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Effect of intestinal *Lactobacillus* starter cultures on the behaviour of *Staphylococcus aureus* in fermented sausage

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Abstract

The effects of *Lactobacillus* strains isolated from intestinal tracts for starter cultures of fermented sausage on the growth rate and enterotoxin production of *Staphylococcus aureus* were studied at two fermentation temperatures of 20°C and 35°C. Initial inoculated populations in the sausage batter were approx. 10^4 cfu/g for *S. aureus* and 10^7 cfu/g for the *Lactobacillus* strain as a starter culture. Samples of sausage were taken during fermentation and analyzed for pH and microbial populations. In control lots without inoculation of *Lactobacillus* strains, staphylococcal enterotoxin was detected during fermentation at each temperature. Of three intestinal *Lactobacillus* strains, *L. rhamnosus* FERM P-15120 and *L. paracasei* subsp. *paracasei* FERM P-15121 inhibited the growth and enterotoxin production of *S. aureus* in sausages during fermentation at both temperatures, although *L. acidophilus* FERM P-15119 could not satisfactorily suppress them. The effect of the two selected strains in meat fermentation (i.e., fermentation time, acid production, inhibition of *S. aureus*) was the same as that of a commercial *L. sake* starter culture for fermented sausage. These results suggest the intestinal *Lactobacillus* strains selected in this study could be utilized as a starter culture to produce new fermented meat products that are microbiologically safe. © 1998 Elsevier Science B.V.

Keywords: Fermented sausage; *Staphylococcus aureus*; Intestinal lactobacilli; Probiotics

1. Introduction

For their therapeutic activities associated with intestinal microbial balance, several *Lactobacillus* species originating from intestinal tracts (e.g., *L. acidophilus* group species) have been utilized as dairy starter cultures and probiotics (Gilliland, 1989; Hull et al., 1992; Arihara and Luchansky, 1994; Itoh, 1994; Charteris et al., 1997). However, these bacteria have not been applied to meat products, although several non-intestinal *Lactobacillus* strains have been utilized for fermented sausages (Hammes, 1990; Berdague et al., 1993; Hammes and Knauf, 1994).

Fermentation of meat has many advantages, and the most important reason for applying these bacteria

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in the production of fermented sausage is their ability to produce a consistent and controlled acidification that inhibits growth of undesirable microorganisms (Bacus, 1984a; Luecke and Hechelmann, 1987). Bacterial starter cultures used in the production of fermented sausage are lactic acid-producing and belong mainly to the genera *Lactobacillus*, *Pediococcus* and *Streptococcus* (Hammes, 1990; Hammes and Knauf, 1994). The major *Lactobacillus* species utilized for meat fermentation are *L. plantarum*, *L. curvatus* and *L. sake* (Hammes, 1990).

For the development of new healthy meat products, several typical probiotic lactic acid bacteria (i.e., *L. gasseri* and *Bifidobacterium* spp.) were applied for meat fermentation in the preceding study (Arihara et al., 1996, 1998). However, these bacteria were relatively sensitive to nitrite and sodium chloride. Since regulations for the maintenance of product safety in Japan require the use of 200 ppm nitrite and 3.3% sodium chloride and processing at a temperature below 20°C, we carried out further screening of probiotic *Lactobacillus* strains for meat fermentation. Of 202 strains of intestinal lactobacilli tested, three strains (*L. acidophilus* FERM P-15119, *L. rhamnosus* FERM P-15120 and *L. paracasei* subsp. *paracasei* FERM P-15121) having nitrite- and sodium chloride-tolerance in a liquid medium were selected (Arihara et al., 1997; Sameshima et al., 1998).

With respect to microbiological safety, *Staphylococcus aureus* has been responsible for food poisoning incidents in many types of food, including fermented sausages (Scheusner and Harmon, 1973; Smith et al., 1983). Given the proper conditions, *S. aureus* can multiply and produce toxin during the initial stage of fermentation. In the meat industry, lactic acid bacteria are widely used as starter cultures for suppressing the growth of *S. aureus* in the manufacturing of fermented meat products (Bacus, 1984a; Marcy et al., 1985). In addition, an industry task force sponsored by the American Meat Institute recommended that fermentation (i.e., pH reduction) be accomplished by the addition of lactic acid-forming bacteria to the formulation and that a pH of 5.3 or less be achieved within a time interval determined by the fermentation temperature (Bacus, 1984a). These recommendations are based on the assumption that *S. aureus* is effectively controlled in an environment of pH 5.3 or less. Therefore, the purpose of this study was to determine the effects of *Lactobacillus* strains originating in intestinal tracts on the growth rate and enterotoxin production of *S. aureus* in meat fermentation.

2. Materials and methods

2.1. Bacterial strains

Four strains of *S. aureus* were used to inoculate fermented sausage. Enterotoxin-producing *S. aureus* strains 101 (enterotoxin A producer), 104 (enterotoxin B producer), 105 (enterotoxin C producer) and 106 (enterotoxin D producer) were from our laboratory collection. These strains, previously tested in our laboratory for enterotoxin formation, were maintained as slant cultures on plate count agar (Difco Laboratories, Detroit, MI, USA). Each of the four strains of *S. aureus* was grown separately in 10 ml of brain heart infusion (Difco) broth at 37°C for 24 h. Cell suspensions of *S. aureus* strains were harvested by centrifugation at 4347 *g* for 20 min, washed twice, and resuspended in 10 ml of saline solution (0.85% NaCl).

Four *Lactobacillus* strains available for utilization as starter cultures were used. Three of them had been previously isolated from human intestinal tracts and identified in our laboratory: *L. acidophilus* FERM P-15119, *L. rhamnosus* FERM P-15120 and *L. paracasei* subsp. *paracasei* FERM P-15121. These strains have been deposited in the Patent Microorganism Depository, National Institute of Bioscience and Human-Technology, Tsukuba, Japan (Arihara et al., 1997). A commercial *L. sake* starter culture was obtained from Chr. Hansen's Laboratorium, Copenhagen, Denmark. Each strain was grown in Bacto APT broth dehydrated (Difco) for 18–20 h at 30°C, harvested by centrifugation at 6158 *g* for 20 min, washed twice and resuspended in 10 ml of saline solution.

2.2. Sausage processing

All of the raw meat obtained from a commercial manufacturer was maintained at 0–2°C during sausage preparation. Sausage batters consisted of lean pork and backfat (4:1) and the following ingredients

(g/kg): NaCl (33), $NaNO_2$ (0.2), sodium ascorbate (0.6), glucose (8), ground white pepper (3) and garlic powder (0.3). Lean pork and backfat were ground through a 3.0-mm plate. The ground meat was kept in a refrigerator at 0°C until use. The ingredients used in this work were weighed into individual bags prior to making the sausage. Cell suspension of *S. aureus* consisting of four enterotoxin-producing strains, and *Lactobacillus* strains as starter culture were added, together with the ingredients, to achieve the proper inoculum level in the sausage batters. All lots of sausage batter were inoculated with each strain of *S. aureus* at approx. (2.5×10^3) cfu/g. The starter cultures were added to achieve approx. 10^7 cfu/g in the sausage batter except for the control lot, which was not inoculated with starter culture (Table 1). All lots were blended in a mixer (TSMV 5L; Wolfking Danmark, Slagelse, Denmark). After being vacuum-mixed for 3 min at 1–3°C, the sausage batters were stuffed into 38-mm diameter fibrous casings (Shikoku Tohcello, Tokushima, Japan) to make individual pieces of sausage weighing approx. 200 g. The sausage pieces were hung in a controlled-temperature, controlled-humidity chamber (PL-4SP; Tabai Espec, Osaka, Japan). All fermentations were carried out at 90–95% RH and at either a low temperature (20°C) or a high temperature (35°C). These fermentation temperatures were selected in consideration of the different fermentation temperatures used for sausage in different countries (e.g., less than 23.9°C for European-style sausages, 27.7–37.7°C for American-style sausages and less than 20°C for Japanese-style sausages) (Bacus, 1984a; Hammes and Knauf, 1994).

2.3. Sampling procedures

Samples for microbiological analysis, staphylococcal enterotoxin assay and pH determination were taken from sausages after stuffing (0 h); from sausages after 12, 18, 24, 36, 48 and 72 h of fermentation at 20°C; and from sausages after 6, 9, 12, 15, 18, 21 and 24 h of fermentation at 35°C.

2.4. Microbiological analysis

Twenty five-gram portions of sausage were homogenized with 225 ml of 0.1% peptone water, and serial 10-fold dilutions were used for microbiological analysis. Viable counts of *S. aureus* were determined on Mannitol salt agar (Nissui, Tokyo, Japan). The lactic acid bacteria was enumerated using plate count agar with bromcresol purple (Nissui) pour plates. Plates were incubated at 37°C for 36 h to recover *S. aureus* and at 30°C for 72 h to recover lactic acid bacteria. The results reported here are the mean counts of two parallel samples.

2.5. Enterotoxin assay

Enterotoxin was assayed by the reversed passive latex agglutination method. Ten-gram portions of samples were homogenized with 90 ml of saline solution, followed by centrifugation at 1429 *g* for 20 min. Detection of enterotoxin was carried out using a staphylococcal enterotoxin-RPLA kit (Denka Biochemical, Tokyo, Japan). Twenty-five milliliters of supernatant and sensitized latex beads solutions of the assay kit were mixed on the microplate, followed

Table 1
Inoculum combinations of *S. aureus* and starter culture for treatment of fermented sausage

Strains	Treatment of lots				
	Control	*L. acidophilus*	*L. rhamnosus*	*L. paracasei* subsp. *paracasei*	*L. sake*
S. aureus	+ [a]	+	+	+	+
L. acidophilus	− [b]	+	−	−	−
L. rhamnosus	−	−	+	−	−
L. paracasei subsp. *paracasei*	−	−	−	+	−
L. sake	−	−	−	−	+

[a] Inoculated.
[b] Uninoculated.

by incubation at room temperature for 20 h. The presence of enterotoxin was determined by comparing the precipitating figures of standard enterotoxins. According to the manufacturer's instructions, the detection limit of the assay is 2 ng/ml. The results reported here are shown as numbers of toxic samples in three parallel samples.

2.6. Chemical analysis

The pH was determined on 1:1 (w/v) double-distilled water extracts using a pH meter with a combination electrode (Model PH 81; Yokogawa Electric, Tokyo, Japan). The results reported here were the mean values of two parallel samples.

3. Results

3.1. Low-temperature fermentation

Changes in pH during fermentation of sausages in the absence or presence of a starter culture are shown in Fig. 1A. The initial pH of all of sausages tested was approx. 5.8. At a fermentation temperature of 20°C, the fastest pH drop was observed in the *L. rhamnosus*, *L. paracasei* subsp. *paracasei* and *L. sake* lots, while the *L. acidophilus* and control lots showed a slower drop. After 24 h of fermentation, the pH values of the *L. rhamnosus*, *L. paracasei* subsp. *paracasei* and *L. sake* lots reached 5.0–5.2, whereas the *L. acidophilus* and control lots maintained higher values.

The viable cell counts of lactic acid bacteria are presented in Fig. 1B. Before fermentation, the number of endogenous lactic acid bacteria in the control lot without inoculation of a starter culture was 4.3 $\log_{10}$ cfu/g. During fermentation, the counts of lactic acid bacteria reached approx. 7.0 $\log_{10}$ cfu/g. Lactic acid bacteria grew at a faster rate in the *L. rhamnosus*, *L. paracasei* subsp. *paracasei* and *L. sake* lots than in the *L. acidophilus* and control lots. After 24 h of fermentation, the counts of lactic acid bacteria in *L. rhamnosus*, *L. paracasei* subsp. *paracasei* and *L. sake* lots reached approx. 8.0 $\log_{10}$ cfu/g, whereas those in *L. acidophilus* lot were 7.4 $\log_{10}$ cfu/g. The higher growth rate was related to the faster drop in pH noted in Fig. 1A. The final counts of lactic acid bacteria in sausages inoculated with *L. rhamnosus*, *L. paracasei* subsp. *paracasei* and *L. sake* were only slightly higher than those observed for the *L. acidophilus* and control fermentations.

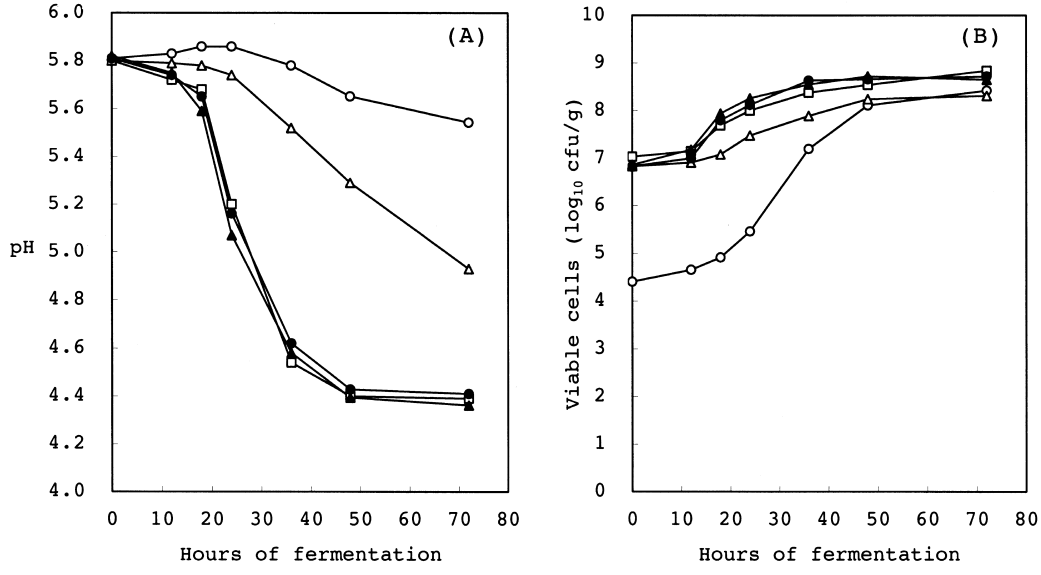

Fig. 1. Changes in pH (A) and levels of lactic acid bacteria (B) in sausages inoculated with *S. aureus* in the absence of a starter culture (○); sausages inoculated with *S. aureus* in the presence of *L. acidophilus* (△), *L. rhamnosus* (□), *L. paracasei* subsp. *paracasei* (●) and *L. sake* (▲) during fermentation at 20°C.

Table 2
Effects of starter culture on the growth rate and enterotoxin production of *S. aureus* in sausages during fermentation at 20°C

Hours of fermentation	Control	*L. acidophilus*	*L. rhamnosus*	*L. paracasei* subsp. *paracasei*	*L. sake*
0	4.2[a](0/3)[b]	4.1(0/3)	4.1(0/3)	4.2(0/3)	4.1(0/3)
12	4.2(0/3)	4.2(0/3)	4.3(0/3)	4.3(0/3)	4.3(0/3)
18	4.3(0/3)	4.3(0/3)	4.2(0/3)	4.3(0/3)	4.2(0/3)
24	4.4(0/3)	4.3(0/3)	4.0(0/3)	4.1(0/3)	4.0(0/3)
36	4.9(0/3)	4.8(0/3)	3.9(0/3)	4.0(0/3)	3.9(0/3)
48	6.1(0/3)	5.5(0/3)	3.9(0/3)	3.9(0/3)	3.7(0/3)
72	7.1(2/3)	6.4(0/3)	3.8(0/3)	3.8(0/3)	3.6(0/3)

[a] Values indicate viable cells ($\log_{10}$ cfu/g) of *S. aureus*.
[b] Number of toxic samples/number of samples assayed.

The effects of a starter culture on the growth rate and enterotoxin production of *S. aureus* in sausages during fermentation at 20°C are shown in Table 2. After inoculation, the viable cell counts of *S. aureus* were 4.1–4.2 $\log_{10}$ cfu/g. The viable cell counts of *S. aureus* in sausages inoculated with *L. acidophilus* and in control sausages increased during fermentation. On the other hand, the viable cell counts of *S. aureus* in sausages inoculated with *L. rhamnosus*, *L. paracasei* subsp. *paracasei* and *L. sake* decreased to below the initial inoculum levels after fermentation. Toxicity was found in samples of control sausages after 72 h of fermentation. During fermentation, no toxicity was detected in sausages inoculated with a starter culture.

3.2. High-temperature fermentation

Changes in pH and the viable cell counts of lactic acid bacteria in sausages during fermentation at 35°C are shown in Fig. 2. The greatest observed difference between the two different fermentation temperatures (20°C and 35°C) was in the fermentation rate. The pH values of the *L. rhamnosus*, *L. paracasei* subsp. *paracasei* and *L. sake* lots dropped to 5.0 at 12 h of fermentation at 35°C. Also, the viable cell counts of

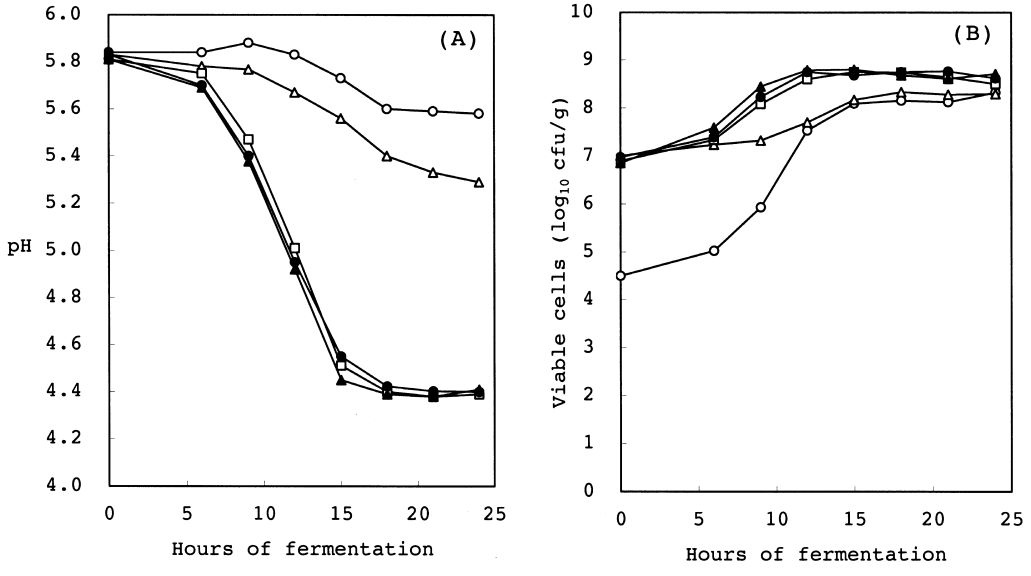

Fig. 2. Changes in pH (A) and levels of lactic acid bacteria (B) in sausages inoculated with *S. aureus* in the absence of a starter culture (○); sausages inoculated with *S. aureus* in the presence of *L. acidophilus* (△), *L. rhamnosus* (□), *L. paracasei* subsp. *paracasei* (●) and *L. sake* (▲) during fermentation at 35°C.

Table 3
Effects of starter culture on the growth rate and enterotoxin production of *S. aureus* in sausages during fermentation at 35°C

Hours of fermentation	Control	*L. acidophilus*	*L. rhamnosus*	*L. paracasei* subsp. *paracasei*	*L. sake*
0	4.2[a](0/3)[b]	4.2(0/3)	3.9(0/3)	4.0(0/3)	4.0(0/3)
6	4.3(0/3)	4.6(0/3)	4.5(0/3)	4.4(0/3)	4.5(0/3)
9	5.1(0/3)	5.0(0/3)	4.9(0/3)	4.8(0/3)	4.7(0/3)
12	6.9(0/3)	6.2(0/3)	5.0(0/3)	4.9(0/3)	4.7(0/3)
15	7.8(3/3)	6.9(0/3)	5.0(0/3)	4.8(0/3)	4.8(0/3)
18	8.6(3/3)	7.9(3/3)	4.9(0/3)	4.8(0/3)	4.7(0/3)
21	8.9(3/3)	8.6(3/3)	4.8(0/3)	4.6(0/3)	4.5(0/3)
24	8.8(3/3)	8.7(3/3)	4.7(0/3)	4.5(0/3)	4.3(0/3)

[a] Values indicate viable cells ($\log_{10}$ cfu/g) of *S. aureus*.
[b] Number of toxic samples/number of samples assayed.

lactic acid bacteria in these lots exceeded 8.0 $\log_{10}$ cfu/g after 9 h of fermentation. On the other hand, the *L. acidophilus* and control lots showed a slower pH drop and maintained a pH value of over 5.2 at the end of fermentation.

Table 3 shows the behaviour of *S. aureus* in sausages during fermentation at 35°C. The viable cell counts of *S. aureus* in sausages inoculated with *L. acidophilus* and in control sausages increased rapidly during fermentation, while the viable cell counts of *S. aureus* in sausages inoculated with *L. rhamnosus*, *L. paracasei* subsp. *paracasei* or *L. sake* increased only slightly until 15 h of fermentation and then decreased to 4.3–4.7 $\log_{10}$ cfu/g. Also, these strains inhibited enterotoxin production, whereas toxicity was found in control and *L. acidophilus* lots after 15 h and 18 h of fermentation, respectively.

4. Discussion

In the meat industry, lactic acid bacteria are widely used as starter cultures for preventing undesirable microorganisms or for flavor development in the manufacture of fermented meat products. In our previous study, a total of 202 intestinal *Lactobacillus* strains were screened for nitrite- and sodium chloride-tolerance in order to find suitable probiotic lactic acid bacteria that can be utilized in the manufacture of fermented meat products (Arihara et al., 1997; Sameshima et al., 1998). Of three strains screened in our laboratory, *L. rhamnosus* and *L. paracasei* subsp. *paracasei* as well as *L. sake*, the commercial starter culture, inhibited the growth of *S. aureus* and enterotoxin production during fermentation of sausages at different experimental temperatures. These were based on temperatures used in the manufacture of different sausage types and on processing regulations relating to fermented sausage in the US, Europe and Japan. The behaviour of *L. rhamnosus* and *L. paracasei* subsp. *paracasei* during fermentation was similar to the reported behaviour of preventing the growth and enterotoxin production of *S. aureus* in fermented meat products (Marcy et al., 1985; Schillinger and Luecke, 1991). Moreover, in our previous study, we found that *L. rhamnosus* and *L. paracasei* subsp. *paracasei* inhibited the growth of pathogenic strains (*Listeria monocytogenes*, *Salmonella* Enteritidis, *Eschericia coli*, *Bacillus cereus*, *Clostridium perfringens* and *Yersinia enterocolitica*) on agar plates containing 2% glucose in an agar drop test (Fleming et al., 1975), although they did not inhibit most of pathogenic strains on agar plates containing 0.2% glucose (Sameshima et al., 1998). The carbohydrate content in the sausage formula is an important factor in determining antimicrobial activity of the lactic acid starter cultures in meat fermentations (Acton et al., 1977; Bacus, 1984b). Acton et al. (1977) recommended the use of at least 0.75% glucose in sausage batters for proper culture growth. Therefore, 0.8% glucose was added to sausage batters in this study.

From the results of this study, we conclude that selected probiotic strains are applicable to meat fermentation to maintain product safety. *L. rhamnosus* FERM P-15120 and *L. paracasei* subsp. *paracasei* FERM P-15121 satisfactorily inhibited the growth of *S. aureus* during fermentation. However, since *L. acidophilus* FERM P-15119 was not suitable for controlling microbial safety, it is most important

to select appropriate starter species/strains of probiotic lactobacilli.

The two strains selected in this study also have been shown to be resistant to gastric acid and bile encountered during passage through the gastrointestinal tract and to give products with satisfactory sensory properties (Arihara et al., 1997; Sameshima et al., 1998). Since intestinal lactobacilli have been reported to show beneficial health properties such as antimicrobial activity, anticancer properties and cholesterol assimilation (Gilliland et al., 1985; Goldin and Gorbach, 1992; Buck and Gilliland, 1994; Itoh, 1994), it is expected that these bacteria can be used for the manufacture of new healthy meat products.

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