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The Screening of Hydrogen Peroxide-Producing Lactic Acid Bacteria and Their Application to Inactivating Psychrotrophic Food-Borne Pathogens

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Abstract. Lactic acid bacteria were isolated from various food samples and evaluated for hydrogen peroxide (H_2O_2) production. Cells suspended in 0.5% (wt/vol) glucose plus 0.5% (wt/vol) lactate (pH 7.0) were incubated for 5 h at 37°C under aeration. Among 193 strains, 27 strains accumulated 201–300 ppm H_2O_2, and 4 strains accumulated more than 301 ppm H_2O_2 in the cell suspensions. Among the 9 high-level H_2O_2-producing strains, 8 strains were identified as *Lactococcus lactis* subsp. *lactis*. The cell-free filtrate from *Lc. lactis* subsp. *lactis* AI 62, which contained approximately 350 ppm H_2O_2, was evaluated for antimicrobial activity against *Enterococcus faecalis*, *Ent. faecium*, enterotoxigenic *Escherichia coli*, *Listeria ivanovii*, *Staphylococcus aureus*, *Yersinia enterocolitica*, and *Aeromonas hydrophila*. After 1 h incubation at 30°C in the cell-free filtrate, the initial viable cell counts of the target bacteria (5.53–6.00 log cfu/mL) were reduced by 0.12–5.00 log units, except in the case of enterococci. The sensitivity varied with the bacterial species and pH. The enterococci were resistant to the treatment. Our results show that H_2O_2 accumulated by lactic acid bacteria in a cell suspension is very effective in reducing the viable cell count of food-borne pathogens.

In recent years, demand has increased among consumers for foods that are high quality, less processed (less intensive heating and minimal freezing damage), less heavily preserved, more natural (free of artificial additives), and safer [14]. This demand has resulted in the emergence of a new generation of chill-stored, minimally processed foods. In such foods, refrigeration is the sole method of preservation. However, psychrotrophic species and strains among the following genera can cause food spoilage at cold temperatures: *Alcaligenes*, *Shewanella*, *Brochothrix*, *Enterococcus*, *Lactobacillus*, *Pseudomonas*, and others [5, 7, 15, 26]. Some foodborne pathogens, such as *Aeromonas hydrophila*, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Clostridium botulinum* type E, can grow below 5°C [24, 26]. Therefore, it is very important to reduce the initial microbial population in order to extend the shelf-life of the food and to prevent foodborne illness. Disinfecting the food with chlorine has been widely used during the preparation of minimally processed vegetables and fruits in order to achieve these objectives [21].

Many reports have demonstrated the production of H_2O_2 by lactic acid bacteria [2, 4, 17, 19, 23, 27, 32, 33]. Some lactobacilli have been reported to inhibit the growth of food spoilage bacteria and pathogens in associative cultures [8, 9, 11, 12, 13, 25, 29]. To date, it has not been shown that the H_2O_2 produced by lactic acid bacteria has a bactericidal effect against spoilage bacteria and pathogens.

This paper describes the development of a method for disinfecting by using an H_2O_2 wash, accumulated by lactic acid bacteria in the cell suspensions, rather than a chlorine wash. We selected strains of lactic acid bacteria that produce high levels of H_2O_2 in cell suspensions and showed that their cell-free filtrates containing H_2O_2 were

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effective in reducing the viable cell counts of psychrotrophic pathogens.

Materials and Methods

Pathogens and culture conditions. Enterotoxigenic *Escherichia coli* (ETEC) ATCC 31705 was obtained from the American Type Culture Collection (Rockville, MD, USA), and *Y. enterocolitica* IID 981 was obtained from the Institute of Medical Science, the University of Tokyo (Tokyo, Japan). *Enterococcus faecalis* JCM 5803, *Ent. faecium* JCM 5804, *Staphylococcus aureus* subsp. *aureus* JCM 2413, *Aer. hydrophila* JCM 1027, and *Aer. hydrophila* JCM 3976 were obtained from the Japan Collection of Microorganisms (Wako, Japan). *Listeria ivanovii* DB1, *L. ivanovii* DB2, and *Y. enterocolitica* YE98013 were obtained from the collection of the Department of Medical Technology, School of Medicine, Hirosaki University. The bacteria were cultivated on a brain–heart infusion agar (BHI agar; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) slant. The *Yersinia* and *Aeromonas* strains were incubated at 30°C, and the others were incubated at 37°C. For the inhibition assay, the bacteria were grown aerobically overnight on a BHI agar plate and were harvested in phosphate-buffered saline (PBS; pH 7.1).

Isolation and identification of lactic acid bacteria. Glucose–yeast extract–peptone (GYP) Chalk agar, and dipyridyl–cycloheximide–sodium azide (DCS)–MRS agar were used for the isolation of the lactic acid bacteria. The GYP Chalk agar contained (per liter) 2.5 g phytone peptone (BBL, Becton Dickinson Microbiology Systems, Rockville, MD, USA), 2.5 g polypeptone peptone (BBL), 10.0 g yeast extract (Oxoid, Basingstoke, UK), 2.0 g lab-lemco powder (Oxoid), 10.0 g glucose, 200 mg $MgSO_4 \cdot 7H_2O$, 10 mg $MnSO_4 \cdot 5H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 10 mg NaCl, 5.0 g calcium carbonate, and 15.0 g agar (pH 6.8). The DCS-MRS agar was MRS agar (Merck, Darmstadt, Germany) supplemented (per liter) with 0.05 g 2,2′-dipyridyl, 0.01 g cycloheximide, and 0.01 g sodium azide.

The organisms were isolated from the following samples: adzuki beans, soybeans, wheat flour, naturally fermented rice, sake koji, green soybeans, tomatoes, corn, cucumbers fermented in brine solution, pumpkins, potatoes, broccoli, Welsh onions, Japanese radishes and their leaves, fermented tea leaves, carrots, Japanese red turnips soaked in vinegar, Japanese eggplants fermented in brine solution, pickled shallots, papaya, watermelons, musk melons, persimmons, avocados, apples, pears, apples fermented in brine solution, pork, chicken meat, sika deer meat, and fermented sausage. The samples were mechanically homogenized in PBS. Appropriate dilutions of the sample homogenates were applied onto GYP Chalk agar or DCS-MRS agar, and the plates were incubated at 30°C for 2–3 days under anaerobic conditions (AnaeroPack®; Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan). The colonies surrounded with a clear zone on the GYP Chalk agar and colonies on the DCS-MRS agar were picked and purified by subculturing on the same media. The isolates were maintained in MRS broth. All 193 isolates were tentatively identified as lactic acid bacteria on the basis of their Gram-positive, non-motile, and catalase-negative characteristics. Identification of the species was performed by using the API manual kit API 50 CHL and API identification program API 50 CHL Ver. 5.0 (bioMérieux, Marcy-l'Etoile, France), according to the manufacturer's instructions. The prefix AI or YS was assigned to the individual strain numbers of the isolates.

Viable cell counts of lactic acid bacteria were determined by plating serial dilutions on MRS agar, and by counting the cfu after incubation at 30°C for 48 h under the anaerobic conditions.

Screening of the hydrogen peroxide producers. The lactic acid bacteria were cultured for 2 days at 30°C in 50 mL of static MRS broth (Merck). The bacterial cells were harvested by centrifugation (6000 rpm for 15 min), and washed three times with PBS. The cells were resuspended in 5 mL of PBS, 0.5% (wt/vol) glucose, or a mixture of 0.5% (wt/vol) glucose and 0.5% (wt/vol) lactic acid (adjusted to pH 7.0 with 0.1 mol/L NaOH). This suspension was incubated for up to 5 h at 37°C in a reciprocal shaker (170 rpm). A sample of this suspension was drawn periodically. After centrifugation (15,000 rpm for 3 min), the amount of H_2O_2 in the supernatant was determined by the method reported by Ito et al. [16]. In a separate experiment, disrupted cells were used for the H_2O_2 production. The bacterial cells were cultured in MRS broth, harvested, and washed with PBS as above. The cells were resuspended in 2.5 mL of PBS and then disrupted by ultrasonication (Tomy UD-200, Tokyo, Japan) for 5 min on ice. The disrupted cell suspension was mixed with an equal volume of PBS, 1.0% (wt/vol) glucose, or a mixture of 1.0% (wt/vol) glucose and 1.0% (wt/vol) lactate (pH 7.0). This mixture was incubated for up to 2 h in a reciprocal shaker (170 rpm). The supernatant was obtained and subjected to the determination of H_2O_2, as above. The results were confirmed in duplicate experiments carried out on different days.

Inhibition assay. *Lc. lactis* subsp. *lactis* AI 62 cells were harvested by centrifugation (6000 rpm for 15 min) from 500 ml of static MRS broth culture, washed with PBS, suspended in 25 ml of PBS, mixed with an equal volume of a mixture of 1.0% (wt/vol) glucose and 1.0% (wt/vol) lactate (pH 7.0), and incubated for 5 h at 37°C in a reciprocal shaker (170 rpm) to produce H_2O_2. The cells were removed by centrifugation (7000 rpm for 10 min), and the resulting supernatant fluid was passed through a filter with a pore size of 0.45 μm. The pH of the cell-free filtrate was adjusted to 4.0, 4.5, 5.0, and 5.5, respectively, and portions of the cell-free filtrate were used for the determination of the H_2O_2 concentration. The other portions of the cell-free filtrate with a specified pH were treated with 5 μg/mL catalase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to decompose the H_2O_2.

One milliliter of the cell-free filtrate with and without treatment with catalase was mixed with 50 μL of the desired pathogen (2.1×10^7 bacteria/mL as a total cell count), and the mixture was incubated for up to 60 min at 30°C. At specified intervals, the samples were withdrawn to evaluate cell viability. A mixture of 1 mL of PBS and 50 μL of each pathogen without incubation served as a control. The viable cell counts of the pathogens were determined as colony-forming units (cfu/mL) by plating a serial dilution of the reaction mixture on the BHI agar. In the case of the mixture without catalase pretreatment, the samples were added by catalase (5 μg/mL) before plating. The plates were incubated aerobically for 24–48 h at 37 or 30°C. The results were confirmed in duplicate experiments performed on separate days.

Results

Conditions for hydrogen peroxide production and the selection of an active hydrogen peroxide producer. The effects of glucose alone and of glucose plus lactate (pH 7.0) on H_2O_2 production were tested by using three isolates of lactic acid bacteria ($4.0–5.0 \times 10^9$ cfu/mL) under continuous shaking. As shown in Fig. 1, a maximum amount of H_2O_2 was obtained in the presence of both glucose and lactate. Therefore, cells of lactic acid bacteria were incubated in a mixture of 0.5% (wt/vol) glucose and 0.5% (wt/vol) lactate in subsequent experiments. The amount of H_2O_2 formed by the ultrasonicated cells was compared with that formed by the intact cells. While similar amounts of H_2O_2 were produced by the

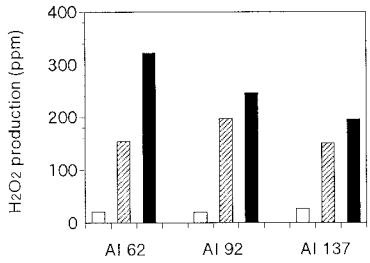

Fig. 1. The effect of glucose alone and that of glucose plus lactic acid on the production of hydrogen peroxide by lactic acid bacteria strain AI 62 (a), AI 92 (b), and AI 137 (c). Bacterial cells were suspended in 1/10 of the culture volume of PBS (□), 0.5% (wt/vol) glucose (▨), or a mixture of 0.5% (wt/vol) glucose and 0.5% (wt/vol) lactate (pH 7.0) (■). The hydrogen peroxide in the suspension was determined after 5 h incubation at 37°C in a reciprocal incubator. The values are the means of duplicate experiments. The results were confirmed in experiments performed on separate days.

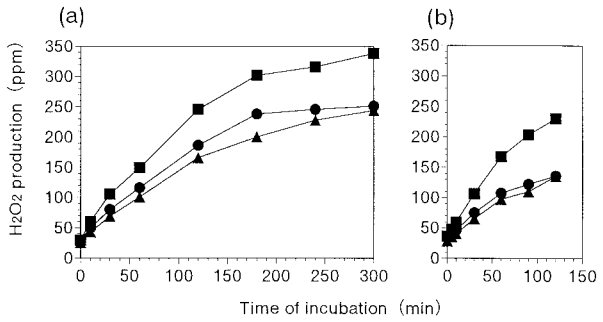

Fig. 2. Production of hydrogen peroxide by intact (a) and ultrasonicated (b) cells of AI 62 (■), AI 92 (●), and AI 137 (▲). The lactic acid bacteria harvested from 2-day static cultures in MRS broth were suspended in a mixture of 0.5% glucose and 0.5% lactate (pH 7.0). The suspensions were incubated for 5 h at 37°C in a reciprocal incubator. The values were the means of duplicate experiments. The results were confirmed in experiments performed on separate days.

ultrasonicated cells and intact cells of AI 62, the intact cells produced more H_2O_2 than did the ultrasonicated cells in the AI 92 and AI 137 (Fig. 2). Therefore, intact cells were used in all of the experiments described below.

Next, 193 isolates of lactic acid bacteria were evaluated in terms of their capacity to produce H_2O_2. As shown in Fig. 3, H_2O_2 production varied among strains; less than 100 ppm (139 strains), 101–200 ppm (23 strains), 201–300 ppm (27 strains), and more than 301 ppm (4 strains). Nine strains that produced higher concentrations of H_2O_2 were identified by the API 50CH kit. Strains AI 52 (coccus isolated from cucumbers fermented in brine solution, with H_2O_2 levels of 241 ppm), AI 62 (coccus from corn, 372 ppm), AI 68 (coccus from pumpkins, 278 ppm), AI 73 (coccus from pork, 268 ppm), AI 91 (coccus from chicken meat, 276 ppm), AI 92 (coccus from the leaves of Japanese radishes, 333

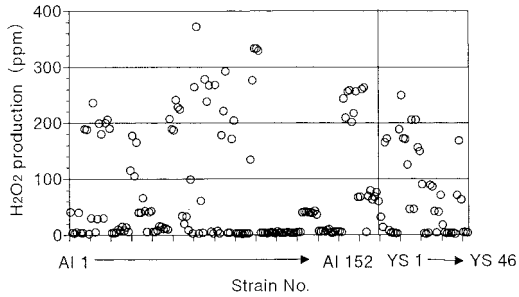

Fig. 3. Production of hydrogen peroxide by 193 isolates of lactic acid bacteria. One hundred fifty strains of AI 1-AI 152 and 43 strains of YS 1-YS 46 were tested. The lactic acid bacteria harvested from 2-day static cultures in MRS broth were suspended in a mixture of 0.5% (wt/vol) glucose and 0.5% (wt/vol) lactate (pH 7.0). The hydrogen peroxide in the suspension was determined after 5 h incubation at 37°C in a reciprocal incubator. The values are the means of duplicate experiments. The results were confirmed in experiments performed on separate days.

ppm), AI 137 (coccus from Japanese radishes, 256 ppm), and AI 145 (coccus from pork, 263 ppm) were identified as *Lc. lactis* subsp. *lactis*. Strain AI 78 (rod from pork, 292 ppm) was identified as *Lactobacillus plantarum*.

Bactericidal effect of hydrogen peroxide produced by *Lactococcus lactis* subsp. *lactis* AI 62 against the psychrotrophic pathogens. To determine the effect of the incubation time and pH on the bactericidal effect, we incubated two *Y. enterocolitica* strains for up to 60 min in an AI 62 cell-free filtrate containing approximately 300 ppm H_2O_2 adjusted to pH 4.5 and 4.0 (Fig. 4). Catalase was added to determine whether the observed bactericidal effect was caused by the pH reduction. The viable cell counts were reduced from approximately 1×10^6 cfu/mL to a less than detectable level after 60 min incubation, except in one case. In both *Y. enterocolitica* strains, the bactericidal effect was enhanced by a longer incubation time and lower pH. The addition of catalase suppressed the bactericidal effect of the AI 62 supernatant at pH 4.5, indicating that H_2O_2 alone was responsible for the bactericidal effect against *Y. enterocolitica*. At pH 4.0, bactericidal activities were observed even after the catalase treatment, indicating a combined effect of low pH and H_2O_2.

In the next experiment, the bactericidal activities of an AI 62 cell-free filtrate containing approximately 350 ppm H_2O_2 against the *Enterococcus*, *Staphylococcus*, *Escherichia*, *Listeria*, *Yersinia*, and *Aeromonas* strains were evaluated by using an incubation time of 60 min at 30°C (Fig. 5). Weak and no bactericidal effects were observed against *Staphylococcus* and *Enterococcus*, respectively (Figs. 5a–c). In the other strains, the bactericidal effect was strongly enhanced at a lower pH (Figs.

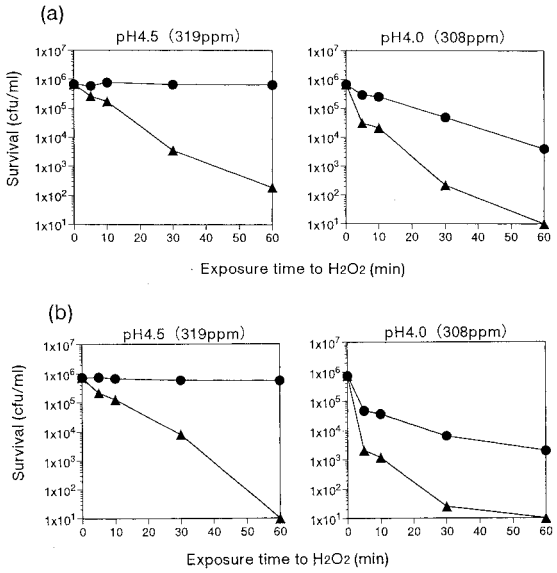

Fig. 4. Effect of the cell-free filtrate of *Lactococcus lactis* subsp. *lactis* containing hydrogen peroxide on the survival of *Yersinia enterocolitica. Y. enterocolitica* IID 981 (a) and YE98013 (b) were suspended in the cell-free filtrate of *Lc. lactis* subsp. *lactis* AI 62, which contained hydrogen peroxide (concentrations are indicated as ppm in parentheses), and were kept for 60 min at 30°C (▲). Survival was determined at specified intervals. A cell-free filtrate of *Lc. lactis* subsp. *lactis* treated with catalase before mixing with *Yersinia* cells was used to determine the effect of the pH (●). The values are the means of duplicate experiments. The results were confirmed in experiments performed on separate days.

5d–h), except in the case of *Aeromonas*, which was very sensitive even in a neutral pH (Figs. 5i and j). With the exception of the strains of *Listeria* and *Yersinia* at pH 4.0, the addition of catalase suppressed the bactericidal effect of the cell-free filtrate. These observations strongly indicated that H_2O_2 was the principal agent of the bactericidal effect in the cell-free filtrate.

Discussion

Several authors have examined the effect of components and aeration on H_2O_2 production by *Lactobacillus* strains. Dahiya and Speck [9] and Villegas and Gilliland [30] indicated that the presence of a carbohydrate source was necessary for the formation of H_2O_2 by lactobacilli. It has been shown that H_2O_2 is also produced from lactate [30]. Collins and Aramaki [8] showed that two of four *L. acidophilus* strains produced a large amount of H_2O_2 (40–55 ppm) with continuous shaking, but only a small amount (5 ppm) without shaking. Marty-Teysset et al. [20] also showed that the concentration of H_2O_2 in aerated cultures was two to three times higher than that in unaerated cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus*. Therefore, we examined H_2O_2 formation by cell suspensions in glucose alone or in glucose plus lactate under aeration.

Villegas and Gilliland [30] reported that the cells of *L. delbrueckii* subsp. *lactis* I produced H_2O_2 at a concentration of 25 μg per 10^9 cfu in buffer (pH 6.5) containing 55.5 mmol/L glucose with constant agitation for 18 h at 5°C. They also showed that this strain produced about 33 and 14 μg H_2O_2 per 10^9 cfu in buffer (pH 6.5) containing 55.5 mmol/L glucose and sodium lactate, respectively, in static incubation at 5°C for about 40 h [30]. However, they made no attempt to determine the H_2O_2 production in the buffer which contained both glucose and sodium lactate. We showed that the *Lc. lactis* subsp. *lactis* AI 62 cells accumulated 300–380 ppm H_2O_2, which corresponds to 60–95 μg per 10^9 cfu, when incubated in 0.5% glucose and 0.5% lactate (pH 7.0) under constant agitation for 5 h at 37°C. Only a few strains have been examined for H_2O_2 production in the lactococci [2], and there are no available data on the amount of H_2O_2 produced by lactococci so far. Our results showed that *Lc. lactis* subsp. *lactis* strains can accumulate H_2O_2 at higher amounts than lactobacilli.

It has been reported that the H_2O_2 produced by *Lactobacillus* strains retarded the growth of *Pseudomonas* strains [8, 25] and inhibited the growth of psychrotrophs [11], *L. monocytogenes* [29], and *S. aureus* [9]. Brashears et al. [6] reported that Trypticase Soy Broth and chicken meat inoculated with the *E. coli* O157:H7 strain and H_2O_2-producing *Lactobacillus lactis* cells exhibited declines in the number of *E. coli* O157:H7 during refrigerated storage. However, in these papers, the authors did not measure the H_2O_2 concentrations formed by the *Lactobacillus* in either the associative cultures or the spent medium. Therefore, it is unclear about the H_2O_2 concentrations that caused the inhibition of the target bacterium.

However, an abundance of data is available on the antimicrobial activity of added H_2O_2. It has been shown that treatment with 0.1% hydrogen peroxide at 54°C for 30 min reduced the total bacterial count in raw milk by 99.999%, and coliform, staphylococcal, salmonellae, and clostridial counts by 100% [22]. The bactericidal and bacteriostatic effects of lower concentrations of H_2O_2 have also been studied. Dahiya and Speck [9] reported that H_2O_2 was bacteriostatic at 6 ppm and bactericidal at 20–35 ppm against *S. aureus* strains at 35°C. Price and Lee [25] reported that H_2O_2 was bacteriostatic at 2–8 ppm and bactericidal at 25–40 ppm against *Pseudomonas* sp. at 30°C. Watson and Schubert [31] reported that 1–60 ppm was bacteriostatic only against *Salmonella typhimurium* at 37°C. Baldry [3] reported that 5–50 ppm H_2O_2 was bacteriostatic for *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus faecalis*, and *S.*

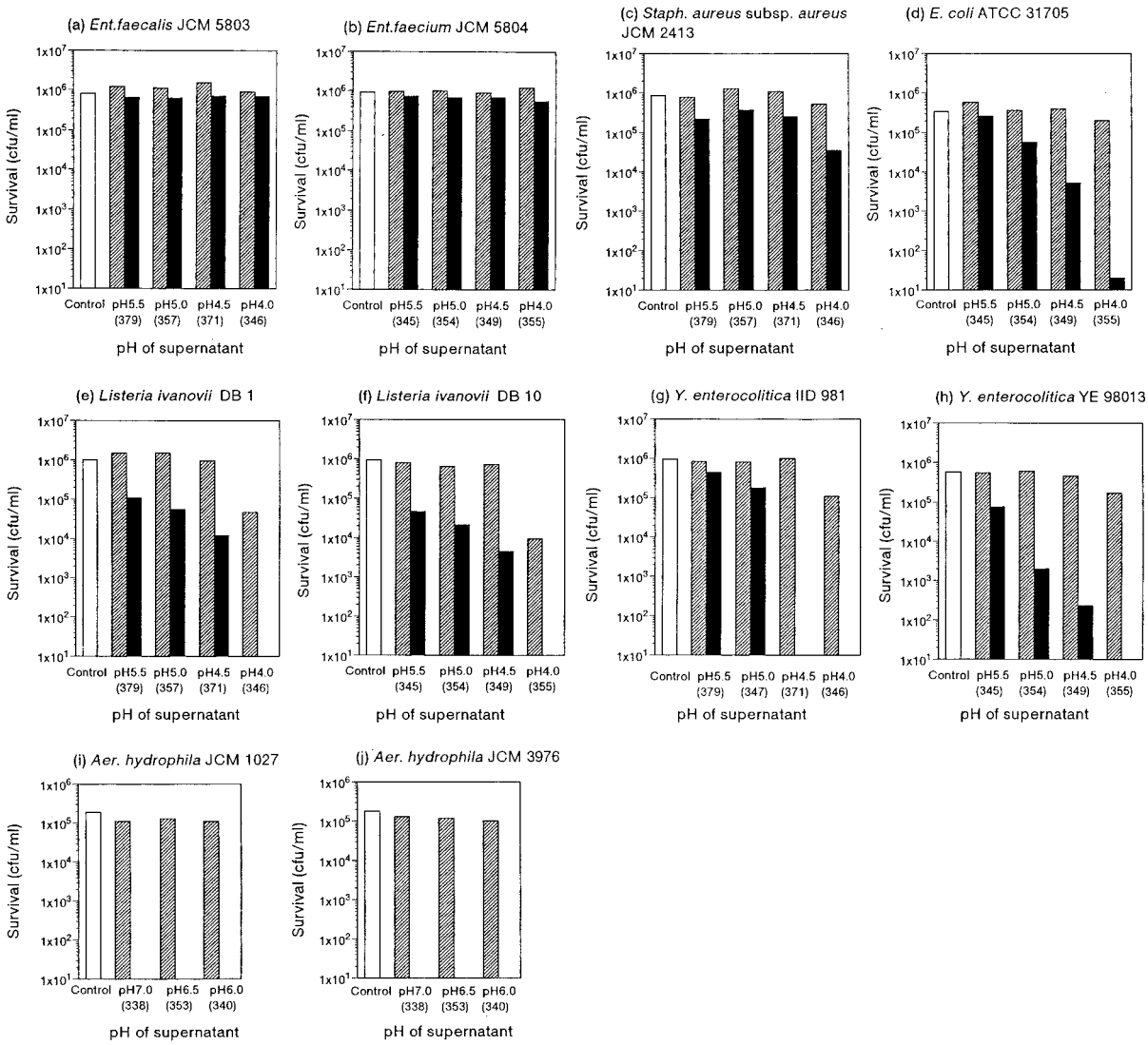

Fig. 5. Effect of the cell-free filtrate of *Lactococcus lactis* subsp. *lactis* containing hydrogen peroxide against the pathogens. The pathogens were *Enterococcus faecalis* JCM 5803 (a), *Ent. faceium* JCM 5804 (b), *Staphylococcus aureus* subsp. *aureus* JCM 2413 (c), enterotoxigenic *Escherichia coli* ATCC 31705 (d), *Listeria ivanovii* DB1 (e), *L. ivanovii* DB10 (f), *Yersinia enterocolitica* IID 981 (g), *Y. enterocolitica* YE98013 (h), *Aeromonas hydrophila* JCM 1027 (i), and *Aer. hydrophila* JCM 3976 (j). The pathogens were suspended in the cell-free filtrate of *Lactococcus lactis* subsp. *lactis* AI 62, which contained hydrogen peroxide (concentrations are indicated as ppm in parentheses), and were kept for 60 min at 30°C (■). The cell-free filtrate treated with the catalase before being mixed with the pathogens was used to determine the effect of the pH (▨). In the control, each pathogen was suspended in PBS instead of in the cell-free filtrate, and samples were taken at 0 min for enumeration (□). The values are the means of duplicate experiments. The results were confirmed in experiments performed on separate days.

aureus between pH 5.0 and 8.0 at 37°C for 5 days. Among these bacteria, only *P. aeruginosa* was more sensitive at a lower pH. Alasri et al. [1] showed that the lowest minimal bactericidal concentrations of H_2O_2 at 37°C were 195 ppm in 4 h for *E. coli*, 391 ppm in 5 h for *S. aureus*, and 98 ppm in 5 h for *P. aeruginosa*.

We showed here that the cell-free filtrate of *Lc. lactis* subsp. *lactis* AI 62 containing 300–380 ppm H_2O_2 had a strong bactericidal effect against psychrotrophic food-borne pathogens such as *Listeria*, *Yersinia*, and *Aeromonas* species, and mesophiles such as *E. coli*. Among the species examined, *Aer. hydrophila* was the most sensitive to H_2O_2. The high sensitivity of *Aer. hydrophila* to H_2O_2 was also demonstrated by Landre et al. [18].

Currently, hydrogen peroxide is widely used in the food industry for the aseptic packaging of fluid foods [28]. In the USA, the use of H_2O_2 as a GRAS substance in milk (0.05%), whey (0.04%), starch (0.15%), and corn syrup (0.15%) is permitted [28]. Other approved uses in

the USA for H_2O_2 are as an oxidizing or bleaching agent for dried eggs, tripe, beef feet, herring, wine, instant tea, and other foods [28]. Certain preparations in which H_2O_2 is the active compound are being marketed as disinfectants for fruits and vegetables [10]. The excess H_2O_2 present after treatment can be inactivated by heat or by the addition of a catalase. Because of this, H_2O_2 has the potential to be used in a variety of ways similar to chlorine in the food industry.

In conclusion, we developed a method for accumulating a bactericidal concentration of H_2O_2 in cell suspensions of lactic acid bacteria. The cell-free filtrate was effective in reducing the viable cell count of psychrotrophic pathogenic bacteria. Our data suggested that the cell-free filtrate of lactic acid bacteria is a suitable washing disinfection agent for the preparation of raw and minimally processed refrigerated foods.

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