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Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility 2—Behaviour of pathogenic and spoilage bacteria in dual species biofilms including a bacteriocin-like-producing lactic acid bacteria

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Abstract

The survival of *Listeria innocua* ATCC 33090, *Staphylococcus aureus* E1S-5 and/or *Hafnia alvei* E1E-25 in dual species biofilms with bacteriocin-like producing lactic acid bacteria (5 *Vagococcus carniphilus*, 3 *Enterococcus faecium*, 1 *Lactobacillus sakei* and 1 *Enterococcus* sp.) was investigated. The aim was to select strains able to repress the growth of undesirable bacteria in biofilms, i.e., the real mode of bacterial attachment.

Two *E. faecium* and 3 *V. carniphilus* species were highly antagonistic to *L. innocua*, *S. aureus* and *H. alvei* repressing their growth by reduction levels able to reach 2, 2.7 and 2.4 $\log_{10}$ cfu/ml compared to the positive control made of sole the target microorganism. Furthermore, planktonic cells were more sensitive to the bacteriocin-like substances than sessile ones.

First results suggest the possibility of selecting bio-preservatives among the endogenous house flora of the studied small-scale facility, that could be implemented on the processing surfaces to repress the growth of undesirable microorganisms.
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Keywords: Antibacterial activity; Lactic acid bacteria; Traditional dry sausages; Small-scale facility; Bacteriocins; Biofilms; Co-culture; Barrier flora

1. Introduction

Biofilms have been of considerable interest in the context of food hygiene. Of special significance is the ability of microorganisms to attach and grow on foods and food-contact surfaces under favourable conditions. Biofilm formation is a dynamic process and different mechanisms are involved in their attachment and growth (Marshall, 1992). Extracellular polymeric substances play an important role in the attachment and colonization of microorganisms to food-contact surfaces (Marshall, 1992).

Various techniques have been adopted for the proper study and understanding of biofilm attachment and control. If the microorganisms from food-contact surfaces are not completely removed, they may lead to biofilm formation and also increase the biotransfer potential (Kumar & Anand, 1998). Therefore, various preventive and control strategies like hygienic plant lay-out and design of equipment, choice of materials, correct use and

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selection of detergents and disinfectants coupled with physical methods can be suitably applied for controlling biofilm formation on food-contact surfaces (Kumar & Anand, 1998). In addition, bacteriocins and enzymes are gaining importance and have a unique potential in the food industry for the effective biocontrol and removal of biofilms. These newer biocontrol strategies are considered important for the maintenance of biofilm-free systems, and as consequence for quality and safety of foods (Kumar & Anand, 1998).

Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes (usually 30–60 amino acids) which have a bactericidal or bacteriostatic effect on other (usually closely related) species (Garneau, Martin, & Vederas, 2002). In all cases, the producer cell exhibits specific immunity to the action of its own bacteriocin. Most bacteriocin biosynthesis occurs during or at the end of exponential growth (Tagg, Dajani, & Wannamaker, 1976). Bacteriocins are generally considered to act at the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipid bilayer (Montville, Winkowski, & Ludescher, 1995). Nisin is the best defined and the only purified bacteriocin produced by LAB that has been approved for use in food products (Hansen, 1994).

Numerous reviews have suggested that pathogens microorganisms in meat fermented foods may be inhibited by some bacteriocin-producing meat LAB (Foegeding, Thomas, Pilkington, & Klaenhammer, 1992; Laukova, Czikkova, Laczkova, & Turek, 1999; Tyopponen, Markkula, Petaja, Suihko, & Mattila-Sandholm, 2003). However, few studies (Leriche, Chassaing, & Carpentier, 1999; Zhao, Doyle, & Zhao, 2002) focused on the application of bacteriocin-producing LAB to control the growth of pathogenic microorganisms in biofilms, i.e., the real mode of microbial attachment to processing surfaces and equipments.

Therefore, we report in this paper on the behaviour of pathogenic and spoilage bacteria in dual species biofilms with bacteriocin-like-producing LAB isolated from the same small-scale facility (except *L. innocua*). The aim was to investigate competitive interactions occurring in the small-scale facility microbial ecosystem between LAB and undesirable bacteria. The final goal was to select an endogenous barrier flora to pathogenic and spoilage bacterial adhesion for this small-scale facility.

2. Materials and methods

2.1. Strains and culture conditions

The 10 LAB (E1L-28, E1L-38, E1L-39, E1L-40, E1L-53, E1L-61, E1L-62, E1L-63, E1L-67 and E1L-69) included in this study were isolated from a small-scale facility producing traditional dry sausages (Chevallier, Talon, Laguet, Labayle, & Labadie, 2001) and were identified in a previous study as 5 *Vagococcus carniphilus* (E1L-61, E1L-62, E1L-63, E1L-67 and E1L-69), 4 *Enterococcus faecium* (E1L-38, E1L-39, E1L-40 and E1L-53), and 1 *Lactobacillus sakei* (E1L-28) (Ammor et al., 2005). *V. carniphilus* species were shown to display antibacterial activity against *Listeria innocua* ATCC 33090, *Staphylococcus aureus* E1S-5 and *Hafnia alvei* E1E-25 also isolated from the same small-scale facility (except *L. innocua*) (Ammor, Tauveron, Dufour, & Chevallier, in press). *E. faecium* species E1L-38, E1L-39 and E1L-40 exhibited antibacterial activity against both *L. innocua* and *S. aureus*, while *E. faecium* E1L-53 was antagonistic to only *L. innocua* (Ammor et al., in press). Finally the *Lb. sakei* E1L-28 was antagonistic to *L. innocua* (Ammor et al., in press). All these LAB, but E1L-53, were characterized as bacteriocins-like-producing (Ammor et al., in press).

LAB were subcultured twice (1% inoculum, 24 h, 30 °C) in 15 ml MRS broth (Biokar Diagnostics, Beauvais, France) and kept frozen at −20 °C in MRS supplemented with 10% glycerol. *L. innocua* ATCC 33090, *S. aureus* E1S-5 and *H. alvei* E1E-25 were subcultured twice (1% inoculum, 24 h, 37 °C) in 10 ml BHI broth (Biokar Diagnostics) and kept frozen at −20 °C in BHI broth supplemented with 30% glycerol.

2.2. Inoculum preparation

Bacterial species were pre-cultivated twice and cultivated in BHI broth at 15 °C for 48 h. Serial dilutions were then performed in BHI broth to obtain approximate cells concentrations of 10^7 cfu/ml for bacteriocin-like-producing LAB and about 10^5 cfu/ml for indicator microorganisms. These cells concentrations were chosen on the basis of cells enumeration results obtained for LAB (on MRS agar), for *S. aureus* (on Baird–Parker agar) and for *H. alvei* (on VRBG agar) while describing the microbial ecosystem of the studied small-scale facility (Chevallier et al., 2001). Otherwise, 15 °C was considered as incubation temperature to simulate the temperature recorded in the studied small-scale facility (Chevallier et al., 2001).

2.3. Biofilm formation

Biofilms were grown according to the method of Ammor, Chevallier, Laguet, Labadie, and Talon (2004). Extra-thick fiberglass disks (diameter: 25 mm, Gelmann Sciences, Michigan, USA) were used as support matrix for biofilm growth. Disks were autoclaved at 121 °C for 15 min prior to use. Twelve disks per indicator microorganism and per assay were laid on Brain Heart Agar (BHA) for 1 h to allow their soaking with

nutrients. Twelve other disks per indicator microorganism and per assay were inoculated with 0.5 ml of bacterial culture. Among these 12 disks, 4 disks were considered as controls for the bacteriocin-producing lactic acid bacterium and 4 others as controls for the indicator microorganism. The last 4 disks were inoculated with mixture, at equal volume, of the bacteriocin-producing lactic acid bacterium and the indicator microorganism. After 5–10 min of contact allowing bacteria attachment, the inoculated disks were rinsed twice for 2 min with saline water (20 ml contained in a Petri plate) under gentle agitation (45 rpm/s) (Orbital agitator 43,000, J.P. Selecta, France) to remove unattached cells. After rinsing, the inoculated disks were placed on the sterile disks already laid on the nutritive medium and incubation was run at 15 °C for 3 days.

2.4. Cell enumeration

Enumeration of both species was performed at 0, 24, 48 and 72 h. The inoculated disks were rinsed for 2 min in a Petri dish containing 20 ml of tryptone salt broth (Biokar Diagnostics) under gentle agitation (60 rpm/s). Disks were then placed in a stomacher bag (AES Laboratoire, Combourg, France) containing 100 ml of tryptone salt broth and stomached with a Lab Blender 400 stomacher (Seward, UK) for 6 min. Decimal dilutions were then performed in saline solution (0.9% NaCl) and pour plated (1% inoculum) simultaneously onto MRS agar (pH 6) and onto the appropriate medium of the indicator microorganism (Baird–Parker for *S. aureus*, Palcam for *L. innocua* and VRBG for *H. alvei*). All these agar media were purchased from Biokar Diagnostics. Plating was performed in duplicate using an automatic spiral plater WASP (Don Whitley Scientific Limited, West Yorkshire, UK) and plates were incubated for 48 h at 30 °C for LAB and at 37 °C for target microorganisms.

After incubation, enumeration was performed using the WASP colony counting system. The inhibitory effect of the bacteriocin-producing LAB was determined by comparing indicator microorganism cells counts from the control and from the dual species biofilm. Experiments were performed in duplicate and the results are displayed as the mean values.

2.5. Survival of indicator microorganisms in dual species planktonic cultures

Indicator microorganisms were investigated for their behaviour when co-cultivated with the bacteriocin-producing LAB in the planktonic form. Four bacteriocin-producing LAB (*E. faecium* E1L-38, E1L-39 and E1L-40 and *Lb. sakei* E1L-28) were co-cultivated with *Listeria innocua* ATCC 33090 or *Staphylococcus aureus* E1S-5 in dual species planktonic cultures.

Bacterial species were pre-cultivated twice and cultivated in BHI broth at 15 °C for 48 h. Serial dilutions were then performed in BHI broth to obtain approximate cells concentrations of about 10^7 cfu/ml for bacteriocin-producing LAB and about 10^5 cfu/ml for indicator microorganisms. Twelve flasks containing 100 ml of BHI broth were considered for each indicator microorganism and for each assay. They were inoculated with 0.5 ml of bacterial culture. Among these 12 flasks, 4 were considered as controls for the bacteriocin-producing LAB and 4 others as controls for the indicator microorganism. The last 4 flasks were inoculated with 0.5 ml mixture, at equal volume, of the bacteriocin-producing lactic acid bacterium and the indicator microorganism. Incubation was run at 15 °C for 3 days and enumeration was performed as described above. Experiments were performed in duplicate and the results are displayed as the mean values.

3. Results

3.1. Survival of L. innocua in dual species biofilms

Table 1 presents $\log_{10}$-reductions of *L. innocua* populations following growth with *Lb. sakei*, *E. faecium* and *V. cariphilus* species in dual species biofilms. In all cases, *L. innocua* population decreased suggesting that antagonism was occurring between this bacterium and the lactic one.

Considering *Lb. sakei* E1L-28, the maximum $\log_{10}$-reduction level of *L. innocua* population was observed within 24 h (0.5 $\log_{10}$ cfu/ml). After 72 h of incubation, counts of *L. innocua* recorded in dual species biofilms were higher than those recorded for the control.

Table 1
Log_{10}-reduction of *L. innocua* populations in dual species biofilms

Hours	Lactic acid bacteria									
	E1L-28	E1L-38	E1L-39	E1L-40	E1L-53	E1L-61	E1L-62	E1L-63	E1L-67	E1L-69
0	0.1	0.0	0.0	−0.2	0.0	0.0	0.0	−0.4	0.2	0.0
24	0.5	0.7	0.7	1.3	0.6	1.2	1.1	0.7	1.2	1.2
48	0.3	0.3	0.9	0.6	2.0	2.0	1.7	0.6	1.4	>2.0
72	−0.3	−0.2	0.5	0.1	>1.0	2.0	0.5	0.4	>1.1	1.4

Considering *E. faecium* species, the maximum reduction of *L. innocua* populations was observed in the presence of E1L-40 within 24 h (1.3 $\log_{10}$ cfu/ml). While E1L-53 showed the lowest $\log_{10}$-reduction level within 24 h (0.6 $\log_{10}$ cfu/ml), it was the most inhibitory of *L. innocua* after 48 and 72 h of growth (2.0 and >1.0 $\log_{10}$ cfu/ml, respectively). Within 72 h, counts of *L. innocua* were approximately equal or higher compared with those of the control in the presence of E1L-38 and E1L-40.

V. carniphilus species E1L69 and E1L-61 were highly antagonistic to *L. innocua* compared to *E. faecium* and *Lb. sakei* species. A reduction of 1.2 $\log_{10}$ cfu/ml was observed within 24 h and 2 $\log_{10}$ cfu/ml or higher after 48 h of growth. However, only E1L-61 was able to maintain a $\log_{10}$-reductions level of about 2 $\log_{10}$ cfu/ml within 72 h. E1L-63 induced the lowest $\log_{10}$-reduction level of *L. innocua* populations recorded after 24, 48 and 72 h of incubation compared with other isolates belonging to *V. carniphilus* species.

In most cases, $\log_{10}$-reduction of *L. innocua* population recorded after 72 h of growth were the lowest compared to those recorded after 24 and 48 h of incubation.

3.2. Survival of S. aureus in dual species biofilms

E. faecium species were generally inefficient against *S. aureus* E1S-5 in dual species biofilms (Table 2). E1L-40 was the only one able to reduce slightly *S. aureus* population by 0.4 $\log_{10}$ cfu/ml. In contrast, counts of *S. aureus* increased in the presence of E1L-39.

V. carniphilus species induced a low reduction of *S. aureus* population within 24 h (Table 2). However, they enhanced $\log_{10}$-reduction levels varying between 0.4 and 1.5 $\log_{10}$ and between 0.9 and 2.7 $\log_{10}$ within 48 and 72 h, respectively. E1L-62 was the most efficient after 48 h of growth, but 24 h after E1L-69 showed the most inhibitory effect.

3.3. Survival of H. alvei in dual species biofilms

V. carniphilus species were strongly antagonistic to *H. alvei*, with each bacterium providing greater than 2.4 $\log_{10}$ cfu/ml differential within 24 h when compared with the control (Table 3). However growth repression of *H. alvei* decreased within 48 h and stabilized at 0.7 $\log_{10}$ cfu/ml after 72 h of culture. E1L-62 was the most efficient isolate against *H. alvei*.

Table 3
Log_{10}-reduction of *H. alvei* populations in dual species biofilms

Hours	Lactic acid bacteria				
	E1L-61	E1L-62	E1L-63	E1L-67	E1L-69
0	0.0	0.0	0.0	0.2	0.2
24	1.0	2.4	0.9	1.2	0.9
48	0.1	1.3	0.2	0.4	0.3
72	0.7	0.7	0.6	0.7	0.7

3.4. Comparison of competitive interactions in planktonic cultures and in biofilms

Table 4 summarizes $\log_{10}$-reduction levels of *L. innocua* population when grown in dual species planktonic cultures of *E. faecium* species E1L-38, E1L-39, E1L-40 and *Lb. sakei* E1L-28. *L. innocua* was much more sensitive to the presence of the bacteriocin-like-producing LAB in planktonic cultures than in biofilms. Levels of $\log_{10}$-reduction varied between 1.4 and 2.7 $\log_{10}$ cfu/ml within 24 and 48 h, while they comprised between 0.3 and 0.9 $\log_{10}$ cfu/ml in biofilms. After 72 h of incubation, target microorganisms were more resistant than within 24 and 48 h.

The same tendency was observed for *S. aureus* since $\log_{10}$-reduction levels comprised between 0.6 and 1.8 $\log_{10}$ cfu/ml within 48 h of growth while they did not exceed 0.2 $\log_{10}$ cfu/ml in biofilms (Table 5). Furthermore *L. innocua* was much more sensitive than *S. aureus* to the presence of the bacteriocin-like-producing LAB.

Table 4
Log_{10}-reduction of *L. innocua* populations in dual species planktonic cultures

Hours	Lactic acid bacteria			
	E1L-28	E1L-38	E1L-39	E1L-40
0	0.0	0.1	0.0	−0.1
24	2.0	2.1	1.4	2.3
48	1.6	2.7	2.2	2.4
72	0.9	1.6	1.4	1.9

Table 2
Log_{10}-reduction of *S. aureus* populations in dual species biofilms

Hours	Lactic acid bacteria							
	E1L-38	E1L-39	E1L-40	E1L-61	E1L-62	E1L-63	E1L-67	E1L-69
0	−0.1	−0.1	−0.6	0.1	−0.1	0.0	0.1	0.1
24	−0.1	−0.3	0.4	0.3	0.4	0.1	0.3	0.3
48	0.2	−0.6	0.1	1.1	1.5	1.0	0.4	1.0
72	0.0	−0.1	0.4	1.7	>2.2	0.9	1.3	2.7

Table 5
Log_{10}-reduction of *S. aureus* population in dual species planktonic cultures

Hours	Lactic acid bacteria		
	E1L-38	E1L-39	E1L-40
0	0.0	0.0	0.0
24	0.0	0.1	0.5
48	1.8	0.6	0.7
72	0.7	0.7	0.8

4. Discussion

L. monocytogenes and *S. aureus* are Gram-positive pathogens that have been involved in outbreaks of food-borne disease in which meat represented the incriminated foodstuff (Lindqvist et al., 2001; Sim et al., 2002). These microorganisms adhere and form biofilms on numerous surfaces (Luppens, Reij, van der Heijden, Rombouts, & Abee, 2002; Mafu, Roy, Goulet, & Magny, 1990). Biofilms may represent an important source of contamination for materials or foodstuffs coming into contact with them (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). Several reports have shown that sessile bacterial cells are more resistant to environmental changes and cleaning or disinfection treatments (Kumar & Anand, 1998). Therefore, there is a growing interest in this mode of growth in food processing that aim to improve the microbiological safety of food.

Biofilm control can be achieved by adopting several strategies like physical and chemical methods (Kumar & Anand, 1998), however, in the case of traditional fermented foods for which fermentation relies on natural contamination by the environmental flora, these strategies may led to inhibition or killing of the technological flora that ensure the fermentation and flavour of the traditional fermented product. In that case, the use of bacteriocin-producing LAB could play a role in controlling colonization by pathogenic and spoilage bacteria in food processing facilities.

Therefore, behaviours of *L. innocua*, *S. aureus* and *H. alvei* were investigated in artificially made biofilms of bacteriocins-like-producing LAB. Except *L. innocua*, all considered bacteria were isolated from the same small-scale facility. The aim of this study was to simulate competitive interactions in the small-scale ecological niche. To restrict the extent of acid production and investigate mainly the bacteriocin-like effect, low-glucose medium, i.e., BHI (0.2% glucose), was used. *L. innocua* was used instead of *L. monocytogenes* since the two microorganisms show similar physiological properties with the difference that the former does not belong to the pathogen species of *Listeria*. Moreover, some papers reported a greater sensitivity of *L. monocytogenes* towards some antibacterial compounds than *L. innocua* (Çon, Gökalp, & Kaya, 2001; Mataragas, Drosinos, & Metaxopoulos, 2003).

L. innocua was sensitive to all considered LAB but at different levels. *Lb. sakei* showed the lowest log_{10}-reduction levels compared to *E. faecium* and *V. carniphilus* species. To our knowledge, it is the first report studying the interaction of a bacterinogenic *Lb. sakei* strain and *Listeria* in biofilms. Alternatively, the ability of enterococcal species to repress the growth of *L. monocytogenes* in biofilms was already reported by Zhao et al. (2002) who showed that 2 *E. durans* species isolated from floor drains inhibited by more than $5 \log_{10}$ cfu/cm^2 *L. monocytogenes* population within 24 h at 37 °C.

Levels of reduction decreased within 72 h confirming previous observations made by some authors who reported an immediate reduction of target cell population by 1 or 3 log cycles upon addition of bacteriocin, with little or no effect upon further incubation (Leriche et al., 1999). Maisnier-Patin, Deschamps, Emin, Gratadoux, and Richard (1995) reported that after the first contact with nisin, *L. monocytogenes* cells became resistant to a solution eight times more concentrated.

S. aureus was practically unaffected by the presence of *E. faecium* species, however in the presence of *V. carniphilus* species, log_{10}-reduction levels of this pathogenic bacterium population were able to reach $2.7 \log_{10}$ cfu/ml. *V. carniphilus* species were also found to be effective against *H. alvei*, the Gram-negative psychrotrophic spoilage bacterium most frequently isolated from meat and meat products (Castano, Garca Fontan, Fresno, Tornadijo, & Carballo, 2002).

Regarding results obtained in broth and in biofilms, planktonic cells of target microorganisms were more sensitive to bacteriocins-like than sessile ones. This is in confirmation with Carpentier and Cerf (1993) who reported that once attached to surfaces bacteria become more resistant to adverse environments.

Furthermore, results obtained in broth were somewhat surprising since all bacteriocin-like substances produced by the considered LAB were unsuccessfully researched in filtered supernatants (Ammor et al., in press). Logically, target microorganism populations would have to be unaffected by the presence of the bacteriocin-like-producing LAB. Since pH values of culture media never decreased below 5.2, the eventuality that the high level of organics acids produced by LAB was at the origin of inhibition was discarded and thus the hypothesis that bacteriocins-like were adsorbed by the filter was more credible than that of the inability to produce inhibitors in liquid culture. Testing different filter types (cellulose acetate, polyethylene sulfate) and pore-sizes (0.22 and 0.48 μm) characterized by a low-binding protein capacity resulted also in inactivation of supernatants residual activity. Thus, the bacteriocin-like substances were adsorbed by filters.

Regarding the results obtained, the use of *E. faecium* E1L-40 and E1L-53 is suggested to control the growth of undesirable microorganisms on material and environmental surfaces. This suggestion is strengthened by the fact that *E. faecium* species has been already suggested as starter cultures for fermentations (de Castro, Montano, Casado, Sanchez, & Rejano, 2002; Foulquie Moreno, Rea, Cogan, & de Vuyst, 2003; Leroy, Foulquié Moreno, & de Vuyst, 2003). However, further characterizations have to be done to be sure of the innocuity of these strains. *V. carniphilus* E1L-61, E1L-62 and E1L-69 also present strong antagonistic performances that could represent a further weapon, according to the hurdle concept, to fight against the settlement of undesirable bacteria on surfaces. However their use is conditioned by the assessment of their clinical significance.

Additional investigations have to be performed to look for the efficiency of these bacteriocin-like-producing LAB in multi-species biofilms. Indeed, Bourion and Cerf (1996) demonstrated the importance of an exopolymer-producing strain of *Pseudomonas aeruginosa* for enhancing the attachment of *L. innocua* and its protection from disinfectants. Thus bacterial response to adverse environments, namely antibacterial compounds, is surely different in mixed cultures than in dual cultures.

5. Conclusion

It was showed in this study that certain bacteriocin-like-producing LAB may repress the growth of some undesirable microorganisms in dual species biofilms and thus may be used as barrier flora against the settlement of these undesirable microorganisms on the processing surfaces equipment in meat small-scale facilities.

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