Jack et al., 1995). Gram-negative bacteria are generally insensitive to bacteriocins from LAB strains because of their outer membrane providing them with a permeability barrier. The sensitivity of Gram-negative bacteria can be increased by sublethal injury of the cells, using for instance high hydrostatic pressure and pulsed electric field as non-thermal methods of preservation (Caplice and Fitzgerald, 1999). In addition, food grade chelating agents such as ethylene-diamine-tetra-acetic acid (EDTA) and citrate can be used to bind magnesium ions in the lipopolysaccharide outer layer of Gram-negative bacteria to render them susceptible to bacteriocins (Holzapfel et al., 1995). Yeasts and fungi are not inhibited by LAB bacteriocins (De Vuyst and Vandamme, 1994b).

The inhibitory spectrum of bavaricin A, reutericyclin, BLIS C57, and plantaricin ST31, produced

by sourdough LAB, is shown in Table 2. Whereas Gram-negative bacteria are not inhibited, a variety of Gram-positive bacteria are sensitive. The producer strains are immune towards their own bacteriocin. Virtually all Gram-positive indicator strains that have been tested are sensitive to reutericyclin; the sensitivity is in the range of 0.05–1 mg l^{-1} of reutericyclin. For comparison, the reutericyclin producer strain *Lb. reuteri* LTH2584 tolerates concentrations of up to 6.4 mg l^{-1} of reutericyclin (Gänzle et al., 2000).

Most LAB strains are sensitive to all four antimicrobial compounds. Among the opportunistic foodborne pathogens and food spoilage bacteria, *Bacilli* are inhibited by BLIS C57, reutericyclin, and plantaricin ST31, but not by bavaricin A. *Staphylococci* are only inhibited by reutericyclin and plantaricin ST31, while *Listeria* strains are inhibited by bavaricin A, BLIS C57, and reutericyclin (Larsen et al., 1993; Corsetti et al., 1996; Gänzle, 1998; Todorov et al., 1999; Gänzle et al., 2000).

The resistance of *Listeria monocytogenes* strains towards bavaricin A has been studied too (Larsen and Nørrung, 1993; Rasch and Knøchel, 1998). Only 3 of the 245 strains examined are resistant to bavaricin A (Larsen and Nørrung, 1993). Rasch and Knøchel (1998) observed that bavaricin A sensitivity correlates with pediocin PA-1 sensitivity of the strains. Pediocin is a class IIa bacteriocin produced by *Pediococcus* spp. Cross-resistance between nisin, a lantibiotic bacteriocin produced by *Lc. lactis* subsp. *lactis* that is commercially available, and pediocin/bavaricin has not been found. Also, reutericyclin showed no cross-resistance with clinical isolates of *Enterococcus faecium* resistant to β-lactam

Table 2
Antimicrobial spectrum of the bacteriocins bavaricin A and plantaricin ST31, the bacteriocin-like inhibitory substance BLIS C57, and the antibiotic reutericyclin from lactic acid bacteria isolated from sourdough[a]

Indicator species	Number of strains inhibited/number of strains tested			
	Bavaricin A	BLIS C57	Reutericyclin	Plantaricin ST31
Gram-positive bacteria				
Lactobacillus	11/25	18/24	23/23	27/39
Carnobacterium	0/1	nd[b]	nd[b]	1/2
Lactococcus	5/15	1/1	nd[b]	0/1
Enterococcus	2/2	0/3	4/4	1/1
Streptococcus	0/2	1/1	nd[b]	1/1
Staphylococcus	0/5	0/1	7/7	1/1
Leuconostoc	4/7	nd[b]	nd[b]	5/10
Bacillus cereus	0/1	0/1	1/1	0/1
Bacillus subtilis	0/1	2/2	9/9	2/2
Other *Bacilli*	0/5	nd[b]	nd[b]	0/1
Listeria	9/10	1/1	2/2	0/3
Pediococcus	2/5	nd[b]	nd[b]	2/3
Clostridium	nd[b]	nd[b]	nd[b]	0/3
Weissella confusa	nd[b]	nd[b]	1/1	nd[b]
Gram-negative bacteria				
Salmonella	nd[b]	0/1	nd[b]	0/1
Citrobacter freundii	nd[b]	nd[b]	nd[b]	0/1
Yersinia enterocolitica	nd[b]	nd[b]	nd[b]	0/1
Klebsiella pneumoniae	nd[b]	nd[b]	nd[b]	0/1
Proteus vulgaris	nd[b]	0/1	nd[b]	0/1
Serratia	nd[b]	0/1	nd[b]	0/1
Escherichia coli	nd[b]	nd[b]	0/7	0/2
Brochotrix thermosphacta	0/1	nd[b]	nd[b]	nd[b]
Pseudomonas fluorescens	nd[b]	0/1	nd[b]	nd[b]

[a] After Larsen et al. (1993), Corsetti et al. (1996), Gänzle (1998), Todorov et al. (1999).
[b] nd, Not determined.

antibiotics, erythromycin, and vancomycin, or with methicillin-resistant *Staphylococci* (Gänzle et al., 2000).

5. Biological advantage

5.1. Additive advantage to foods

The use of nisin in several food products is well known and generally accepted (Delves-Broughton et al., 1996). The practical use of other LAB bacteriocins or bacteriocin-producing LAB strains in various food matrices such as milk products (Fenelon et al., 1999; Laukova et al., 1999a; McAuliffe et al., 1999), meat products (Hugas et al., 1998; Laukova et al., 1999b; Schobitz et al., 1999; Siragusa et al., 1999; Aymerich et al., 2000; Callewaert et al., 2000), and fish (Duffes et al., 1999, Nilsson et al., 1999; Duffes et al., 2000; Messi et al., 2000) is currently a point of interest. Most research is done on controlling the growth of the food-borne pathogens *L. monocytogenes* and *S. aureus*, although the presence of LAB bacteriocins in food products can also contribute to the inhibition of the outgrowth of spoilage bacteria and to the preservation of the overall organoleptic properties of the food.

The role of bacteriocins in sourdough under practical conditions remains to be determined. The use of LAB bacteriocins or bacteriocin-producing sourdough LAB strains that are active against *Bacillus* species can be advantageous, since these species can cause spoilage of bread by rope formation, and may constitute a health risk. Rope formation occurs principally in wheat breads that have not been acidified, or in breads with high concentrations of sugar, fat, or fruits (Beuchat, 1997). The acidification and production of acetic acid by heterofermentative lactobacilli in sourdough delay growth of *B. subtilis* in bread, provided that the raw material and technological pre-fermentation conditions are optimal (Gänzle, 1998). Ropiness is mainly caused by *B. subtilis*, but *B. licheniformis*, *B. megaterium* and *B. cereus* have also been associated with ropy bread. Initially the spoilage is noticed as an unpleasant odour, followed by a discoloured, sticky and soft breadcrumb, caused by the breakdown of starch and proteins by microbial amylases and proteases, and by the production of extracellular, slimy polysaccharides (Rosenkvist and Hansen, 1995).

5.2. Physiological advantage to host

Since some LAB bacteriocins isolated form sourdough are found inhibitory towards food-borne pathogens such as *L. monocytogenes*, *B. subtilis* and *S. aureus*, their application either as food additive or by using bacteriocin-producing, sourdough LAB strains as starter or protective culture, may contribute to the production of safer and healthier products. For example, food-borne illness related to consumption of ropy bread is considered unlikely to happen due to the slimy appearance of the crumb. However, loaves with high counts of *B. subtilis* and *B. licheniformis*, showing no rope symptoms, may cause (diarrhoea and/for vomiting (Rosenkvist and Hansen, 1995).

Additionally, bacteriocins may lead to a reduction of the use of some traditional, chemical preservatives that are being questioned because of their possible harmful effects towards the consumer, e.g. the use of calcium propionate in bread that is banned in Germany (Voysey and Hammond, 1993).

6. Applications

The antimicrobials described above, isolated from sourdough, may be produced in or used commercially as additives to sourdough or to other foods requiring preservation. Nisin was the first bacteriocin with a ‘Generally Regarded As Safe (GRAS)’ status for its use in specific foods (Delves-Broughton et al., 1996), and it is still the only bacteriocin applied in most countries. Other LAB bacteriocins still need to be approved as food additives for future use as food biopreservatives. Therefore, bacteriocin-producing LAB strains will afford the best opportunities for the application of bacteriocins in the near future (Holzapfel et al., 1995; Caplice and Fitzgerald, 1999; Ennahar et al., 1999).

6.1. As food additive

The role of purified LAB bacteriocins, including those isolated from sourdough, in sourdough fermentations under practical conditions remains to be deter-

mined. Application of purified bavaricin A has yet been tested in two other cases.

The potential of bavaricin A addition to preserve brined shrimps (containing ca. 3% NaCl) has been analysed by Einarsson and Lauzon (1995). Typically, the product contains NaCl and an acidulant, as well as benzoic and sorbic acids in concentrations from 0.05% to 0.1%, with pH values ranging from 5.0 to 6.0. Since consumers have raised concerns about traditional preservatives such as benzoic and sorbic acids, replacements are sought. In the experiment, the shrimps were preserved during the whole storage period (59 days) using a benzoate–sorbate solution (0.1% (w/w) each). Crude bavaricin A resulted in a shelf life of 16 days as opposed to 10 days without preservatives. In comparison, carnocin UI49, a lantibiotic bacteriocin produced by *Carnobacterium piscicola* UI49, did not extend the shelf life, while nisin extended the shelf life to 31 days.

Also, the prevention of histamine formation in cheese by addition of bacteriocin-producing LAB starters has been tested (Joosten and Nunez, 1996). Several cases of cheese-related outbreaks of amine poisoning have been reported, and histamine is implicated in most of them. Unfortunately, bavaricin A does not show inhibitory activity towards any of the 13 amine-forming lactobacilli tested. On the other hand, all strains are susceptible to nisin and to most bacteriocins of enterococcal origin (Joosten and Nunez, 1996).

6.2. As starter culture, co-culture or protective culture

For a starter culture, metabolic activity (e.g., acid production) has technological importance, while antimicrobial action may constitute a secondary effect; for a protective culture, the functional objectives are the inverse. Application of a protective culture should in the first instance be considered as an additional safety factor, with the potential of improving the microbiological safety of the food. Their implementation should support good manufacturing practices, thereby reducing risks of growth and survival of food-borne pathogens and food spoilage organisms (Holzapfel et al., 1995).

Up to date, neither the in vitro (in medium) nor the in situ (in food) inhibitory activities of any of the LAB bacteriocins isolated from sourdough nor of the antibiotic reutericyclin have been tested. However, screening of the *lactobacilli*, isolated from the same batch of sourdough rye extract as the one where the reutericyclin producer strain was derived from, showed that these strains are rather resistant to reutericyclin with a minimum inhibitory concentration of 0.3–0.7 mg l^{-1} as compared to other sourdough isolates with minimum inhibitory concentration of 0.1–0.2 mg l^{-1} (Gänzle et al., 2000). This demonstrates that reutericyclin may be produced in sourdough to exert selective pressure on competitors of the producer strain (Gänzle et al., 2000).

6.3. Possible applications

Appropriate bacteriocin-producing LAB starter cultures can be used in sourdough fermentations to control the growth of food-borne pathogens, such as *B. subtilis* that is the dominant *Bacillus* species in bread (Rosenkvist and Hansen, 1995), or spoilage organisms, by microbial interactions. Starter cultures that are isolated from sourdough stand possibly the best chance to be effectively used in sourdough fermentation processes, because it is likely that they are highly adapted to this particular food matrix. *Bacillus* spores can survive the baking process where the temperature in the centre of the crumb remains at a maximum of 97–101 °C for a few minutes. Hence, the bacteriocins, especially those that are active towards *B. subtilis*, must be resistant to these temperatures during baking. The antimicrobials BLIS C57, reutericyclin, and plantaricin ST31 are active towards *B. subtilis* in culture media. The bacteriocin plantaricin ST31 is the most heat resistant compound. Reutericyclin activity could be lost at baking temperatures. The heat resistance of BLIS C57 has not been determined yet. Hence, if the conditions prevailing in sourdough would support growth of *Lb. plantarum* ST31, this strain might be used to control rope formation in bread.

Also, LAB bacteriocin production may be interesting for selection of more competitive starters for implantation and stability of sourdough (Todorov et al., 1999). Therefore, a screening of LAB strains from different sourdoughs for bacteriocin production is most promising to isolate strains that are adapted to this food ecosystem and that may produce bacteriocins in situ. Recently, after an extensive screening, we

found four interesting bacteriocin-producing sourdough LAB strains, namely one *Lb. sanfranciscensis* strain, one *Lb. brevis* strain and two *Lb. pontis* strains that produce antibacterial, proteinaceous substances (Messens, W., Verluyten, J., Schrijvers, V., Paramithiotis, S., Vancanneyt, M., Tsakalidou, E. and De Vuyst, L., unpublished results).

According to Eckner (1992), a possible use of bacteriocins could be foreseen in pasta. Pasta dough is susceptible to contamination and growth of *S. aureus*. If the production conditions of pasta dough allow growth and bacteriocin production by *Lb. reuteri* LTH2584, *Lb. plantarum* ST31 or novel sourdough LAB strains, these strains could be used as starter or protective culture to control growth of *S. aureus*.

6.4. Limitations

Some food applications may not be suitable for the use of LAB bacteriocins, either because of chemical or enzymatic activity present in the food, food processing treatments, or physical food characteristics like high viscosity or the presence of particulates. A limited effectiveness may also be seen because of low bioavailability and stability of the molecules and due to narrow antibacterial specificity and activity ranges. When protective cultures are relied upon in situ production of the bacteriocins instead of a direct addition of bacteriocin preparations, there is even greater uncertainty introduced, because of growth conditions, the microenvironment, and the metabolic state of the bacteriocin-producing organism (Eckner, 1992). However, as pointed out before, it is most promising to isolate bacteriocin-producing LAB strains that are adapted to this food ecosystem and that may produce bacteriocins in situ.

Spontaneous resistance development of the target organisms may also reduce the application potential of LAB bacteriocins. Although only a few strains of *L. monocytogenes* were resistant to bavaricin A, possibilities exist that such resistant strains might be favoured through selection, and might be responsible for cross-resistance (Larsen and Nørrung, 1993). To avoid the growth of resistant strains, the use of a combination of LAB bacteriocins in biopreservation has been suggested (Rasch and Knøchel, 1998). Moreover, the use of different hurdles such as minimum processing, bacteriocins or other inhibitory compounds such as organic acids and novel molecules like reutericyclin, and new preservation techniques, for instance high hydrostatic pressure and pulsed electric field, is most promising (Ennahar et al., 1999).

It is, however, questionable if reutericyclin will be allowed for food applications, and, more interestingly, will be accepted by both the food processor and consumer. Currently, antibiotics do not possess a good reputation, in particular in food and feed uses. On the other hand, bacteriocins are antibacterial peptides that are naturally present in several foods that in turn are consumed for many years. Because of their proteinaceous structure, bacteriocins can be unstable in a food environment, and when eaten the human digestive system will easily degrade them. Therefore, appropriate LAB bacteriocins, added to or produced in the food, may be of great importance for food biopreservation in the near future.

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