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Growth Inhibition of *Streptococcus mutans* by Cellular Extracts of Human Intestinal Lactic Acid Bacteria

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The in vitro growth of *Streptococcus mutans* was completely inhibited by water-soluble extracts from cells of various intestinal lactic acid bacteria identified as *Streptococcus faecium*, *Streptococcus equinus*, *Lactobacillus fermentum*, and *Lactobacillus salivarius*. The growth inhibition was dependent on the concentrations of the extracts. In contrast, the extracts did not inhibit the growth of the major indigenous intestinal lactic acid bacteria isolated from humans. These lactic acid bacteria were not acutely toxic in mice.

Inhibition of the growth of *Streptococcus mutans*, which has been considered a major pathogen of dental caries, is thought to be one of the most important factors in preventing dental caries. However, continuous and long-term use of germicides, antibiotics, or bacteriocins is inadequate, for these agents involve the risks of disturbing the intestinal microflora and of other adverse effects on human health (4, 10). Therefore, an inhibitor of *S. mutans* growth without adverse effects is desired. Investigations of intestinal microbiology indicate that indigenous intestinal lactic acid bacteria are safe and useful for our health (5).

This study was initiated to isolate human intestinal lactic acid bacteria which inhibit the growth of *S. mutans* and to examine them for the prevention of dental caries without adverse effects on human health when used practically. In this paper, we show that cellular extracts of intestinal streptococci and lactobacilli isolated from the feces of healthy humans completely inhibited the growth of *S. mutans*.

MATERIALS AND METHODS

Bacteria. *S. mutans* 8148, serotype c, was supplied by S. Hamada, Department of Dental Research, National Institute of Health, Tokyo, Japan. The other 15 strains of *S. mutans*, serotypes a to g, were from the Department of Microbiology, Tokyo Dental College. Stock cultures on Todd-Hewitt agar were stored at 4°C and transferred weekly. *Lactobacillus fermentum* AD0002, *Lactobacillus salivarius* AD0001, *Streptococcus faecium* AD1050, and *Streptococcus equinus* AD8005 were selected from about 8,000 strains of lactic acid bacteria isolated from the feces of healthy humans. The feces were diluted in 0.85% NaCl and cultured on LBS agar, a selective medium for lactobacilli (BBL Microbiology Systems, Cockeysville, Md.), or KMN agar (11) at 37°C for 48 to 72 h. Colonies formed on the agar plates were restreaked for purification, and purified colonies were inoculated into Rogosa broth (3) and Rogosa agar. The isolates were stored at 4°C on Rogosa agar or Todd-Hewitt agar (Difco Laboratories, Detroit, Mich.) for 2 weeks.

Preparation of hot water-soluble extracts of lactic acid bacteria. The purified lactic acid bacteria were inoculated into 2 liters of Rogosa broth at a final concentration of ca. 10^6 cells per ml and cultured at 37°C for 24 h. The bacterial cells were harvested by centrifugation at 8,000 × *g* for 10 min and washed twice with 500 ml of 0.85% NaCl. After being washed, the harvested cells were suspended in 20 ml of distilled water and heated at 115 to 121°C for 15 min. Water-soluble extracts were obtained by centrifugation at 8,000 × *g* for 10 min, and the supernatant was adjusted to pH 7 and freeze-dried.

Treatment with human saliva. About 10 ml of saliva was collected from healthy humans, and insoluble matter was removed by centrifugation at 2,300 × *g* for 10 min. The saliva was freeze-dried and then dissolved in 5 ml of distilled water. The concentrated saliva was sterilized with a membrane filter (pore size, 0.2 μm; Millipore Corp., Bedford, Mass.). The sterile saliva was added to assay media at the original concentration.

Assay for growth inhibition. The hot water-soluble extracts from lactic acid bacteria were added to the basal media (Rogosa broth, Todd-Hewitt broth, or GAM broth; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), and the concentrations of the extracts (0 to 30 mg/ml) in the basal media were adjusted with sterile 0.85% NaCl. *S. mutans* or other bacteria were inoculated into these media at ca. 10^6 cells per ml, and growth was observed by measurement of viable cells or turbidity.

Identification of lactic acid bacteria. The lactic acid bacteria, the cellular extracts of which inhibited the growth of *S. mutans*, were identified by colony morphology on selective agar media, the shape of the cell, Gram staining, and biochemical and physiological properties (1, 2, 6).

Acute toxicity of heat-treated dead cells of lactic acid bacteria. ICR male mice (Charles River Japan, Inc., Atsugi, Kanagawa, Japan) weighing ca. 30 g were used (five mice per sample). A 1.5-g sample of the dried heat-treated dead cells of the lactic acid bacteria was suspended in 0.85% NaCl, and the volume was adjusted to 4.5 ml. The suspension was orally administered to the mice (0.6 ml per mouse). Two weeks after administration, the mice were sacrificed, and their internal organs were examined. During the 2 weeks, the body weight of the mice was measured every day.

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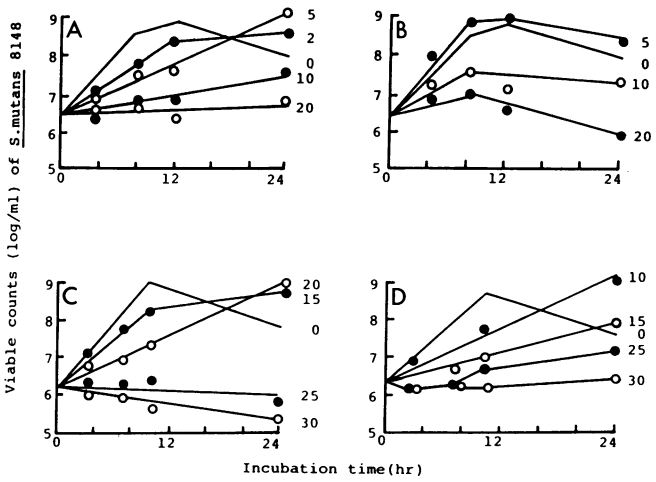

FIG. 1. Inhibition of the growth of *S. mutans* 8148 by extracts of human fecal streptococci and lactobacilli. *S. mutans* 8148 was inoculated at 10^6 cells per ml (viable cell concentration) into Rogosa broth containing the bacterial extracts at various concentrations (milligrams per milliliter; numbers at right of each graph) and was cultured at 37°C. The extracts were from *S. faecium* AD1050 (A), *S. equinus* AD8005 (B), *L. fermentum* AD0002 (C), and *L. salivarius* (D).

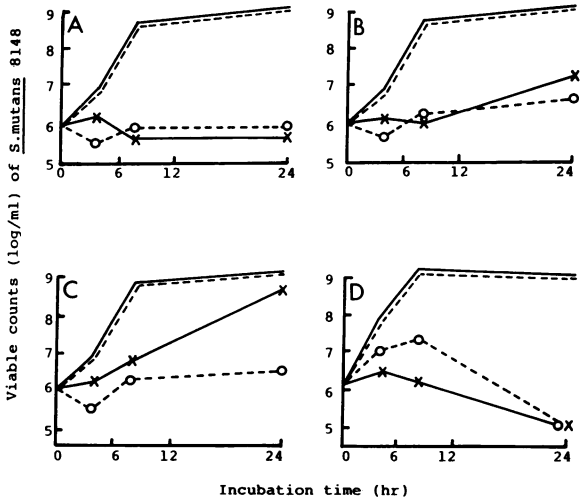

FIG. 2. Influence of human saliva on the inhibition of the growth of *S. mutans* 8148 by cellular extracts of lactic acid bacteria. The growth of *S. mutans* in Rogosa broth with water (control), the cellular extracts, the cellular extracts and human saliva, or human saliva at 37°C was observed. The concentration of the extracts was ca. 30 mg/ml. The extracts were from *L. salivarius* AD0001 (A), *S. faecium* AD1050 (B), *L. fermentum* AD0002 (C), and *S. equinus* AD8005 (D). Symbols: ——, control; ○, cellular extract; ×, cellular extract and human saliva; – – –, human saliva.

RESULTS

Four strains of intestinal lactic acid bacteria, the cellular extracts of which inhibited the growth of *S. mutans* 8148, were found. These strains were identified as *S. faecium*, *S. equinus*, *L. salivarius*, and *L. fermentum*.

Inhibition of growth of *S. mutans*. The growth patterns of *S. mutans* 8148 in the media with added cellular extracts are shown in Fig. 1. Growth inhibition by the cellular extracts was concentration dependent. The growth of *S. mutans* 8148 was completely inhibited by the addition of 20 to 30 mg of the concentrated extracts per ml. At lower concentrations, the growth was slowed. The extract of *S. faecium* AD1050 had growth-inhibiting effects at more than 2 mg/ml. The extract of *L. salivarius* AD0001 slowed down the growth of *S. mutans* at 10 mg/ml. The extracts of *S. equinus* AD8005 and *L. fermentum* AD0002 had bactericidal effects at concentrations higher than 20 to 25 mg/ml. At lower concentrations, the extract of *S. faecium* AD1050 had the strongest inhibitory effect of the four strains. The inhibitory effects on the growth of strains of *S. mutans*, serotypes a to g, are summarized in Table 1. The growth of *S. mutans* of any serotype was inhibited by the extracts.

TABLE 1. Inhibition of growth of *S. mutans* of various serotypes by extracts of human fecal streptococci and lactobacilli

S. mutans		Inhibition[a] by extract (ca. 30 mg/ml) of:			
Serotype	Strain	*S. faecium* AD1050	*S. equinus* AD8005	*L. fermentum* AD0002	*L. salivarius* AD0001
a	OMZ61	++	++	++	++
a/g	AHT	++	+	++	++
b	BHT	++	++	++	++
b	107P	++	++	++	++
c	8148	++	++	++	++
c	PS-14	++	++	++	++
c	JC-2	++	++	++	++
c	Ingbritt	++	++	++	++
d	B-13	++	++	++	++
d	OMZ176	++	++	+	++
e	AT-10	+	+	++	++
e	LM-7	++	++	++	++
f	OMZ175	++	++	++	++
f	QP50-1	++	++	++	++
g	K1R	++	++	+	++
g	6715	++	++	++	++

[a] ++, No growth was observed or maximal growth was less than 10% of that of the control; +, the growth rate was down, but growth reached the maximal growth level of the control in 24 h.

Influence of saliva on the inhibition of growth of *S. mutans*. The inhibitory effect of the cellular extracts of *S. faecium* AD1050 and *L. salivarius* AD0001 was not influenced by human saliva. However, the inhibitory effect of the cellular extract of *S. equinus* AD8005 appeared to be a little strengthened in the presence of human saliva, and the inhibitory effect of the cellular extract of *L. fermentum* AD0002 was weakened in the presence of human saliva (Fig. 2). Human saliva did not influence the growth of *S. mutans* (Fig. 2).

Influence of the cellular extracts on intestinal lactic acid bacteria. We examined the influence of the cellular extracts on the in vitro growth of various lactic acid bacteria, especially human intestinal lactic acid bacteria (Table 2). The cellular extracts did not inhibit the growth of strains of *S. faecium*, *Streptococcus faecalis*, *Lactobacillus acidophilus*, *L. fermentum*, and *Bifidobacterium* spp., which are indigenous lactic acid bacteria in humans, with the exception that the extract of *S. equinus* AD8005 slowed down the growth of *S. faecalis*. The growth of *Streptococcus salivarius*, *Streptococcus sanguis*, and *Streptococcus bovis* was slowed down or completely inhibited by cellular extracts of *S. faecium* AD1050, *S. equinus* AD8005, and *L. salivarius* AD0001.

Acute toxicity of heat-treated dead cells of lactic acid bacteria. No mice died after oral administration of the heat-treated dead cells of the lactic acid bacteria, and we noticed nothing unusual in the mice. The concentration of

TABLE 2. Influence of cellular extracts which inhibited the growth of *S. mutans* 8148 on the proliferation of lactic acid bacteria and *Escherichia coli*

Organism	Inhibition[a] by extract (ca. 30 mg/ml) of:			
	S. faecium AD1050	*S. equinus* AD8005	*L. fermentum* AD0002	*L. salivarius* AD0001
Streptococcus salivarius AD10001	++	++	−	++
S. sanguis IID5224	+	+	+	++
S. bovis AD4002	++	++	+	++
S. avium AD2001	−	+	−	−
S. durans AD3001	−	+	−	−
S. faecalis AD9001	−	+	−	−
S. faecium AD1005	−	−	−	−
Lactobacillus fermentum AD0003	−	−	−	−
L. acidophilus ATCC 9338	−	−	−	−
L. casei ATCC 393	−	−	−	−
Bifidobacterium adolescentis ATCC 15705	−	−	−	−
B. infantis AD20001	−	−	−	−
B. bifidum AD20002	−	−	−	−
B. breve AD20003	−	−	−	−
Escherichia coli ATCC 11246	−	−	−	−

[a] −, Growth was not inhibited; +, the growth rate was down, but growth reached the maximal growth level of the control in 24 h; ++, no growth was observed or maximal growth in 24 h was less than 10% of that of the control.

the heat-treated dead cells of the lactic acid bacteria was more than 6 g (dry weight) per kg of body weight (Table 3).

DISCUSSION

It was found that cellular extracts of human intestinal lactic acid bacteria had a strong growth-inhibiting effect specific for *S. mutans*. We also examined the inhibition of the growth of bacteria other than *S. mutans* to determine how the cellular extracts of the lactic acid bacteria influence the human intestinal microflora, especially lactic acid bacteria. The inhibition of the growth of human intestinal lactic acid bacteria by the cellular extracts was bacterial species specific (Table 2). The major intestinal lactic acid bacteria were not inhibited by the extracts, with the exception that the extract of *S. equinus* AD8005 slowed down the growth of *S. faecalis*. Therefore, it is expected that the extracts will not disturb the human intestinal microflora very much.

Indigenous intestinal lactic acid bacteria are thought to be safe and beneficial for human health; lactic acid bacteria such as *S. faecium* and *L. fermentum*, which were isolated for the present experiment, exist in many kinds of daily food, especially fermented stuffs, and have been used for processing food (7–9) and manufacturing drugs which have well-known alleviation effects on gastrointestinal disorders.

TABLE 3. 50% Lethal doses of heat-treated dead cells of lactic acid bacteria orally administered to mice

Lactic acid bacteria	50% Lethal dose (g/kg of body wt)
S. faecium AD1050	>6.60
S. equinus AD8005	>6.39
L. fermentum AD0002	>6.62
L. salivarius AD0001	>6.47

In these experiments, as expected, no acute toxicity of the heat-treated dead cells of the four lactic acid bacterial strains was found. The heat-treated dead cells were administered into mice to satiation, and the 50% lethal dose, more than 6 g of heat-treated dead cells per kg of body weight, was equivalent to more than 1 to 1.5 g of dried cellular extracts per kg of body weight.

There may be several different kinds of substances in the cellular extracts that inhibit the growth of *S. mutans*; these may have different modes of inhibitory action, including concentration dependency, different influences from various species of lactic acid bacteria, and different reactivities to human saliva.

Thus, it could be concluded that the cellular extracts of the lactic acid bacteria isolated have inhibitory effects on the growth of any serotype of *S. mutans* and that the cellular extracts do not lose their inhibitory effects on the growth of *S. mutans* in the presence of human saliva. We think that these extracts may be useful for preventing human dental caries because of their strong effectiveness and their safety in humans. Further studies on the effects of these extracts on the human microflora in vivo are indicated.

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