Short communication

Antimicrobial effect of lactic acid producing bacteria culture condensate mixture (LCCM) against Salmonella enteritidis

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Received 9 January 2004; received in revised form 17 August 2004; accepted 19 November 2004

Abstract

The antimicrobial effects of a lactic acid producing bacteria culture condensate mixture (LCCM) were assessed against Salmonella enteritidis. In the presence of LCCM, bacterial growth was assessed in vitro by the measurement of optical density (OD) and viable bacterial counting. At concentrations of 1.25 and 2.5% LCCM, OD values were significantly lower than that of the control broth, and at concentrations of 5 and 10% LCCM, OD values did not increase for the entire period of experiment. At 8 h after incubation, the viable bacterial numbers in 5% and 10% LCCM-containing broths were remarkably lower than that in the control broth. This antimicrobial ability of the LCCM was fundamentally attributed to causing cell death rather than inhibiting growth. Even when the pH of LCCM-containing broth was adjusted to 7.2, the number of viable bacteria was significantly lower in the broths containing LCCM over 2.5% than that in control broth at 8 h after incubation. However, the OD value of each culture in the presence of each concentration of the LCCM increased over 1.0 at the completion of the experiment. The in vivo antimicrobial effects of the LCCM against S. enteritidis were also assessed. In S. enteritidis-infected mice, the LCCM decreased both the viable bacteria found in the feces and the mortality rate of the mice. These findings showed that the LCCM might have an antimicrobial ability against S. enteritidis.

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Keywords: Antimicrobial; Salmonella enteritidis; Lactic acid producing bacteria culture condensate mixture (LCCM); pH

1. Introduction

Salmonella infection is one of the most common food-borne diseases worldwide. In the United States, an estimated 1.41 million cases and more than 500 human deaths occur annually (Mead et al., 1999). Approximately 95% of the human Salmonella infections are food-borne, corresponding to approximately 30% of deaths caused by food-borne infections in the United States (Mead et al., 1999).
Antimicrobial chemotherapeutic agents have been widely used to control gastrointestinal infections. However, the widespread use of antibiotics is now being discouraged due to problems including the emergence of drug-resistant strains and chronic toxicity (Dundas et al., 1999; Cetinkaya et al., 2000; Mody et al., 2003). In addition, antibiotics are often responsible for acute diarrhea due to the loss of normal intestinal microbes as well as pathogenic organisms (Van der Waaij et al., 1982). As alternatives, lactic acid bacteria (LAB) such as lactobacilli and bifidobacterium or their derivates have been administered. LAB have antimicrobial effects against enteropathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Vibrio cholerae*, and *Salmonella enteritidis* through producing organic acid, hydrogen peroxide, and bacteriocins or through inhibiting the cell adhesion of pathogenic organisms in vitro (Drago et al., 1997; Todoriki et al., 2001; Bevilacqua et al., 2003; Santos et al., 2003).

We isolated *Lactobacillus plantarum* strains CBT-LP2 and CBT-LP3 from *kimchi*, *Pediococcus pentosaceus* CBT-PP1 from goat’s milk, and *Lactococcus lactis* CBT-P7 from cow’s milk. These probiotics were cultured in an appropriate broth, condensed by vacuum evaporation, and lyophilized. The dried powders were then mixed in different weight ratios to contain flat activity to many indicator strains. In the present study, the in vitro and in vivo antimicrobial ability of this lactic acid producing bacteria culture condensate mixture (LCCM) was evaluated against *S. enteritidis*.

### 2. Materials and methods

#### 2.1. Microorganisms

*S. enteritidis* CCARM 8037 (Culture Collection of Antibiotic Resistance Microbes, Seoul, Korea) was used. This organism was grown in tryptic soy (TS) agar (Difco Laboratories, Sparks, MD, USA) at 37 °C for 24 h. A single colony was inoculated into 10 ml of a fresh TS broth in a 15 ml conical tube and cultured in a shaking incubator (NB 205, N-Biotec, INC., Bucheon, Kyoungki-Do, Korea) at 200 rpm and 37 °C.

#### 2.2. Isolation of lactic acid bacteria

In order to isolate LAB, swabs of *kimchi*, goat’s milk, and cow’s milk were inoculated on MRS agar (Difco, Sparks, MD, USA). Following an incubation period of 3–4 days at 25 °C, bacteria were isolated from single colonies and tested for inhibitory effects on four indicator pathogens [*E. coli* O157:H7 ATCC 43894 (American Type Culture Collection, Rockville, MD, USA), *Staphylococcus aureus* KFRI 240 (Korea Food Research Institute, Sungnam city, Kyoungki-Do, Korea), *Shigella flexineri* KFRI 445, *S. paratyphi* A ATCC 11511] by agar overlay methods as described previously (Janes et al., 1999). Briefly, tested LAB strains were spot-inoculated onto the MRS agar and incubated at 37 °C for 24–48 h. After the formation of colonies, these agar plates were overlaid with 7 ml of soft (0.75% agar) TS agar containing a set of four indicators (5 × 10⁷ CFU/ml of each strain) and were incubated at 37 °C for 18–24 h. The antimicrobial activity of each LAB strain was assessed by the size of the clear zone in the indicator lawn.

The antimicrobial activity of the LAB culture supernatants was also assayed by the spot-on-lawn method (Dubreuil et al., 1985). The lactic acid bacteria were cultured overnight at 37 °C and the culture supernatants were obtained by removing the cells by centrifugation at 2700 × g for 30 min. TS agar plates were overlaid with 7 ml of soft (0.75% agar) TS agar containing 5 × 10⁷ CFU/ml of a set of indicators and then dried for 30 min at 37 °C. Each culture supernatant was double-diluted serially. 10 µl of the final solution was then spotted on this agar plate. After incubating for 24–48 h at 37 °C, the formation of an inhibitory clear zone around the spot was checked. The reciprocal of the greatest inhibitory dilution was used to calculate the arbitrary activity unit (AU) per ml. Four strains with the highest levels of inhibitory activity were selected and characterized using an API 50 CHL kit (bioMerieux Vitek, Hazelwood, MO, USA). In addition, 16 S rRNA gene of each bacterium was sequenced. These bacteria were identified as two of *L. plantarum* (CBT-LP2 and CBT-LP3 (Cellbio-tech, Kimpo, Kyunggido, Korea)), one of *P. pentosaceus* (CBT-PP1), and one of *L. lactis* (CBT-P7).
2.3. Preparation of lactic acid producing bacteria culture condensate mixture (LCCM)

Each selected bacterium was grown overnight in a liquid medium containing enzymatically hydrolyzed casein (3.0%), glucose (2.0%), and lactose (4.0%) at 37°C under anaerobic conditions. The bacterial cells were removed by centrifugation at 2700×g for 30 min and the culture media was concentrated 20-fold into a solid by vacuum evaporation and lyophilization. The dried powders were mixed different weight ratios to contain flat activity to the indicator strains. The antimicrobial activity of the final LCCM was 800 AU/g for all indicators when assessed by spot-on-lawn methods. The LCCM was stored at 4°C until use. At the time of use, the LCCM powder was dissolved to a final concentration of 1.25, 2.5, 5, and 10% (w/v) with TS broth and the pH of each solution was measured.

2.4. In vitro assessment of antimicrobial effect of the LCCM

*Salmonella enteritidis* was grown in TS broth to an optical density (OD) of 0.85–1.0 at 600 nm (>5×10⁸ CFU/ml). 200 μl of the culture was added to conical tubes containing 10 ml of each concentration [1.25, 2.5, 5, and 10% (w/v)] of the LCCM and to a tube containing 10 ml of TS broth as a control. The tubes were incubated with shaking at 200 rpm at 37°C. 1 ml of each culture was taken at 0, 1, 2, 3, 4, 6, and 8 h postincubation and centrifuged at 5000 rpm for 10 min. The supernatants were discarded and the bacterial cells were resuspended in 1 ml of fresh TS broth. Finally, the OD was measured at 600 nm. In addition, viable bacteria were counted by plating onto the TS agar at 8 h postincubation. All tests were repeated twice.

It was examined whether the low pH influenced the antimicrobial effect of LCCM. Tubes containing 10 ml of various concentrations of the LCCM were adjusted to pH 7.2 by the addition of NaOH. As control cultures, TS broth without the LCCM was also adjusted to pH 3.0, 4.0, 5.0, 6.0, and 7.2 by the addition of NaOH. Subsequently, 200 μl of the cultures was added to each tube containing 10 ml of each pH-adjusted TS broth and incubated at 37°C. The OD was measured at 0, 2, 4, 6, and 8 h postincubation using the same method as described above. At 8 h postincubation, viable bacteria were counted by plating onto TS agar. This process was also repeated twice.

2.5. Animals

Five-week-old specific pathogen-free (SPF) female BALB/c mice (Seoul National University Laboratory Animal Center, Seoul, Korea) were used for *S. enteritidis* infection. They were housed in polycarbonate cages in isolators and fed a commercial pellet diet with water ad libitum. Food and water were removed from the cages 5 h prior to inoculation of the bacteria and/or LCCM. All animal experimentation was performed in accordance with the Regulation and Guideline for the Care and Use of Laboratory Animals of Seoul National University (Seoul, Korea).

2.6. Protective effect of the LCCM against *S. enteritidis* in mice

One microliter containing 5×10⁷ CFU of *S. enteritidis* was added to each tube containing 9 ml of the LCCM at various concentrations (2.5, 5, and 10%) or of the TS broth (positive control). The tubes were then vortex-mixed and used as inocula. The mice were immediately administered 0.5 ml of each inoculum (2.5×10⁶ CFU of *S. enteritidis* per mouse) via the intragastric route while the negative control mice were administered with the same volume of TS broth. Subsequently, the mice of each treated group were administered 0.5 ml of the LCCM of their respective concentration once a day for 4 days. The mice of the positive and negative groups received 0.5 ml of TS broth. Bacteria from fecal samples were counted by plating on XLD (Difco) agar at 0.5, 1, 2, 3, and 4 days postinoculation (pi) and mortality was for 21 days pi.

2.7. Statistical analysis

The significance of differences between the experimental and control groups was determined by Duncan’s Multiple Range Test (SAS ver. 8.2; SAS Institute Inc., Cary, NC, USA) and student’s *t*-test.
Fig 1. In vitro growth curve of *S. enteritidis* in the LCCM-containing broths and TS broth (A), in the broths containing the LCCM adjusted to pH 7.2 by the addition of NaOH (B) *S. aureus* (B), and in each TS broth adjusted to pH 3.0, 4.0, 5.0, 6.0, and 7.2 (C), respectively. Data show the mean±S.D. of the three experiments performed. In panels A and B, each closed mark indicates the control (TS broth without LCCM) (–), 1.25% (–), 2.5% (–), 5% (–), and 10% (–) LCCM; each open mark indicated pH 7.2, 6.0, 5.0, 4.0, and 3.0, respectively. In panels D, E, and F show each result of the corresponding experiment in panels B and C, respectively.
3. Results and discussion

3.1. In vitro assessment of antimicrobial effect of the LCCM

The ability of the LCCM to inhibit the growth of *S. enteritidis* in vitro was evaluated and the results are shown in Fig. 1. Just before *S. enteritidis* was added to each tube, the pH levels of the solutions were measured at 4.03–4.19 (10% LCCM), 4.32–4.52 (5% LCCM), 4.96–5.17 (2.5% LCCM), 6.02–6.27 (1.25% LCCM), and 7.24 (control broth without LCCM). At the beginning, the OD increased in proportion to the concentration of the LCCM, because the LCCM has a dark-brown hue.

The growth of *S. enteritidis* was effectively inhibited by the presence of the LCCM (Fig. 1A). At concentrations of 1.25 and 2.5% LCCM, OD values increased with time, but were significantly lower than growth in TS broth without the LCCM (p<0.05). At concentrations of 5 and 10% LCCM, OD values did not increase for the entire period of the experiment (Fig. 1A). At 8 h after incubation, viable bacterial cells were counted by plating on TS agar. The numbers were significantly lower in LCCM-containing broths than that in the control (TS broth without LCCM) (p<0.05, Fig. 1D). The numbers in 5% and 10% LCCM-containing broths were 4.924±0.338 and 4.470±0.340 log_{10} CFU/ml. At the beginning, bacteria added to each tube were over 10^7 log_{10} CFU/ml. Accordingly, the antimicrobial effect of the LCCM might be fundamentally attributed to cause cell death rather than growth inhibition.

To identify the antimicrobial mechanism of the LCCM against *S. enteritidis*, we first considered the low pH due to the production of lactic acid; the pH of each broth was inversely proportional to the concentration of the LCCM. Based on this consideration, the pH of each broth was adjusted to 7.2 by the addition of NaOH. 200 µl of *S. enteritidis*-culture was subsequently added to each broth. As a result, the OD values of each culture in the presence of each concentration of the LCCM increased to over 1.0 at 8 h after incubation, similar to that of the control (Fig. 1B). Although, at 8 h after incubation, the bacterial number was significantly lower in the broths containing the LCCM over 2.5% than that in the control broth, the numbers in the 5% (9.09±0.017 log_{10} CFU/ml) and 10% (9.08±0.025 log_{10} CFU/ml) LCCM adjusted to pH 7.2 were much higher than that in the natural LCCM of each corresponding concentration [4.924±0.338 (in 5% LCCM) and 4.470±0.340 (in 10% LCCM) log_{10} CFU/ml] (Fig. 1E). These findings indicate that a low pH of the LCCM plays a critical role in the antimicrobial effect, even though LCCM may have bacteriocin-like substances. Previous findings (Panchayuthapani et al., 1995) have also shown that some bacteriocin-producing *Lactobacillus* strains, although able to inhibit a variety of pathogenic bacteria, do not inhibit the growth of both *Salmonella* sp. and *V. cholerae* when the effect of acid was excluded.

### Table 1

The bacterial counts from fecal samples of mice intragastrically administered with 0.5 ml TS broth containing 2.5×10^6 CFU of *S. enteritidis*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Log_{10} CFU/g feces</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
</tr>
<tr>
<td>Negative control</td>
<td>ND</td>
</tr>
<tr>
<td>Positive control</td>
<td>5.51±0.09(5)</td>
</tr>
<tr>
<td>Treated groups</td>
<td></td>
</tr>
<tr>
<td>2.5% LCCM</td>
<td>4.64±0.51(4)</td>
</tr>
<tr>
<td>5% LCCM</td>
<td>4.57±0.25(5)</td>
</tr>
<tr>
<td>10% LCCM</td>
<td>ND</td>
</tr>
</tbody>
</table>

All groups originally consisted of five mice.

a Negative control mice were administered with 0.5 ml of TS broth. Subsequently, the mice of each treated group were administered with 0.5 ml of the LCCM of their respective concentration once a day for 4 days and the mice of positive and negative groups received 0.5 ml of TS broth.

b Not detected.

c Values in parentheses represent the number of mice from which bacteria were isolated.
The growth pattern of *S. enteritidis* was determined in TS broth (without LCCM) at different pH levels. OD values were higher in proportion to the pH of the broth at each point of the experiment. OD values increased with time in the broths with a pH greater than 4.0. However, OD values were constant in the broth of pH 3.0 (Fig. 1C). At 8 h after incubation, the bacterial count in each TS broth at different pH, except that with pH 3.0, was over 8.0 log\textsubscript{10} CFU/ml (Fig. 1F). It is noteworthy that the pH levels of 5% and 10% LCCM were measured at 4.32–4.52 and 4.03–4.19, respectively, and the bacterial number in each broth containing 5% and 10% LCCM was below mean 5.0 log\textsubscript{10} CFU/ml at 8 h after incubation, which was much less than that in TS broth at pH 4.0. These findings mean that factors other than pH, such as bacteriocin and hydrogen peroxide, might play a part in the antimicrobial ability of the LCCM.

### 3.2. Protective effect of the LCCM against *S. enteritidis* in mice

Table 1 shows the bacterial count from the fecal samples. The number of isolated bacteria was dose-dependently less in the mice inoculated with an admixture of the LCCM and the bacteria than in the mice that received only the bacteria (positive control). However, the bacteria were steadily isolated in the mice that received 2.5% and 5% LCCM over the first 4 days. The bacteria were isolated from only one mouse of the group administered with 10% LCCM at 4 days pi. The limitation of the bacterial count was 4.00 log\textsubscript{10} CFU/g feces. Mortality was assessed for 21 days pi. Daily administration of the LCCM or TS broth in each group was terminated when the first death occurred in the positive control group at 5 days pi. All mice of the positive control group died within 7 days of injection (Fig. 2). Although all mice in the 2.5 and 5% LCCM-treated group eventually died, mortality occurred between 9 and 11 days pi, later than that of the positive control group. This might be because the presence of 2.5 and 5% LCCM did not completely inhibit the growth of *S. enteritidis* and the bacterial growth rapidly progressed after the LCCM administration was terminated (4 days pi). Within the 10% LCCM-treated group, one mouse died on day 14 pi and another on day 18 pi, while the rest survived until 21 days pi (Fig. 2).

In conclusion, in vitro and in vivo experiments showed that the LCCM has antimicrobial effect against *S. enteritidis*. Ingestion of the LCCM after a meal will be helpful for preventing *S. enteritidis* infection.

### Acknowledgements

This work was supported by the Brain Korea 21 Project. We would like to express our thanks to Dr. Yeonhee Lee (Department of Biology and CCARM, Seoul Women’s University) for his wonderful advice and providing the pathogens used in this study.

### References


