AN ASSAY FOR DETECTION OF ACTIVITY OF PLASMID ENCODED BACTERIOCIN IN FERMENTED FOOD

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SUMMARY

This paper describes a procedure for demonstration of plasmid encoded bacteriocin activity of lactic acid bacteria in fermented food. The activity of two bacteriocin producing *L. sake* strains, Lb 706 (sakacin A) and Lb 45 (lactocin S) were examined in dry sausage, fermented carrots and in Norwegian Saint Paulin cheese. The assay is based on cured, sensitive variants (Bac^-,Imm^-) of the bacteriocin producers transformed with pVS2, conferring chloramphenicol resistance. Fermented products were made with a mixture of the producer organism and the bacteriocin sensitive, antibiotic resistant variant. By comparing numbers of antibiotic sensitive and resistant colonies one can specifically monitor the fate of the sensitive strain. Production of active bacteriocin was demonstrated in dry sausage and in fermented carrots.

INTRODUCTION

Several strains of lactic acid bacteria produce bacteriocins, a heterologous group of antibacterial proteins usually characterized by a narrow host range, low molecular weight and remarkable physiological stability. Bacteriocins may inhibit growth of pathogens and spoilage organisms during food processing and food fermentations, and may thus be of interest to the food fermentation industry. The demonstration of bacteriocin production in synthetic media does not necessarily reflect production and activity in fermented foods. Nutrient availability, temperature, pH, inoculum size and other factors will all influence production (DAESCHEL, 1990). This paper reports a procedure to demonstrate production and effect of plasmid encoded bacteriocins in fermented food in a system designed to eliminate all environmental influence, since the indicator and the producer strain are derived from the same bacterium and thus should react equally to changes in the environment.

MATERIALS AND METHODS

Bacterial strains: The two bacteriocin producing strains and their cured, sensitive variant are described in Table 1.

Table 1. *Lactobacillus sake* strains used in this study.

Strain	Origin	Phenotype	Obtained from	Reference
L. sake Lb 706	Vacuum packed beef	Bac^+, Imm^+	F.-K. Lücke	Schillinger & Lücke, 1987
L. sake Lb 706-B	Cured variant of Lb 706	Bac^-, Imm^-		Schillinger & Lücke, 1989
L. sake Lb 45	Vacuum packed meat	Bac^+, Imm^+	MATFORSK	Mørtvedt & Nes, 1990
L. sake Lb 45H	Spontaneous variant of Lb 45	Bac^-, Imm^-		

To assure that the sensitive variant and the parent strain had identical growth characteristics, their sugar fermenting patterns were checked with a API 50 CH Lactobacillus kit (Bio Meriaux SA, France).

Microbial analysis

Bacto Lactobacilli MRS agar was used to measure total bacterial number. Plates were incubated at 25 or 30 °C for 2 days for Lb 706

and Lb 45 respectively. MRS agar containing 5µg Cm/ml was used to monitor Bac$^-$, Imm $^-$, Cm^R variants.

Bacteriocin production was detected by:

a. Deferred antagonism as described by JOERGER & KLAENHAMMER (1986) and

b. Well diffusion assay according to SCHILLINGER & LÜCKE (1989).

Electroporation

The plasmid introduced, pVS2, is a general lactic acid bacteria cloning vector conferring chloramphenicol and erythromycin resistan (WRIGHT et al 1987). Electroporation was performed with the cured, sensitive *Lactobacillus sake* variants to render them Cm-resista Cells were made electro-competent by growth in MRS with 1% glycine, and harvested at A_{600}^{1cm} 0,6. Cells were washed in 10 mM MgC and 30 % (w/v) polyethyleneglycol (PEG 1500) and finally concentrated in PEG 1500 (100x). Electroporation conditions were: 1.5 k 400 Ω and 25 µF using the Bio Rad Gene pulser and 2 mm electroporation cuvettes (Bio Rad Lab., Richmond, CA.)(AUKRUST BLOM, 1992).

Plasmid stability studies

For stability studies of pVS2 in Lb 706-B and Lb 45H, MRS-broth cultures grown with 5µg Cm/ml were used for inoculation (1 % Each culture was transferred 4 subsequent times in Cooked Meat media (Difco) without antibiotic pressure, supplemented with 3 % NaCl and 0,7 % glucose to imitate conditions in fermented sausages. Over night cultures and 4 days old cultures were analysed o MRS-agar, and grown colonies were further replicaplated to MRS agar containing 5 µg Cm/ml. Colonies failing to grow had lost th plasmid.

Growth studies in milk and several fermented products

Preparation of lactobacilli cultures: MRS broth cultures were concentrated in peptone water (Oxoid) or 10 % skim milk (Difco) t about 1-2·10^9 cfu/ml before addition to sausage mix, carrot brine or cheese milk respectively. Fermented products were made wit mixed cultures of *L. sake* Lb 45 + Lb 45H (pVS2) (1:1) or *L. sake* Lb 706 + 706-B (pVS2) (1:1), with controls containing only th bacteriocin sensitive, Cm-resistant variants.

Preparation of dry fermented sausages: The basic initial sausage mixture contained (% w/w): Beef (50.4), pork (18.3), lard (25.1), salt (3.3), ascorbate (0.4), dextrin (0.3) and glucose (0.4). The level of inoculation in fresh sausage was about 10^7 cfu/g. Each of th four batches were divided into 10 sausages of about 200 g by hand. After the initial fermentation phase (2 days at 24 °C and 92% relative humidity(rh), 2 days at 20 °C and 88 % rh, 2 days at 18 °C and 85 % rh) the sausages were ripened at 15 °C and 85 % rh fo 30 days. The sausages were analyzed the first six days and then on day 9, 16 and 31.

Production of fermented carrots: Carrots were thoroughly washed and cut in 1.5 mm slices by a food processor. Sterile brine consisting of 1 % NaCl, 50 mM $CaCl_2$ and 0.2 % hydrolyzed casein (Merck) was added and carrots were submerged in the brine i 2 l sterile glass jars. Each of 4 jars was with concentrated lactobacilli cultures inoculated to about 10^7 cfu/ml and kept at 25 °C.

Norwegian Saint Paulin cheese: Ten 1.5-2.0 kg Norwegian Saint Paulin type cheeses were made from pasteurized milk containing 2.6 % fat with addition of 0.5 % acid hydrolyzed casein (Merck), 1 % glucose and 0.5 g $MnSO_4$ g/l. Four experimental vats and one control vat, each containing 200 l, were produced in the same day. After heating to coagulation temperature of 33 °C, each vat was inoculated with 0.5 % regular cheese starter culture. The experimental vats were in addition inoculated to about 2-5·10^6 cfu o lactobacilli/ml cheese milk. Renneting (60 ml/vat), draining of whey (100 l), addition of $NaNO_3$ (20 g), further heating to 37 °C, pressing snf salting to about 1.5% NaCl, as prescribed for this cheese variety. The Saint Paulin cheese was ripened at 17 °C. pH and microbial analysis of the cheese were performed every day for ten days and after 14 and 17 days of ripening. Grated cheese was mixed to a slurry in a Warring blender following standard procedures for microbial examination.

RESULTS AND DISCUSSION

Stability of the plasmid pVS2 in the sensitive strains grown without antibiotic pressure was examined, revealing a high degree of stability. Less than 1 % of the colonies tested after 4 subsequent transfers in modified Cooked Meat media had lost their Cm resistance (pVS2).

Production of dry fermented sausages resulted in a normal pH drop to about 4,7-4,9 within a week of storage. Lactocin S production could be detected after 2 days (Figure 1). There was about 50 fold reduction of cfu/g between growth of Lb 45H alone, compared to growth in a mixed culture with Lb 45 (Figure 1), indicating a significant bacteriostatic effect. Deferred assay showed an increase in number of lactocin S producing colonies from 50 % of total count in fresh sausage to 93 % on day 3.

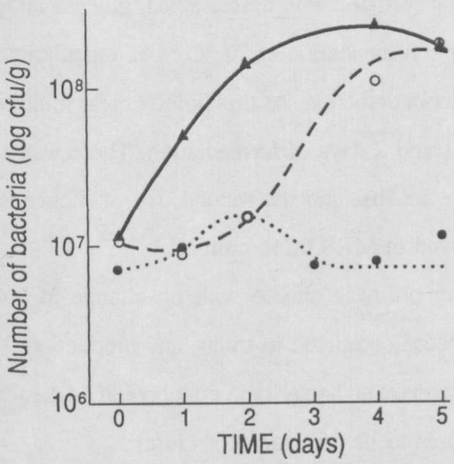

Figure 1. Effect of bacteriocin production in fermented sausage. Total bacterial count of Lb 45 + Lb 45H (pVS2) on MRS (—▲), sensitive strain (Bac$^-$) in a mixed culture with Lb 45 (Bac$^+$) on MRS agar with chloramphenicol (....,●) and sensitive strain strain alone - Lb 45H (pVS2) (---,○)

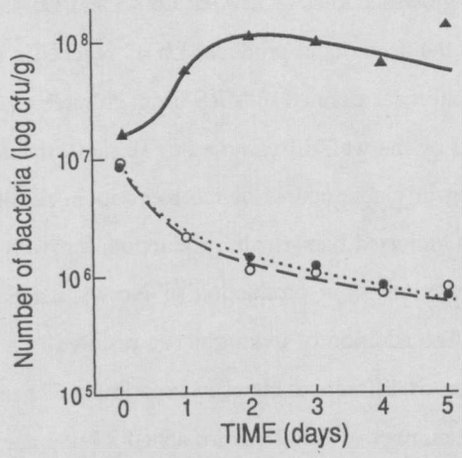

Figure 2. Effect of bacteriocin production in fermented sausage. Total bacterial count of Lb 706 + Lb 706-B (pVS2) on MRS (—▲), sensitive strain (Bac$^-$) in a mixed culture with Lb 706 (Bac$^+$) on MRS agar with chloramphenicol (....,●) and sensitive strain strain alone - Lb 706-B (pVS2) (---,○)

The effect of sakacin A production was masked by a poor growth of *L. sake* Lb 706-B (Figure 2). Growth inhibition of Lb 706-B (pSV2) as sole culture in sausage was uneexpected. With deferred assay only 4 % of the colonies were bacteriocin sensitive after one day of growth in mixed culture. Our results indicate that the growth characteristics of Lb 706-B in sausage is different from those of Lb 706, while investigation of sugar fermenting pattern by API and NaCl tolerance studies reverted no differences.

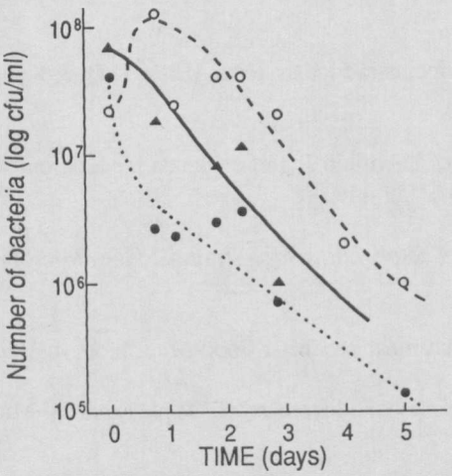

Figure 3. Effect of bacteriocin production in fermented carrots. Total bacterial count of Lb 45 + Lb 45H (pVS2) on MRS (—▲), sensitive strain (Bac$^-$) in a mixed culture with Lb 45 (Bac$^+$) on MRS agar with chloramphenicol (....,●) and sensitive strain strain alone - Lb 45H (pVS2) (---,○).

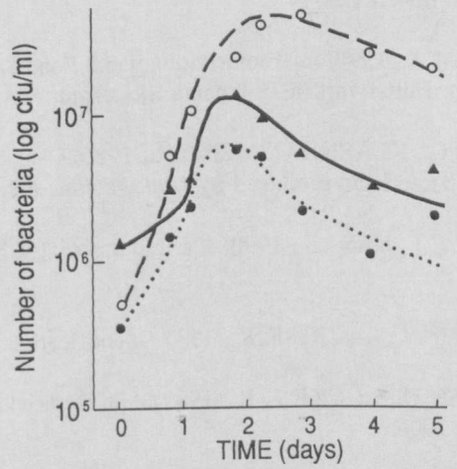

Figure 4. Effect of bacteriocin production in fermented carrots. Total bacterial count of Lb 706 + Lb 706-B (pVS2) on MRS (—▲), sensitive strain (Bac$^-$) in a mixed culture with Lb 706 (Bac$^+$) on MRS agar with chloramphenicol (....,●) and sensitive strain strain alone - Lb 706 B (pVS2) (---,○)

SCHILLINGER et al (1991) reported however that growth of Lb 706 in minced meat and comminuted cured raw pork reduced viable count of *Listeria monocytogenes* with about one log cycle.

During fermentation of carrots, both strains produced active bacteriocin. A pronounced effect was observed with Lb 45 in Figure 3. There was about one log cycle reduction in number of sensitive cells (Bac^-) compared with total count of both strains on MRS agar after less than 19 h growth. Deferred assay of the MRS colonies also indicated a relative drop in proportion of bacteriocin sensitive cells within the first 27 h of incubation. The sakacin A producing capacity of *L. sake* Lb 706 in brine is shown in Figure 4. A weak effect is observed after about 40 h of incubation.

Preliminary growth studies of *L. sake* Lb 45 and Lb 45H in UHT milk with 4 % fat fortified with amino acids, glucose and $MnSO_4$ showed that the bacteriocin producer Lb 45 reached a level of $1\text{-}2 \cdot 10^8$ cfu/ml after 8 h incubation at 30 °C (4 % inoculation), which is the maximal level attained in MRS broth cultures usually associated with bacteriocin detection. At this point a clear inhibition zone was detected by the well diffusion assay (2 mm), the zone increased in size after 1 and 2 days of fermentation. There was a drop of about 1.5 log in viable counts of the bacteriocin sensitive strain Lb 45H between the first and the second day of incubation. Both observations indicated bacteriocin production, but with a lower activity than observed in MRS broth cultures.

This assay was tested in production of Norwegian Saint Paulin cheese, obtaining ordinary cheese with no change in production parameters. The addition of extra glucose resulted in a slight lowering of pH in cheese compared to traditional production, but there were no observed differences between experimental cheese and control. In cheese, however, no bacteriocin effect could be demonstrated, although the number of lactobacilli reached a level normally sufficient for bacteriocin to be detected (10^8 cfu/g).

This assay may prove useful in demonstrating bacteriocin activity in fermented food , since additional anti-microbial activity and environmental influence can be neglected.

ACKNOWLEDGEMENT

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