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EFFECT OF BACTERIOCIN PRODUCING STARTER CULTURES ON SURVIVAL OF *LISTERIA MONOCYTOGENES* IN A PEPPERONI MODEL SYSTEM.

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Introduction.

Although a major outbreak of listeriosis related to meat consumption has not been identified, the association of *Listeria monocytogenes* with meat products, including cured and fermented products, is well documented[1,2]. Recent investigations showed that *L. monocytogenes* is able to survive microenvironmental conditions associated with the fermentation and drying of meat[3,4].
Inhibition of *L. monocytogenes* by the bacteriocin pediocin PA-1 produced by *Pediococcus acidilactici* has been shown to provide an enhanced control against this pathogen in fermented meat products[4,5]. The bacteriocin bavaricin produced by a *Lactobacillus bavaricus* strain MI401 has also shown to have bacteriostatic effect against *L. monocytogenes*[6]. The present study was designed to investigate whether an *in situ*-produced pediocin PA-1 analogous from a commercial meat starter, *P. acidilactici* CHCC3261, had inhibitory effect against growth of *L. monocytogenes* and whether any synergistic effect was attainable by simultaneous addition of a bavaricin producing *L. bavaricus* MI401 starter culture in a Pepperoni model system.

Materials & Methods.

Bacterial inocola for Pepperoni meat fermentation. *L. monocytogenes* V80 (raw meat isolate, serotype 1/2b) was grown in brain heart infusion (BHI) broth at 37°C. Bacteriocin-producing (Bac^+) *P. acidilactis* CHCC3261, bacteriocin-negative (Bac^-) *P. acidilactici* CHCC3261 and bacteriocin-positive *Lactobacillus bavaricus* MI401 (bread isolate) were grown in MRS broth at 30°C and subsequently added 20% glycerol and stored at -50°C until further use.
Plasmid-curing, using acriflavin hydrochloride (Gonzales & Kunka, 1983), was accomplished to obtain the non-bacteriocin producing *P. acidilactici* CHCC3261 (bac^-) strain used in the present experiment. DNA-fingerprint, API-profile and growth- as well as pH-profile were performed to ensure that the obtained bac^- strain did not differ from the bac^+ strain.

Bacteriocin detection and assay. Bacteriocin was extracted from samples by mixing 10 g of meat with 10 ml 0.02 N HCl and subsequent homogenization for 30 s. pH was adjusted to 4.5 and extracts were incubated at 30°C for 30 min during which samples were regulary shaken. Subsequently, extracts were centrifuged (1700 x g, 20 min, 4°C), the liquid state was taken and adjusted to pH 6.0 before final filtration (0.45 μm filter). An agar diffusion assay was used to determine the bacteriocin titer. Serial twofold dilutions of the obtained extraxts were spotted (50 μl) into wells (d 6 mm) of an agar inoculated with the indicator bacteria *Lactobacillus sake* NCFB 2714 ($1x10^6$ CFU/ml) and incubated at 24 h at 30°C. The titer was defined as the reciprocal of the highest dilution exhibiting complete inhibition of the indicator within a zone of at least 1 mm times 20 and was expressed in activity units per gram meat (AU/g).

Pepperoni model system. Pork shoulder (38%) and beef back (38%) was grounded with backfat (24%) through a 3 cm and subsequent a 0.3 cm plate. A cure mixture of salt (3.2%), glucose (0.53%), spices (2.1%), ascorbate (500 ppm) and $NaNO_2$ (60 ppm) was added to ground meat and mixed. The appropriate starter culture(s) (10^7 or $2{\cdot}5x10^6$ CFU/g) or pediocin powder (507 AU/g) and *L. monocytogenes* ($6x10^4$) were mixed and immidiately added to the cured meat. The minced meat was filled into sterile bottles which subsequently were incubated at 40°C for 24 h followed by 38 h at 20°C in a waterbath to simulate the conditions of production of Pepperoni .

Sampling and evaluation. Pepperoni meat was sampled at selected times to determine *L. monocytogenes* and lactic acid bacteria populations by aseptically removal of 10 g from each sample. Samples were diluted 1:9 with 0.1% peptone-water and homogenized in a stomacher for 30 sec. *L. monocytogenes* was enumerated after microaerophilic incubation at 37°C for 48 h on Palcam agar. A detection limit of < 10 *L. monocytogenes* per g meat were obtained by plating samples of 200 μl from the 10^1-dilution on five Palcam plates. Lactic acid bacteria was enumerated on MRS agar after incubation at 30°C, 48 h and 45°C, 24 h, respectively. pH of meat samples were monitored by using a combination electrode (Xerolyt, Ingold) and a pH meter (Metrohm).

Results & Discussion.

The total amount of lactic acid bacteria in the different samples during incubation as found on MRS agar at 30°C for 48 h and at 45°C for 24 h showed no appreciable difference (data not shown). This indicate that *P. acidilactici* CHCC3261 outgrew *L. bavaricus* MI401 during the incubation in samples incubated with both, as the latter is not able to grow at 45°C. This was further confirmed by direct microscopy of samples, which did not showed presence of any characteristic stick-formed bacteria at the end of the incubation.
An increase of one log unit in the numbers of *L. monocytogenes* was found during the first 12 h incubation in samples without added starter culture and no further increase was seen during the remaining incubation period (Figure 1). No inactivation of *L. monocytogenes* during the incubation was observed when the bac^- *P. acidilactici* CHCC3261 strain was added as starter culture or when pediocin powder was added to the Pepperoni meat. However, when the bac^+ *P. acidilactici* CHCC3261 strain alone or in combination with the bacteriocin producing *L. bavaricus* MI401 were used as starter cultures the number of *L. monocytogenes* dropped from $6x10^4$ to below the detection limit (10^1) within 12 h incubation (Figure 1). The observed inactivation of *L. monocytogenes* during incubation of samples added *P. acidilactici* CHCC3261 or the combination of *P. acidilactici* CHCC3261 and *L. bavaricus* MI401 as starter cultures cannot be ascribed to any pH effect during the incubation, as pH decreased and reached the same level as control samples with addition of bac^- *P. acidilactici* CHCC3261 as starter culture (Figure 2). Hence, the observed inactivation of *L. monocytogenes* in these samples

can be ascribe to *in situ*-produced bacteriocin. Table 1 gives the bacteriocin activity in samples inoculated with bacteriocin producing bacteria or pediocin powder at selected times during the incubation period. No bacteriocin could be detected in samples without added bacteriocin producing starter cultures. No difference in bacteriocin activity was measured in samples added *P. acidilactici* CHCC3261 or the combination of *P. acidilactici* CHCC3261 and *L. bavaricus* MI401 as starter cultures during the early stage of the fermentation. However, a high bacteriocin activity was found in samples inoculated 62 h with both bacteriocin producing strains which indicate enhanced bacterocin production at this stage of the fermentation. This phenomenon might be due to bacteriocin production from the *L. bavaricus* MI401 during the late stage of the fermentation, as the temperature at this point is close to the optimum temperature for bacteriocin production of *L. bavaricus* MI401 (~20°C), while this strain does not contribute to the bacteriocin activity found during the earlier stage the fermentation, where thefermentation temperature was far from the optimum temperature for bacteriocin production of *L. bavaricus*

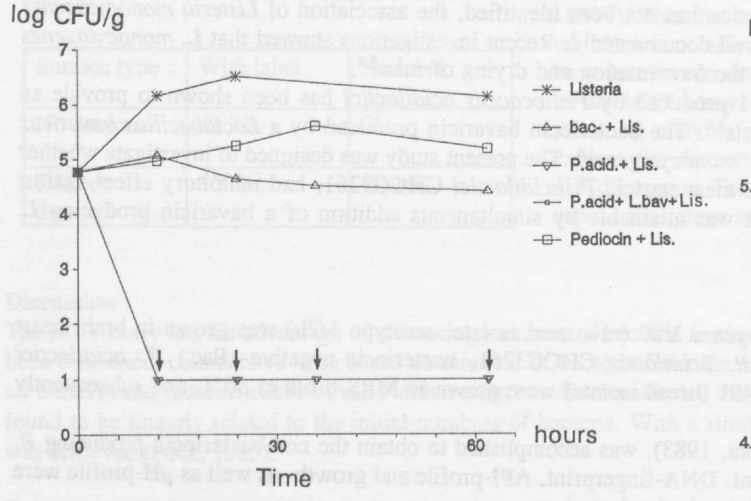

Figure 1. Development in *L. monocytogenes* during fermentation of Pepperoni inoculated with $6x10^4$ CFU/g sample and added starter culture(s) or pediocin powder. bac⁻: non-bacteriocin producing *P. acidilactici* ; Listeria ≡ Lis.: *L. monocytogenes* V80. ↓ indicate that less than 10 CFU/g was found in samples.

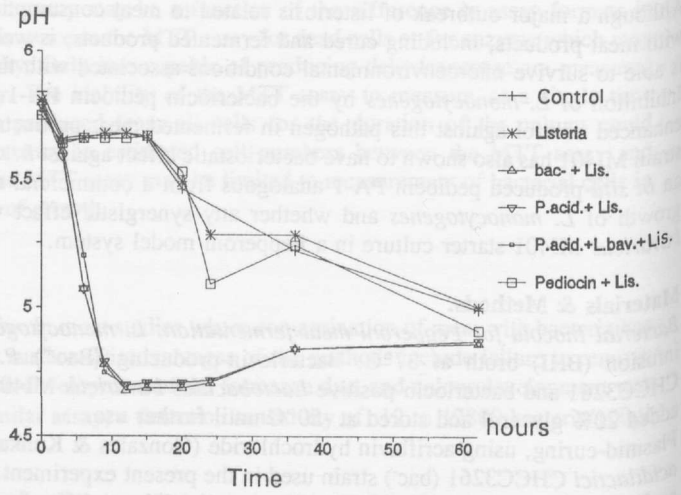

Figure 2. Progress in pH during fermentation of Pepperoni inoculated with $6x10^4$ CFU/g sample and added starter culture(s) or pediocin powder. Control: Without addition of starter culture or pediocin powder.

MI401. Due to the limited data this observation has to be taken with a pinch of salt as it also conflict with observations with regard to total lactic bacteria during the fermentation and direct microscopy of samples (see above). Bacteriocin activity was also observed in samples added pediocin powder directly. However, as no effect on *L. monocytogenes* was found, the powder might be non active under the given conditions or uneven distributed in samples as indicated by the fact that no activity was found in samples after 12 h incubation.

Time 24 h at 40°C and 38 h at 20°C	*P. acid.* + *L.mono.*	*P. acid.* + *L. bav.* + *L.mono.*	Pediocin + *L. mono.*
	Bacteriocin activity AU/g		
12	325	325	-
24	150	75	325
36	75	75	75
62	75	325	75

Table 1. Bacteriocin activity in samples added *P. acidilactici* CHCC3261; *P. acidilactici* CHCC3261 and *L. bavaricus* MI401, or pediocin powder during fermentation of Pepperoni.

Conclusion.

In situ-produced bacteriocin from *P. acidilactici* CHCC3261 was able to contribute to enhanced control of *L. monocytogenes* in the production of Pepperoni sausages. Simultanous addition of *P. acidilactici* CHCC3261 and the bacteriocin producing *L. bavaricus* MI401 did also inactivate *L. monocytogenes* in the present Pepperoni model system, however no synergistic effect was observed due to the experimental design.

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