STABILITY AND ANTIBACTERIAL ACTIVITY OF BACTERIOCIN PREPARATIONS IN PORK MODEL SYSTEMS

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Background

Pathogenic microorganisms are common in raw pork in Mexico City and hence could be found in processed products. Lactic acid bacteria and their metabolites have some antibacterial effect, and are used to control pathogens in foods. In previous studies, Kuri *et al.* (1996) suggested that the survival of *Salmonella* spp. in Mexican fermented meats was related to the effectiveness of lactic fermentation and isolated and characterized *Salmonella* spp. (Kuri *et al.* 1995) and lactic acid bacteria strains from temperature abused pork and chorizo. Further studies with those strains showed that the antagonistic activity against *Salmonella* was mainly due to acid production, however bacteriocin production was detected, and bacteriocins were further characterized. Bacteriocins or bacteriocin producing cells could be an aid to control pathogens in meats, but information on specific applications is still needed.

Objective

The aim of this work was to evaluate the stability and antibacterial properties of a bacteriocin from *Lactobacillus sake* MX133 isolated from chorizo for application to control pathogenic bacteria in pork.

Methods

Pork muscle homogenates were used as model systems. Minced porcine *Longissimus dorsi* (10 g) purchased from a local butcher was mixed with 10 mM phosphate-citrate buffer (pH 6.8 and pH 4.0). The mixture was homogenized with an Ultra Turrax homogenizer (D 10N probe, Janke and Kunkel, Staufen, Germany) and the volume adjusted to 100 ml with the appropiate buffer. Connective tissue was removed from the emulsion by filtering through a strainer. This meat stock was further tenfold diluted and sterilised by filtering through a 0.2 μm low protein binding membrane (Acrodisk, Gelman Sciences, Ann Harbor, MI, USA).

Bacteriocin stock was prepared from an acidified (pH 2) supernatant of an overnight culture of *Lactobacillus sake* MX133. After decanting the pH was adjusted to 6.5, and it was maintained at 75°C for 15 min. After ammonium sulphate precipitation and dialysis against 0.1 M citrate-phosphate pH 6.6 buffer, this antibacterial preparation (960 AU ml^{-1}) was used as bacteriocin stock.

To assess the effect of proteases on the bacteriocin, stock solution was subjected to the action of the following proteolytic enzymes (Sigma); pepsin, pronase E, α-chymotrypsin, proteinase K, lipase, lysozyme and papain prepared with 10 mM phosphate-citrate pH 6.8 buffer at a concentration of 2 mg ml^{-1}, and an extra set of solutions in 10 mM phosphate-citrate pH 4.0 buffer for pepsin and muscle preparation pH 4.0. To rule out any possible non enzymatic effect, a heat inactivated enzyme control was included for each enzyme. An aliquot of each enzyme was heated in Eppendorff tubes (75°C, 30 min) and cooled in an ice bath. Then, 50 μl of bacteriocin stock solution (including a blank of buffer) was added with 50 μl of enzyme solution (final concentration of 2 mg ml^{-1}) in the wells of a microplate. (including buffer as control) and incubated at 37°C for 2 h. Activity units were assessed from serial twofold water dilutions. In a subsequent test, the pH of the pepsin and pork pH 4.0 bacteriocin solutions was adjusted to pH 4.0 (with 3% phosphoric acid) before incubation, and further neutralized (1 N NaOH) before the bioassay.

Alternatively, a method based on diffusion from agar wells was used with *L. innocua* NCTC11288 (10^7 cfu ml^{-1}) as indicator strain in BHI soft agar with three aligned equidistant wells filled with 33 μl of bacteriocin stock solution or enzyme solution (1 mg ml^{-1}) and the corresponding buffer. After pre-diffusion and incubation at 37°C for 24 h, the inhibition areas were registered. The activity of the enzyme solutions used was tested with a proteolytic activity assay (Yamaguchi *et al.* 1982) and calpain activity was assessed with the method of Etherington *et al.* (1987) and Alarcon-Rojo *et al.* (1995) incubating at 30°C for 30 min using casein as a substrate.

The effect of bacteriocin concentration on different bacterial concentrations was studied with an assay in microplates with BHI broth or pork extract mixed with BHI broth (1:1), standardized inoculum was added in 5, 50 and 150 μl amounts and the bacterial load in the inoculum was obtained by plating out onto BHI agar. BHI broth was used as a negative control in one row. Bacteriocin stocks of 980 AU ml^{-1} for 22'cin and 1920 AU ml^{-1} for 133'cin were two-fold diluted with 50mM sodium-phosphate buffer pH 6.5. A line of wells with buffer was the control for bacteriocin, and water was used as blank. After incubation at 37°C, seven readings of optical density (O.D.) at 405 nm were taken every 30 min and then after 20 and 24 h.

Results and Discussion

The action of proteases on the bacteriocin preparation was clearly observed (Figure 1) The bacteriocin that diffused through the gel matrix outwards from the well, lost their antibacterial properties in the area where the protease also diffused, and this is observed as an indentation of the inhibition area beside the well that contained the protease solution. The activity of all the protease preparations was verified using casein, and both pH 6.8 and pH 4 pork homogenates also presented some proteolytic activity (28.2 and 11.8 AU g^{-1} calpain, and 2.2 and 0.0 AU g^{-1} casein).

After treating the bacteriocin preparation (960 AU ml^{-1}) with the enzymes, no residual activity was detected for pepsin, pronase E, α-chymotrypsin and papain, while just 30 and 120AU ml^{-1} for lipase and proteinase K.

Thus the bacteriocin studied was susceptible to the action of some hydrolytic enzymes, as a result of their proteinaceus nature, as previously observed (Ray and Daeschel 1992). It was therefore necessary to verify if the endogenous muscle proteases had any effect on the antibacterial activity of bacteriocins from potentially anti-pathogenic cultures. The same test showed that for lysozyme and the meat homogenates the residual activity was not reduced by more than one twofold dilution. Although caseinolytic and calpain activity of the meat extracts (pH 6.8) were confirmed *in vitro,* proteolytic activity of meat is normally attributed to calpains and other lysosomal enzymes (cathepsins) (Alarcón-Rojo and Dransfield 1995) and temperature and pH may influence the susceptibility of their

inhibitors and activators. The activity of proteases from meat is dependant on local *in situ* concentrations of cofactors and inhibitors and the mechanism is still not clear (Dransfield, 1994). Therefore it was then considered that a more reliable approach to assess bacteriocin stability on meat was to evaluate the bacteriocin activity after treatment with a meat extract at pH 6.8 and 4.0 as this range represents the pH occurring in normal and PSE (pale-soft-exudative) pork meat immediately post-mortem and after acidification. Morover, the action of all the proteolytic enzymes in meat against the bacteriocin under the conditions of the test would be revealed. The methods applied involved disrupting the structure of the meat tissue and the intricate mechanisms of proteolysis were possibly altered, however proteolytic activity was still detected.

Figure 2 shows that the bacteriocin from *Lb. sake* MX133 was able to reduce the rate of growth ($O.D.^{405nm}$) of the indicator bacteria in both synthetic media and the pork model, compared with the control (BHI broth) without bacteriocin. The concentration of inoculum and bacteriocin showed no significant difference on inhibition of growth. Therefore the culture broth composed with pork homogenate performed as an adequate media to demonstrate the antibacterial action of both bacteriocins indicating that the meat extract was not compromising bacteriocin activity against indicator strains when compared to culture media without meat.

Previous observations (not shown) indicated that the antibacterial performance of bacteriocins in liquid media could be unstable, and because their application is intended in meat or meat products, a method involving a solid media was applied to measure the inactivation of bacteriocins by proteases. As illustrated in Figure 1, pork muscle extracts with calpain activity were unable to inactivate the bacteriocin of *Lb. sake* MX133.

In a both a liquid and a semisolid system containing active meat proteases a bacteriocin preparation remained active, therefore the contribution of this bacteriocin could be considered valuable in meat systems. Previous studies indicated that *Lb. sake* MX133 inhibited some listeria, pediococci, lactobacilli and enterococci, but not *Salmonella* spp. However, combining a resistant fast-fermenting strain with either the bacteriocin or the producing cells, biocontrol of a wider range of undesirable microflora in muscle foods could be achieved.

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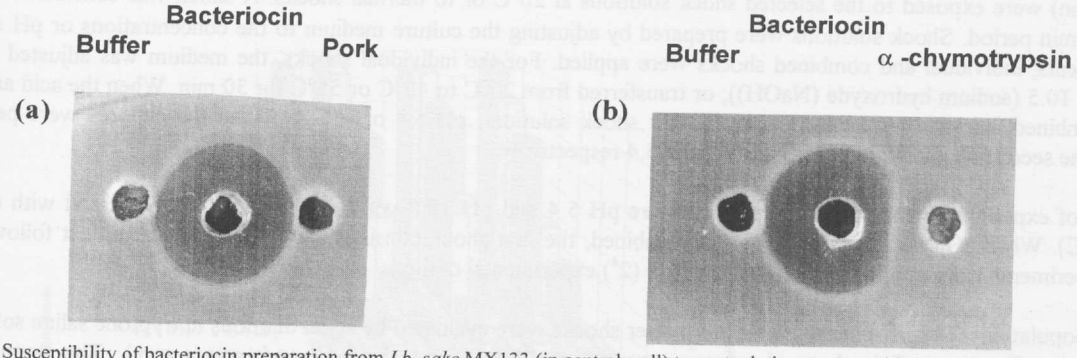

Figure 1. Susceptibility of bacteriocin preparation from *Lb. sake* MX133 (in central well) to proteolytic enzymes added into right well (a) preparation from pork and (b) α -chymotrypsin with phosphate-citrate buffer (pH 6.8) as control in left well, tested by the agar wells method against *Listeria innocua* NCTC11288 (10^7 cfu ml^{-1}) as indicator strain in BHI soft agar

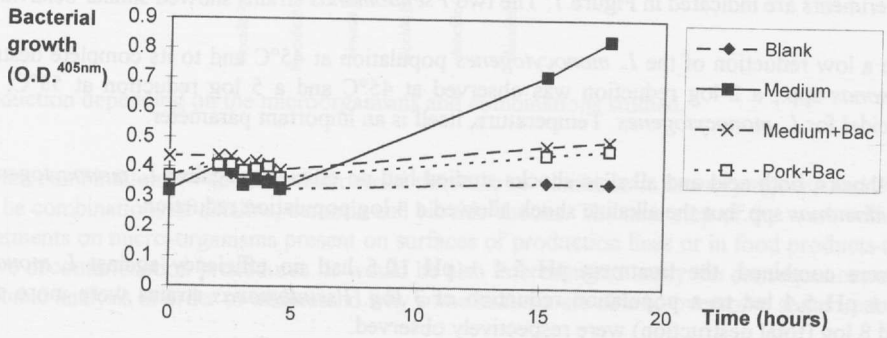

Figure 2. Growth of *Listeria innocua* NCTC11288 (10^7 cfu ml^{-1}) in the presence of bacteriocin (480 AU ml^{-1}) inoculated in a medium containing pork homogenate (Pork + Bac) or in BHI medium (Medium + Bac). The control was BHI (Medium) without antibacterial and the blank was water.