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Enhancing the antilisterial effect of *Lactobacillus curvatus* CWBI-B28 in pork meat and cocultures by limiting bacteriocin degradation

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Abstract

This work focused on *Listeria monocytogenes* growth inhibition and growth rebound in raw and cooked pork meat inoculated with *Lactobacillus curvatus* strains. During storage of raw meat homogenates in the presence of the bacteriocin-producing strain *Lactobacillus curvatus* CWBI-B28wt, the *Listeria monocytogenes* cfu count was initially reduced to an undetectable level, but a growth rebound occurred after two weeks, coinciding with loss of 70% of the bacteriocin activity present at the end of week 2. The *Listeria* growth rebound was suppressed when proteolysis of bacteriocin was countered by the absence of proteases (bacteriocin addition to cooked meat) or the presence of 1% soy flour (added to provide competing substrates). Further experiments confirmed that bacteriocin is sensitive to the action of proteolytic enzymes isolated from both *Lactobacillus curvatus* CWBI-B28wt and the meat matrix. Bacteriocin proteolysis thus emerges as a cause of *Listeria* growth rebound.

Keywords: Bacteriocin; *Lactobacillus curvatus*; *Listeria monocytogenes*; Commercial pork meat; Proteases

1. Introduction

Lactic acid bacteria such as *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus*are involved in processing all types of fermented meats (De Vuyst & Vandamme, 1994). It is well known that such bacteria have the potential to produce numerous antimicrobial compounds, e.g. organic acids and bacteriocins (Hammes & Hertel, 1996). Protective starter cultures can be used to suppress growth of undesirable bacteria and foodborne pathogens like *Listeria monocytogenes*. They can also be used to control the fermentation process by inhibiting growth of competing microflora (Vogel, Pohle, Tichaczek, & Hammes, 1993). In Europe, the starter cultures used to manufacture fermented sausages mainly contain *L. sakei* and *L. curvatus* (Hammes & Hertel, 1996). These cultures grow during the initial stage of fermentation, and stringent conditions inhibit subsequent growth of other bacteria. Yet *L. monocytogenes* rebound remains a major problem (Johnson, Doyle, & Cassens, 1990; Leistner, 1995). It has been reported that bacteriocins produced by several lactic acid bacteria exert a transitory bactericidal effect against *L. monocytogenes*, often followed by re-growth of *Listeria* cells in bacteriocin-supplemented food models (Benkerroum, Daoudi, & Kamal, 2003; Bhatti, Veeramachaneni, & Shelef, 2004; Bouttefroy & Milliere, 2000). This growth rebound might be due to factors that severely limit growth of bacteriocin-producing cells (e.g. restricted nutrient availability), to decreased bacteriocin action as a result of adsorption onto food particles, fats, and proteins, to the presence of a curing agent, to the emergence of bacteriocin-resistant cells, and/or to bacteriocin degradation by proteases of food and/or microbial origin (Schillinger, Kaya, & Lücke, 1991). Most of these factors and their effects on bacteriocin

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activity have been studied more extensively than the influence of bacteriocin degradation attributed to proteolytic activity.

This paper focuses on the role of proteases present in both *L. curvatus* CWBI-B28 strains and the pork-meat matrix in *Listeria* rebound.

2. Materials and methods

2.1. Bacteria and growth media

The *L. curvatus* strains used to inoculate pork-meat mixtures were CWBI-B28wt (wild type), an antilisterial bacteriocin-producing strain described by Benkerroum et al. (2005), and CWBI-B28mt (mutant type) a bacteriocin-non-producing derivative of CWBI-B28wt obtained by mutagenesis. *L. monocytogenes*, sensitive to the bacteriocin produced by *L. curvatus* CWBI-B28wt and previously isolated from bacon, was used to artificially contaminate meat samples.

L. curvatus CWBI-B28wt and CWBI-B28mt were grown on de Man, Rogosa and Sharp medium (MRS broth) (Biokar, Beauvais, France), *L. monocytogenes* was regularly spread over Palcam agar (Oxoid, Beauvais, France) plates and activated at the time of its use in Tryptic Soy Broth (TSB, Biokar, Beauvais, France). All strains were stored at −80 °C in their respective media with added glycerol (40%).

2.2. Lyophilized cell-adsorbed bacteriocin preparation

A crude preparation of freeze-dried *L. curvatus* CWBI-B28wt bacteriocin was obtained as described previously (Benkerroum et al., 2005). Briefly, *L. curvatus* CWBI-B28 was grown at 37 °C in MRS broth (20 l) in a 100-l fermentor (Biolafite, France). The fermentor was operated without aeration, with moderate stirring (80 rev min^{-1}), and at controlled pH and temperature. The pH was maintained constant at 6.5 by automatic addition of 2.5 M NaOH. At this pH, the bacteriocin of *L. curvatus* CWBI-B28wt was shown to adsorb maximally onto producer cells (Benkerroum et al., 2005).

After 30 h of fermentation, the culture was heat-treated at 70 °C for 30–35 min to allow cell and protease inactivation (data not shown). Next, 8 l supernatant was centrifuged at 4 °C and 4000*g* for 90 min in a Beckman centrifuge (Avanti J-25I; Beckman, CA, USA).

The resulting concentrate was suspended in 1800 ml supernatant kept at 4 °C for 30–35 min to allow adsorption of bacteriocins onto producer cells. Then a second centrifugation for 25 min at 4000*g* and 4 °C was carried out to eliminate the supernatant containing any non-adsorbed bacteriocins. The pellet concentrate was then suspended in 150 ml supernatant, allowed to crystallize overnight at −80 °C, and lyophilized (Koeltechniek Louw B.V.B.A, Rotselaar, Belgium). The powder obtained was aliquoted, vacuum-packed, and preserved at −20 °C.

2.3. Using *L. curvatus* CWBI-B28wt or a lyophilized cell-adsorbed bacteriocin to control *L. monocytogenes* growth on pork meat (lean bacon)

2.3.1. Meat preparation and inoculation

Raw pork meat (lean bacon) was obtained from various Belgian commercial producers. The listed characteristics of the meat products are: moisture (60%); protein (15%); fat (13%); minerals (5%); carbohydrates (7%); pH at 24 h(5.65). Twelve blocks of 50 g were cut off aseptically and transferred to sterile Stomacher bags. Deionized water (50 ml) was added and six 50-g pieces were homogenized for 3 min at 260 rpm (Stomacher 400, Seward, London, UK). The homogenate was transferred to sterile bottles, then *L. monocytogenes* and *L. curvatus* CWBI-B28wt or CWBI-B28mt (10^3 cfu/g for each bacterium in 2 × 50 g) were co-inoculated at the same time under a laminar flow hood (Clean Air, VWR, Belgium). A control containing only *L. monocytogenes* at an initial concentration of 10^3 cfu/g in 2 × 50 g was included.

Another six 50-g pieces were heat-treated (121 °C for 10 min) to obtain cooked meat, then homogenized and transferred to sterile bottles as above, prior to addition of *L. monocytogenes* alone (10^3 cfu/g in 2 × 50 g), *L. monocytogenes* plus *L. curvatus* CWBI-B28wt (10^3 cfu/g for each bacterium in 2 × 50 g), or *L. monocytogenes* (10^3 cfu/g) plus lyophilized cell-adsorbed bacteriocin (0.01 g/g in 2 × 50 g).

Each treated homogenate of raw or cooked meat was incubated for 4 weeks at 4 °C.

These experiments on meat were carried out in parallel with an *in vitro* experiment in MRS broth. *L. monocytogenes* and either the CWBI-B28wt or the CWBI-B28mt *L. curvatus* strain were co-inoculated into flasks containing sterile MRS broth (100 ml) at the final concentration of about 10^6 cfu/ml for each bacterium. The flasks were incubated for 72 h at 37 °C.

Each experiment was performed twice and each determination was done in triplicate. Data are presented as means of two independent experiments with SD.

2.3.2. Meat sampling and analysis

At sampling, after 0, 7, 14, and 28 days of incubation, 20-g samples (crushed meat) were diluted with 10 ml sterile saline solution (0.85% sodium chloride) and homogenised in a Stomacher bag. In a parallel *in vitro* MRS broth experiment, 1-ml samples were taken aseptically at regular intervals and homogenized with 9 ml saline.

2.3.2.1. Microbiological analysis. Growth of the inoculated strains was determined on the basis of viable counts after homogenization in peptone water, as described by Katla et al. (2001). A decimal dilution series was prepared and at specific time intervals, samples were taken to perform microbial counts. *L. monocytogenes* was enumerated after incubation for 48–72 h at 37 °C and lactic acid bacteria were enumerated after 24–48 h of incubation at 37 °C, after homogenization in peptone water, as described by Katla et al. (2001).