Evaluation of Anti-Oral Pathogen Activity and Safety of *Lacticaseibacillus paracasei* TISTR 2688, Isolated from Fermented Termite Comb.

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Abstract

Objective: This study aimed to evaluate the potential of *Lacticaseibacillus paracasei* TISTR 2688 to be used in the field of oral health care.

Materials and methods: The strain TISTR 2688 was assessed for its antimicrobial activity using agar well diffusion assay, ability to suppress plaque formation on prosthetic teeth, tolerance to lysozyme, antibiotic susceptibility and acute oral toxicity based on Organization for Economic Co-operation and Development (OECD) Guidelines.

Results: Cell-free supernatant of the strain TISTR 2688 possessed inhibitory activity against *Actinomyces vericosus, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum subsp. polymorphum, Porphyromonas gingivalis, Prevotella intermedia, Streptococcus mutans, S. sanguinis,* and *S. sobrinus,* but exhibited no antifungal activity against *Candida albicans.* In terms of safety, TISTR 2688 showed the phenotypic antibiotic susceptibility to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin and tetracycline. The acute oral toxicity assay in rats with TISTR 2688 at 1×10^{10} CFU/kg body weight demonstrated no mortality results. In addition, no toxicity or evidence of gross pathological alterations was observed.

Conclusion: Due to its antibacterial activity against some oral pathogens and safety in term of antibiotic susceptibility test together with no acute oral toxicity, *L. paracasei* TISTR 2688 tend to have potential for development as an oral health care product.

Keywords: Acute oral toxicity, Antibacterial activity, Antibiotic susceptibility, Oral pathogens, Probiotics, TISTR 2688.

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Introduction

Oral diseases, especially dental caries and periodontal diseases, are prevalent at all ages and have become a major global public health burden. An important cause of oral diseases is an imbalance of microflora in the oral cavity due to poor oral hygiene. The overgrowth of oral pathogens causes various pathologies beginning with the accumulation of plaque on the surfaces of teeth and gingiva. If untreated, tooth caries occur, followed by gingivitis, periodontitis and tooth loss (1). In addition, oral diseases are linked to some non-communicable diseases (NCDs), especially diabetes mellitus (DM) and cardiovascular disease. Despite oral self-care and many commercially available oral-care products, including mouthwashes, dental caries and periodontal diseases persist globally. In addition, the use of oral-care products containing antimicrobial agents may have undesired consequences such as side effects, the disturbance of the oral microflora balance and, notably, antimicrobial resistance (2). Hence, alternative methods with greater efficacy and fewer undesirable effects are still required for maintaining oral health.

Various lactic acid bacteria have been isolated and evaluated for their benefits in oral health. A number of studies have demonstrated the in vitro antibacterial activities of *Lactobacillus* spp. against an important bacterium for early caries initiation, *Streptococcus mutans* (3,4), and the periodontal pathogens (5). Some strains of *L. paracasei* were proven to reduce the release of IL-6, IL-8 and prostaglandin E2 (PGE2) from monocytes (6). *In vitro* and *in vivo* studies of probiotic efficacy for the treatment of various oral diseases were summarized by Chugh et al. (7). The probiotics most commonly used for oral health include *L. rhamnosus* GG, *L. paracasei* SD1, *L. acidophilus, Bifidobacterium adolescentis, B. animalis, Propionibacterium freudenreichii, Enterococcus faecalis, E. faecium and Streptococcus salivarius.* All of them were isolated from conventional sources such as animal milk (8), human breast milk (9) or caries-free subjects (4-5). To date, there have been no reports on the potential of probiotics from non-human sources to be used for the treatment of dental caries or periodontal diseases.

L. paracasei TISTR 2688 was isolated from fermented termite comb, fermented liquid obtained from 10-days fermentation of termite comb mixed with cooked sticky rice and water from washing rice. It was assessed for probiotic characteristics and safety properties including hemolytic activity and antimicrobial resistance according to the Notification of the Ministry of Public Health Re: Use of Probiotic Microorganisms in Foods B.E. 2011. It was found that the strain TISTR 2688 exhibited resistance to simulated gastric and simulated small intestinal juices, good adherence to the Caco-2 and HT-29 human colon cell lines, an absence of hemolytic activity and susceptibility to antibiotics according to a disk diffusion assay. Interestingly, TISTR 2688 showed strong antibacterial activity against *Staphylococcus aureus, S. epidermidis, Escherichia coli, Salmonella* Typhimurium, *S.* Enteritidis, *Listeria monocytogenes* and clinically isolated *Helicobacter pylori* PT14 (10). Therefore, TISTR 2688 was selected for the further investigation of its antibacterial activity against oral pathogens and safety characteristics.

Materials and Methods

1. Microorganisms and Growth Conditions

L. paracasei TISTR 2688 was grown in de Man, Rogosa and Sharpe (MRS) medium (Merck, Darmstadt, Germany), pH 6.8, supplemented with 0.05% L-cysteine HCl (Merck, Germany). Incubation was carried out in an anaerobic jar (Thermo Scientific™, USA) containing an AnaeroPack® (MCG, Tokyo, Japan) at 37 °C for 24–48 h.

The indicator strains used in this study were oral pathogens including *Actinomyces vericosus* ATCC 43146, *Aggregatibacter actinomycetemcomitans* ATCC 700685, *Fusobacterium nucleatum* subsp. *polymorphum* ATCC 10953, *Porphyromonas gingivalis* ATCC 3327, *Prevotella intermedia* ATCC 25611, *Streptococcus mutans* ATCC 25175, *S. sanguinis* ATCC BAA-1455, *S. sobrinus* ATCC 27351 and *Candida albicans* ATCC 10231. All the indicator microorganisms were stored at -80°C in 40% glycerol until use. *C. albicans* ATCC 10231 was grown in Sabouraud dextrose broth or agar (Merck, Germany), while the other indicator microorganisms were anaerobically grown on 5% sheep blood agar at 37 °C for 24–54 h.

2. Preparation of Cell-Free Supernatant of TISTR 2688

A 48 h-old culture of TISTR 2688 grown in MRS broth, pH 6.8, supplemented with 0.05% L-cysteine HCl, was centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was collected, sterilized through a 0.22 µm filter membrane (Merck, Germany) and kept at -20°C until use.

3. Determination of Antimicrobial Activity of TISTR 2688 by Agar Well Diffusion Method

Colonies of the indicator microorganisms grown anaerobically on suitable media—5% sheep blood agar or Sabouraud dextrose agar were suspended in brain heart infusion (BHI) broth (Merck, Germany) to make the inoculum. After adjusting the turbidity to match that of a 0.5 McFarland standard, the inoculum was evenly applied on the surface of 20 mL of either 5% sheep blood agar or Sabouraud dextrose agar. Then, 6 mm wells were made in the test agar using a sterile cork borer. Each well was loaded with 60 µL of the cell-free supernatant. Normal saline was used as the negative control. After a suitable incubation period, the diameters of the inhibition zones observed around the wells were measured in millimeters (mm) (11).

4. Inhibition of Biofilm Formation on Prosthetic Teeth by TISTR 2688

An inoculum of S. mutans ATCC 25175 was prepared by suspending a few colonies of 48 h-old culture grown on 5% sheep blood agar in BHI broth. Its turbidity was subsequently adjusted to match that of a 0.5 McFarland standard. Each sterile prosthetic incisor teeth were added into each well of 12-well culture plates (Corning®, USA). Then, 2 mL of mixture containing 1 mL of double-strength BHI broth supplemented with 0.5% sucrose and 1 mL of cell-free supernatant was added into each well. After adding the *S. mutans* ATCC 25175 inoculum (100 µL/well), the plate was anaerobically incubated at 37 °C for 3 days. BHI broth was used as a negative control. The mixture was changed daily. At the end of the incubation time, each prosthetic incisor teeth was gently washed 3 times with Dulbecco's phosphate buffered saline (DPBS, Gibco®, USA). The amount of *S. mutans* ATCC 25175 presented on each tooth surface was enumerated by the standard plate count method using 5% sheep blood agar as the growth medium.

5. Tolerance of TISTR 2688 to Lysozyme

Cell pellets were prepared by centrifuging an overnight culture of TISTR 2688 at 5000 rpm for 10 min. The pellets were washed twice with DPBS and subsequently suspended in DPBS to obtain a cell concentration of 10^9 CFU/mL. The resistance to lysozyme was determined by adding 100 µL of TISTR 2688 inoculum to 3 mL of phosphate buffered saline (Merck, Germany), pH 7.2, containing 1 mg/mL lysozyme (GoldBio®, USA). After incubation at 37 °C for 180 min, the numbers of TISTR 2688 at 0 and 180 min were enumerated by viable plate counts using MRS agar containing 0.05% L-cysteine HCl (12).

6. Antibiotic Susceptibility of TISTR 2688

Eight antibiotics were used for testing: ampicillin (AMP), chloramphenicol (C), clindamycin (CD), erythromycin (E), gentamicin (CN), kanamycin (K), streptomycin (S) and tetracycline (TE). The susceptibility of TISTR 2688 to the antibiotics was measured by determining the minimal inhibitory concentrations (MICs) using MIC test strips (Liofilchem, Italy) following the procedure described in the manufacturer's instructions with a slight modification. Briefly, TISTR 2688 inoculum was prepared by directly suspending colonies from 48 h-old TISTR 2688 anaerobically grown on MRS agar containing 0.05% L-cysteine HCl in normal saline solution (0.85% NaCl). After adjusting the turbidity to match that of a 0.5 McFarland standard, the inoculum was evenly applied on the surface of 25 mL Hi-sensitive agar plate (Himedia, India) using cotton swabs. Each antibiotic strip was gently placed on the inoculated plates. After incubation, the MIC value for each antibiotic was read and compared to the microbiological cut-off values described by the European Food Safety Authority (EFSA) (14). *L. rhamnosus* GG DSM 33156 was also comparatively tested. MIC values higher than the microbiological cut-off values were taken as indicating resistance.

7. Acute Toxicity Evaluation of TISTR 2688

The acute toxicity of TISTR 2688 was evaluated in rats according to OECD Guidelines for Testing of Chemicals, Test Guideline (TG) No. 420: Acute Oral Toxicity—Fixed Dose Method, 2001. Five female Sprague Dawley rats (Nomura Siam International Co. Ltd., Bangkok, Thailand) aged 7 weeks with initial weights of 188–226 g were employed. After 1 week of acclimatization and before experimentation, all the rats were fasted overnight for 16 h. Then, they were orally fed with live TISTR 2688 suspended in UHT milk at a concentration of 1×10^{10} CFU/kg body weight. Following treatment, the rats were closely observed for any clinical signs or toxicological symptoms (such as convulsions, tremors, diarrhea, salivation, lethargy, sleep, coma and mortality) during the first 4 h. After that, observation was performed daily for 14 days. The body weights of all the rats were recorded shortly before the administration of TISTR 2688 and at the end of each week. At the end of the experiment, the animals were humanely sacrificed by CO_2 asphyxiation. Gross pathological examinations of internal organs were performed. The animal experiment was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at TISTR, and the Animal Ethics Committee at TISTR approved the study protocol (Approval No. TS-63003).

8. Statistical Analysis

The experimental results are expressed as mean ± standard deviation (SD). The data were subjected to analysis of variance (ANOVA) with Tukey's honestly significant difference test with statistical significance at $p < 0.05$. All the analyses were carried out using IBM SPSS Statistics 23.

Results

1. Antimicrobial Activity of TISTR 2688 against Oral Pathogens

The antimicrobial activity of cell-free TISTR 2688 supernatant against nine oral pathogenic indicator microorganisms was determined via a well diffusion assay. The supernatant showed antagonistic activity against eight of the oral pathogenic indicators tested (Table 1). The inhibition zones ranged from 11.33 ± 0.58 to 18.33 ± 0.58 mm. *P. intermedia* ATCC 25611 was found to be the most susceptible strain (inhibition zone = 18.33 ± 0.58 mm). By contrast, the cell-free supernatant of *L. paracasei* TISTR 2688 did not exhibit antifungal activity against C. albicans ATCC 10231.

Table 1. Antimicrobial activities of cell-free supernatant (CFS) of TISTR 2688 against various pathogenic indicator microorganisms.

Pathogenic Indicator Microorganisms[1]	Size of Inhibition Zones (mm)[1]	
	CFS[2]	Neutralized CFS[3]
A. vericosus ATCC 43140	12.33 ± 0.58*	Not detected
A. actinomycetemcomitans ATCC 700685	11.33 ± 0.58*	Not detected
F. nucleatum subsp. polymorphum ATCC 10953	13.67 ± 0.58	Not detected
P. gingivalis ATCC 33277	11.67 ± 0.58*	Not detected
P. intermedia ATCC 25611	18.33 ± 0.58*	Not detected
S. mutans ATCC 25175	14.67 ± 0.58	Not detected
S. sanguinis ATCC BAA-1455	14.00 ± 0.00	Not detected
S. sobrinus ATCC 27351	12.00 ± 0.00*	Not detected
C. albicans ATCC 10231	0.00 ± 0.00*	Not detected

[1]Results are presented as mean ± SD obtained from three experiments. Symbol * in a column show significant differences ($p < 0.05$) compared with S. mutans ATCC 25175.

[2]CFS = Cell-Free Supernatant prepared from 48 h-old culture of TISTR 2688 grown in MRS broth, pH 6.8, supplemented with 0.05% L-cysteine HCl as mentioned in materials and methods.

[3]Neutralized CFS = Cell-Free Supernatant prepared from 48 h-old culture of TISTR 2688 grown in MRS broth, pH 6.8, supplemented with 0.05% L-cysteine HCl and then neutralized to pH 7 with 1 M NaOH.

2. Inhibition of Biofilm Formation on Prosthetic Teeth

After a 3-day incubation of prosthetic incisor teeth in a mixture containing *S. mutans* ATCC 25175 and cell-free TISTR 2688 supernatant, the concentrations of *S. mutans* ATCC 25175 on the prosthetic incisor teeth were compared to those on control prosthetic incisor teeth grown in BHI broth. It was found that the concentrations of *S. mutans* ATCC 25175 detected on the prosthetic incisor teeth grown in the mixtures were less than 10 CFU/tooth (1 log CFU/tooth), whereas those counted from the control group were 7.9 ± 0.07 log CFU/tooth (Table 2). These results indicate the ability of TISTR 2688 to inhibit *S. mutans* ATCC 25175 biofilm formation on prosthetic incisor teeth.

Table 2. Ability of *L. paracasei* TISTR 2688 to inhibit biofilm formation in vitro and to tolerate lysozyme.

Assays	Number of Bacteria Detected			
	S. mutans ATCC 25175 (log CFU/Tooth)		TISTR 2688 (log CFU/mL)	
	Control	Cell-Free Supernatant	0 min	180 min
Inhibition of biofilm formation	7.9 ± 0.07*	1*	ND	ND
Tolerance to lysozyme	ND	ND	8.05 ± 0.01	8.34 ± 0.01

ND = not tested. Results are presented as mean ± SD obtained from triplicates. * indicates statistically significant difference ($p < 0.05$).

3. Tolerance of TISTR 2688 to Lysozyme

In this study, the resistance of TISTR 2688 to lysozyme was also investigated. After 180 min of exposure to 1 mg/mL lysozyme, the number of TISTR 2688 (8.34 ± 0.01 log CFU/mL) did not decrease from the initial amount at 0 h (8.05 ± 0.01 log CFU/mL) (Table 2).

4. Antibiotic Resistance Phenotype of TISTR 2688

Eight types of antibiotics clinically important for medical treatment were chosen as a basic minimum requirement for the assay (14). Based on the microbiological cut-off values, TISTR 2688 was susceptible to all the antibiotics tested (Table 3).

Table 3. Antibiotic susceptibility phenotype of TISTR 2688 isolated from fermented termite comb. Antibiotic susceptibility was interpreted according to microbiological cut-off values specified in EFSA (14).

Test Bacteria	MIC Values (μg/mL)							
	AMP	**C**	**CD**	**E**	**CN**	**K**	**S**	**TE**
TISTR 2688	0.38	4	2	0.25	3	24	48	3
	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)
DSM 33156	0.38	4	0.50	0.125	6	48	16	0.25
	(S)	(S)	(S)	(S)	(S)	(S)	(S)	(S)

Microbiological cut-off value of ampicillin (AMP) = 4 μg/mL, chloramphenicol (C)= 4 μg/mL, clindamycin (CD) = 1 μg/mL, erythromycin (E) = 1 μg/mL, gentamicin (CN) = 32 μg/mL, kanamycin (K) = 64 μg/mL, streptomycin (S) = 64 μg/mL and tetracycline (TE) = 4 μg/mL; S = susceptible and R = resistant

TISTR 2688 isolated from fermented termite comb showed the same antibiotic susceptibility pattern as *L. rhamnosus* GG DSM 33156, a commercial probiotic isolated from the human intestine. The latter has been granted qualified presumption of safety (QPS) status in Europe and is used worldwide as a food ingredient and dietary supplement.

5. Acute Toxicity of TISTR 2688

Concerning the 3 Rs (replacement, reduction, and refinement) policy of the European Union (EU) on using animals in toxicity tests, the results obtained from in vitro cytotoxicity testing suggest that TISTR 2688 is a safe substance. Therefore, only a high concentration of TISTR 2688 was used in this study. The results revealed that the oral administration of TISTR 2688 at 1×10^{10} CFU/kg body weight showed no treatment-related mortality in rats throughout a 14-day observation period. The physical observation of all the tested rats throughout this study indicated that none of them showed signs of toxic effects such as changes in skin and fur, changes in eyes and mucous membranes, behavioral changes, tremors, salivation, diarrhea and coma. The body weights of individual rats gradually increased. The body weight gains detected weekly on days 7 and 14 were 45.8 ± 1.32 and 62.2 ± 3.83 g, respectively. Gross pathological examinations did not reveal significant changes in the organs (Table 4).

Table 4. Body weight gain, mortality, toxicity signs and gross pathological examination of female rats orally treated with single dose of *L. paracasei* TISTR 2688 at 1 × 10^{10} CFU/kg body weight.

Items	Day 7	Day 14
Body weight gain (g)	45.8 ± 1.32	62.2 ± 3.83
Mortality (%)	0.00	0.00
Toxicity signs	None	None
Gross pathological examinations	Not tested	Normal

Note: Results are presented as mean (n = 5)± SD. Average body weight of treated rats on day 1, 7 and 14 were 206.20 ± 13.83, 252.60 ± 13.25 and 268.40 ± 12.86 g, respectively.

Discussion:

To investigate the possibility of oral-health applications, *L. paracasei* TISTR 2688 was first screened for antagonistic activity against oral pathogens involved in dental caries, periodontitis and oral candidiasis. It was found that the cell-free supernatant of TISTR 2688 could inhibit the oral bacterial pathogens tested. Some reports have indicated antimicrobial activity for *L. paracasei* isolated from humans against oral pathogens. *L. paracasei* SD1 displayed strong inhibitory effects against *S. mutans, S. sobrinus, S. sanguinis, P. gingivalis and A. actinomycetemcomitans* (5). It was proven that the inhibitory activity of *L. paracasei* SD1 was due to paracasin SD1 (16). Rossoni et al. (4) demonstrated that culture filtrates from *L. paracasei* 25.4, *L. paracasei* 20.3 and *L. paracasei* 11.6 isolated from the oral cavities of caries-free subjects could decrease the growth of *S. mutans* UA159. It was proposed that the antibacterial activity of the culture filtrates might be due to metabolites secreted from these bacteria. To investigate whether the antimicrobial property of TISTR 2688 was due to bacteriocin or not, neutralized cell-free supernatant at pH 7 was tested for antimicrobial activity against the indicator microorganisms. No inhibition zone was observed. The results imply that the antimicrobial activity of cell-free supernatant obtained from TISTR 2688 might be due to organic acids such as lactic acid or acetic acid (17). In addition, the cell-free supernatant of TISTR 2688 could effectively inhibit *S. mutans'* formation of biofilms on prosthetic incisor teeth. Its antagonistic activity and ability to inhibit biofilm formation indicate the potential of TISTR 2688 as an antiplaque agent.

Lysozyme in the oral cavity is responsible for the antimicrobial function of saliva. Though salivary lysozyme has shown antagonism to *S. mutans* (18), many lactic acid bacteria have been found to resist lysozyme. *L. reuteri* and *L. vaginalis* isolated from poultry intestines showed >90% survival after exposure to 100 µg/mL of lysozyme for 120 min (19), while five isolates of *L. plantarum* obtained from fresh leaves showed >69% viability after 180 min exposure to 100 µg/mL lysozyme (20). The lysozyme concentration in human saliva is generally less than 100 µg/mL (21-22). In our study, TISTR

2688 showed 100% viability after exposure to lysozyme at a concentration of 1 mg/mL, which is 10 times greater than the concentrations tested in the other studies. The results indicate the strong ability of TISTR 2688 to overcome the severe conditions created by salivary lysozyme in the mouth.

Based on the recommendation of the Joint FAO/WHO Working Group on Guidelines for the Evaluation of Probiotics in Food (23), TISTR 2688 was also studied for antibiotic resistance patterns. In the case of *L. paracasei*, the vancomycin resistance is considered safe because it is intrinsic and cannot be transferred to other bacteria (24). By contrast, resistance to ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, kanamycin, streptomycin and tetracycline are regarded as acquired resistance, which can cause the spread of antibiotic resistance genes to other bacteria via horizontal gene transfer (25–26). Therefore, lactic acid bacteria exhibiting transferable antibiotic resistance should not be used as starters, probiotics or ingredients in foods or dietary supplements. Based on this criterion, TISTR 2688 was found to be safe for use in humans in terms of antibiotic susceptibility. No phenotype of transferable antibiotic resistance was detected.

Conclusion:

L. paracasei TISTR 2688 isolated from fermented termite comb has been proven to possess antagonistic activity against oral pathogenic bacteria and to prevent plaque formation by *S. mutans* on prosthetic incisor teeth. In addition, TISTR 2688 was susceptible to antibiotics clinically important for medical treatment and showed no sign of acute oral toxicity in female rats at the concentration of 1×10^{10} CFU/kg body weight.

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