Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Antibacterial activity of viable and heat-killed probiotic strains against oral pathogens

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Significance and Impact of the Study: Our study provides insights into the antipathogenic efficacy of different probiotic species and their potential roles in developing functional foods to improve oral health. We showed that the probiotic strains *Lactobacillus salivarius* subsp. *salicinius* AP-32, *L. rhamnosus* CT-53, *L. paracasei* ET-66 and *Bifidobacterium animalis* subsp. *lactis* CP-9 have great potential for use in the development of functional foods to improve oral health. Since active probiotics may provide strong and long-term protection, the development of functional food products should favour the use of viable bacteria.

Keywords
antibacterial, functional health food, heat-killed, hydrogen peroxide, oral, pathogen, probiotic, viable.

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2019/2281: received 19 December 2019, revised 10 January 2020 and accepted 10 January 2020

doi:10.1111/lam.13275

Abstract

Probiotics can stabilize gut flora, regulate intestinal immunity and protect the host from enteric diseases; however, their roles in oral health have received little attention compared to their roles in gut health. Nowadays, the prevalence of sugar-sweetened foods and abuse of antibiotics contribute towards dysbiosis of oral microbiota and drug resistance development in oral pathogens, resulting in various intractable oral diseases. We screened the antibacterial activities of viable and heat-killed probiotic strains against the oral pathogens *Streptococcus mutans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*. The probiotic strains *Lactobacillus salivarius* subsp. *salicinius* AP-32, *L. rhamnosus* CT-53, *L. paracasei* ET-66 and *Bifidobacterium animalis* subsp. *lactis* CP-9 displayed strong antipathogenic activities, whereas heat-killed AP-32, CT-53 and ET-66 displayed high levels of pathogen inhibition. The antibacterial activities of these probiotics were not associated with their H_2O_2 production; *L. acidophilus* TYCA02 produced high levels of H_2O_2 but merely exhibited moderate antibacterial activities. Oral tablets containing probiotics showed positive inhibitory effects against oral pathogens, particularly those containing viable probiotics. Our results indicate that probiotics prevent the growth of oral pathogens and improve oral health, providing insights into the antipathogenic efficacy of different probiotic species and their potential role in functional foods that improve oral health.

Introduction

Probiotics such as lactobacilli and bifidobacteria are beneficial microbes that mainly colonize the intestinal tract, which is inhabited by 500–1000 different microbial species. Imbalance of intestinal microbiota can result in infective diseases. Probiotics are known to stabilize gut microflora, regulate the immune system, prevent infection and maintain human health (Pickard *et al.* 2017; Azad *et al.* 2018). Therefore, studying the role of probiotics in gut microbiota can aid in the development of novel treatments for these diseases (de Almada *et al.* 2015). The

majority of these physiological functions of probiotics are directly or indirectly dependent on their antimicrobial activities; for instance microbes can produce short-chain fatty acids and distinct antimicrobial molecules that inhibit the growth of various enteric pathogens, including *Clostridium difficile* (Baktash *et al.* 2018), *Staphylococcus aureus* (Wang *et al.* 2014), *Escherichia coli* and *Klebsiella pneumonia* (Nagpal *et al.* 2018). Several functional probiotics can even secrete hydrogen peroxide (H_2O_2) to fight against *Salmonella* (Pridmore *et al.* 2008), whereas lactic acid bacteria (LAB) secrete antibacterial proteins that exert highly effective antipathogenic activities (Gaspar *et al.* 2018). Thus, maintenance of viability and probioactives is essential for probiotics to perform their functions and confer health benefits to consumers (Champagne *et al.* 2018).

Streptococcus mutans, *P. gingivalis*, *F. nucleatum* and *Aggregatibacter actinomycetemcomitans* are notorious oral pathogens. *Streptococcus mutans* grows alongside other oral microbes and forms biofilms to adhere to periodontal tissues, causing dental caries and dental plaques (Forssten *et al.* 2010), whereas *P. gingivalis* produces proteinase and digests collagen, leading to periodontitis (Griffen *et al.* 1998). *Fusobacterium nucleatum*, a critical component of periodontal plaque, also plays a role in periodontal disease (Signat *et al.* 2011), whereas oral *A. actinomycetemcomitans* infection is often associated with aggressive periodontitis and halitosis (Amou *et al.* 2014). Nowadays, various antibiotics, mouthwashes and dental gels are used to treat these oral diseases (Arteagoitia *et al.* 2018; Haque *et al.* 2019), however, multiple antibiotic-resistant oral pathogens can arise due to the abuse of these drugs. Therefore, using probiotics to control the growth of oral pathogens and maintain the stability of the oral microbiota is a promising option for preventing antibiotic resistance when treating oral diseases (Mahasneh and Mahasneh 2017).

To improve the viability and stability of probiotics in functional food products, prebiotics, polyols and non-bovine milk are applied in the food industry. However, these supplements may significantly alter the sensory properties of foods (Ranadheera *et al.* 2018; Kalicka *et al.* 2019). This has triggered the emergence of novel concepts such as paraprobiotics and postbiotics. Inactivated probiotic cells and refined probioactives from probiotics may also confer health benefits to consumers (Champagne *et al.* 2018). Paraprobiotics and postbiotics are relatively stable in comparison to live probiotics and can be used in a wide pH range and heat-processed foods. Furthermore, they provide a better quality control and a safer alternative for manufacturers and vulnerable individuals (Almada *et al.* 2016; Barros *et al.* 2019). Therefore, more studies on paraprobiotic and postbiotic application in functional foods are urgently required.

In this study, we evaluated the antibacterial activities of different probiotic isolates against *S. mutans*, *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* and investigated the inhibitory role of heat-killed probiotic strains (paraprobiotics) against oral pathogens. Our study provides insights into the antipathogenic efficacy of different probiotic and paraprobiotics and their potential roles in functional health foods to improve oral health.

Results and discussion

Lactobacillus salivarius subsp. *salicinius* AP-32, *L. rhamnosus* CT-53, *L. paracasei* ET-66 and *B. animalis* subsp. *lactis* CP-9 show strong antibacterial activity against oral pathogens

Due to the slow progress in the discovery of novel antibiotics and rapidly emerging antibiotic resistance in pathogens, living therapeutics have attracted increasing attention recently (Kota *et al.* 2018). Probiotics are natural symbiotic microbes that reside in the gut, skin and mucosal tissue, and their use in oral healthcare is considered promising due to their moderate effects and natural quality (Rastogi *et al.* 2011). To investigate the role of probiotic isolates in oral health and to develop functional probiotic products against infectious oral diseases, we coincubated 12 distinct probiotic strains with *S. mutans*, *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* and evaluated the size of the pathogen inhibition zones produced. *Lactobacillus salivarius* subsp. *salicinius* AP-32, *L. rhamnosus* CT-53, *L. paracasei* ET-66 and *B. animalis* subsp. *lactis* CP-9 displayed strong antibacterial activity against the four oral pathogens, with inhibition zones larger than 2 cm in width. In contrast, *L. reuteri* GL-104, *L. rhamnosus* F-1 and *L. helveticus* RE-78 displayed low inhibition scores (Table 1 and Fig. S1). These results suggest that functional oral health products should use the AP-32, CT53, ET-66 and CP-9 strains.

Antipathogenic activity may not be associated with high probiotic H_2O_2 production

Next, we evaluated H_2O_2 production in these probiotic strains to examine the possible mechanisms of their antibacterial activities and teeth whitening functions. Surprisingly, high H_2O_2 production did not effectively reflect the inhibition of oral pathogens. Although *L. acidophilus* TYCA02 secreted high levels of H_2O_2 in PIPES buffer after incubation for 5 h (42·4 and 10·6 mg l^{-1} respectively), the strain only displayed moderate oral pathogen inhibition (Fig. 1). Moreover, *L. rhamnosus* CT-53, ET-66 and CP-9

Table 1 The inhibition scores of viable lactic acid bacteria against four distinct oral pathogens

Strains	AP-32	GL-104	CT-53	RE-78	TYCA02	ET-66	BB-115	CP-9	GL-156	F-1
Streptococcus mutans	3	0	3	2	3	3	3	3	3	2
Porphyromonas gingivalis	2	1	2	2	2	3	3	3	3	2
Fusobacterium nucleatum	3	2	3	2	2	3	2	3	2	2
Aggregatibacter actinomycetemcomitans	3	0	3	1	2	3	2	3	2	1
Total	11	3	11	7	9	12	10	12	10	7

Scores denote: 0 = <1-cm wide inhibition zone; 1 = 1- to 2-cm-wide inhibition zone; 2 = 2- to 3-cm wide-inhibition zone; 3 = ≥ 3-cm-wide inhibition zone.

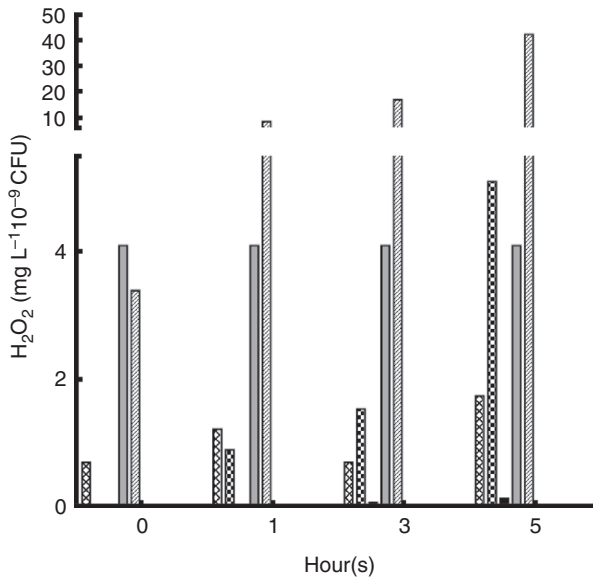

Figure 1 Analysis of probiotic hydrogen peroxide production. The selected probiotic strains (*Lactobacillus salivarius* AP-32, *L. reuteri* GL-104, *L. rhamnosus* CT-53 and F-1, *L. paracasei* ET-66 and GL-156, *L. helveticus* RE-78, *L. acidophilus* TYCA02 and *B. animalis* subsp. lactis BB-115 and CP-9) were washed and incubated in PIPES buffer, and the concentrations of the hydrogen peroxide produced were analysed at the indicated time points using hydrogen peroxide test strips. The bar graph data represent the mean of two independent experiments ((▨) AP-32; (▩) GL-104; (■) CT-53; (▢) RE-78; (▨) TYCA02; (▦) ET-66; (▨) BB-115; (▧) CP-9; (▥) GL-156; (▤) F-1).

did not produce H_2O_2, but displayed high bactericidal activities, suggesting that H_2O_2 may not be a major antibacterial compound secreted by the probiotic strains. These results indicate that TYCA02 could be a functional probiotic strain for teeth whitening and that H_2O_2 may not be a key factor in the prevention of oral diseases.

Heat-killed *L. salivarius* subsp. *salicinius* AP-32, *L. rhamnosus* CT-53 and *L. paracasei* ET-66 exhibit strong inhibition of oral pathogens

As described in Fig. 1, H_2O_2 secreted from viable probiotic strains played no predominant role in the inhibition of oral pathogens; therefore, we examined the antipathogenic activities of heat-inactivated probiotics. The heat-killed probiotics exerted poor antibacterial activities against *S. mutans* and *A. actinomycetemcomitans,* which displayed survival rates of almost 100% in the presence of most of the inactivated probiotic strains. Heat-killed CT-53 was the only functional strain with antibacterial activities, reducing the survival rate of *S. mutans* by approximately 40% (Fig. 2a). The survival rate of *A. actinomycetemcomitans* was slightly reduced in the presence of heat-killed TYCA02, ET-66 and F-1 (Fig. 2d), whereas *P. gingivalis* and *F. nucleatum* were susceptible to most of the heat-killed probiotic strains. Notably, heat-killed AP-32 and CP-9 inhibited *P. gingivalis* by almost 100%, whereas heat-killed GL-104, CT-53 and F-1 inhibited *P. gingivalis* by 70–80% and ET-66 and *L. paracasei* GL-156 inhibited *P. gingivalis* by 50–60% (Fig. 2b). Heat-killed AP-32, GL-104, RE-78 and GL-156 suppressed *F. nucleatum* survival by 90–100%, whereas inactivated CT-53, ET-66, *B. animalis* subsp. *lactis* BB-115 and F-1 inhibited *F. nucleatum* by 50–75% (Fig. 2c). Overall, heat-killed AP-32, CT-53 and ET-66 displayed the highest oral pathogen inhibition scores (Table 2).

Viable CP-9 displayed strong growth-inhibitory effects against *S. mutans* and *F. nucleatum*, whereas significant reduction of antibacterial activity was observed after heat inactivation, indicating that CP-9 mainly inhibits these oral pathogens through the production of heat-sensitive antimicrobial molecules or competition for nutrients. Conversely, it has been reported that *L. salivarius* BGHO1, isolated from the human oral cavity, produces salivaricin LS1 and LS2 to suppress the growth of pathogenic bacteria, including *Streptococcus* and *Salmonella* (Busarcevic *et al.* 2008; Messaoudi *et al.* 2013). AP-32 may inhibit oral bacteria by producing salivaricin, which is resistant to heat treatment as its antipathogenic activity remains strong after boiling in a water bath for 15 min (Busarcevic *et al.* 2008). This suggests that salivaricin plays an essential role in the suppression of oral pathogens by AP-32. In addition, to fight intestinal bacteria, *L. rhamnosus* produces antimicrobial peptides, which are heat-stable molecules (4 kDa) that are active over a wide

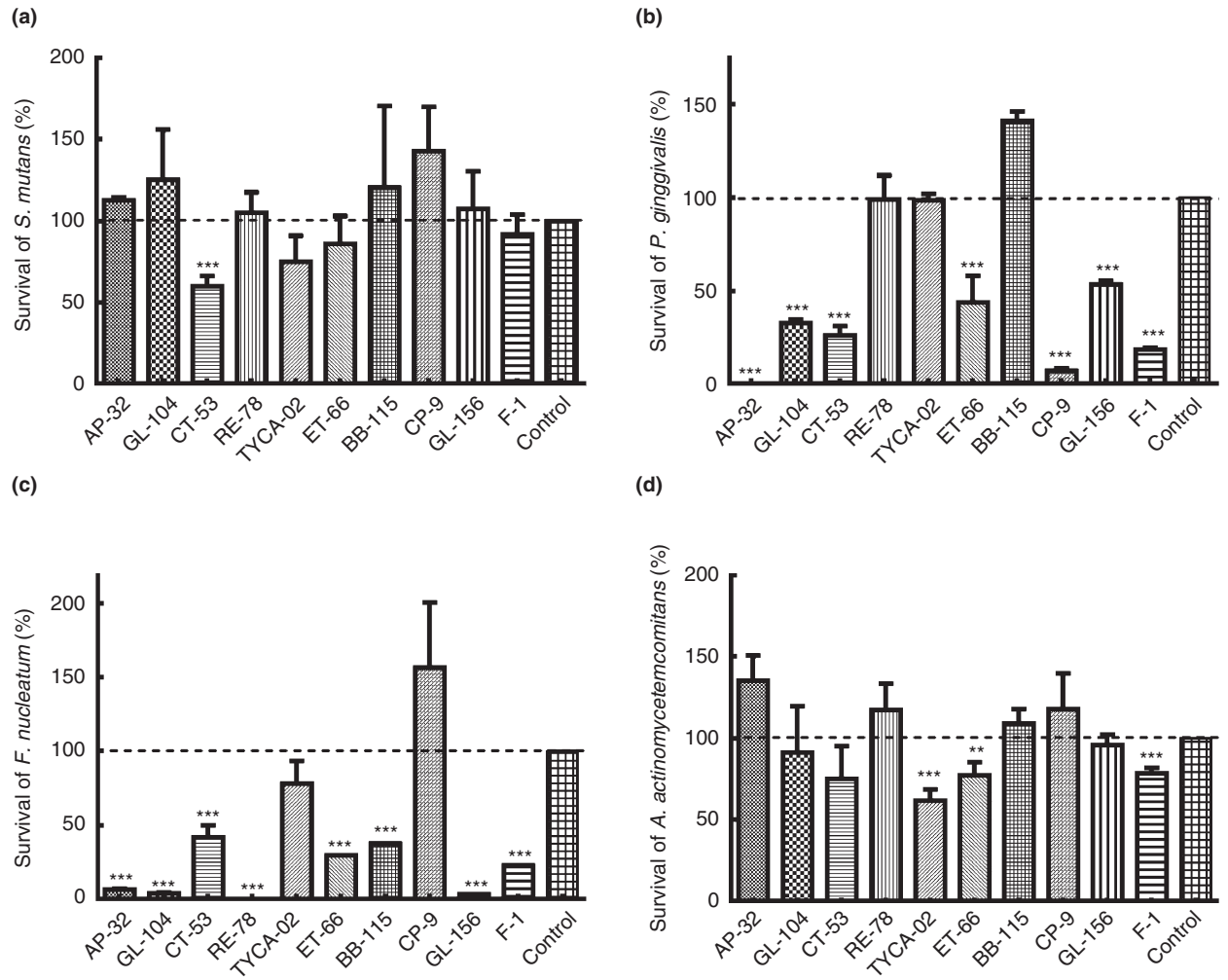

Figure 2 Bacteriostatic activity of heat-killed probiotics. Probiotic cells were heated to 100°C for 1 h, and the heat-killed probiotic cells (10^9 per ml) were co-cultured with oral pathogens (MOI = 0·01) at 37°C for 48 h. (a) *Streptococcus mutans*, (b) *P. gingivalis*, (c) *F. nucleatum* and (d) *A. actinomycetemcomitans* survival rates were calculated using a CFU assay with reference media control. The bar graph data represent the mean ± SD of three independent experiments. The dashed line on the graphs indicates the survival rate of the media-only control. Statistical analyses were performed using two-tailed *t*-tests, and significant differences were observed compared to the media control (***P* < 0·01; ****P* < 0·005).

Table 2 The inhibition scores of heat-killed lactic acid bacteria against four distinct oral pathogens

Strains	AP-32	GL-104	CT-53	RE-78	TYCA02	ET-66	BB-115	CP-9	GL-156	F-1
Streptococcus mutans	0	0	2	0	1	1	0	0	0	0
Porphyromonas gingivalis	4	3	3	0	0	4	0	4	2	3
Fusobacterium nucleatum	4	4	2	4	1	4	3	0	4	2
Aggregatibacter actinomycetemcomitans	0	0	1	0	1	2	0	0	0	1
Total	8	7	8	4	3	11	3	4	6	6

Scores denote: 0 = 0% inhibition rate; 1 = ~10–40% inhibition rate; 2 = ~40–60% inhibition rate; 3 = ~60–80% inhibition rate; 4 = ~80–100% inhibition rate.

pH range (Oscariz and Pisabarro 2001). CT-53 may secrete these molecules to inhibit the growth of oral pathogens. Furthermore, ET-66 may kill bacteria by producing Paracin 1·7, a heat-resistant bacteriocin first discovered in *L. paracasei* isolated from Chinese sauerkraut juice. The bacteriocin shows a wide range of antibacterial activities and suppresses the growth of various gram-positive and -negative bacterial pathogens, such as *Staphylococcus* and *Salmonella* (Ge *et al.* 2016). However, further studies on the isolation and identification of the effective antibacterial molecules in AP-32, ET-66 and CT-53 are required. Overall, these findings suggested that heat-killed AP-32, CT-53 and ET-66 produce heat-stable antibacterial molecules, which can be widely applied to heat-processed foods.

Oral tablet containing viable probiotics shows higher inhibitory efficacy against oral pathogens

On the basis of the results of the antipathogenic assays, we selected AP-32, CP-9 and ET-66 as potential food additives and produced oral tablets to improve oral health. The antibacterial analyses revealed that the oral tablets containing viable probiotics killed oral pathogens more efficiently than the vehicle controls, reducing the survival of the four oral pathogens by approximately 80–95% (Fig. 3a). Furthermore, the oral tablets containing heat-inactivated AP-32 and ET-66 displayed moderate antibacterial activities against *P. gingivalis* but exhibited reduced or no antibacterial activities against *S. mutans*, *F. nucleatum* and *A. actinomycetemcomitans* (Fig. 3b). Despite strong inhibition of *F. nucleatum* by both heat-killed AP-32 and ET-66, the tablet failed to suppress the growth of *F. nucleatum*. This was probably due to the effects of additive excipients in tablets that may neutralize antibacterial materials or facilitate the growth of pathogens. Active probiotics could constantly produce antibacterial molecules and compete with the pathogens to acquire nutrients to maintain their physiological activities. This not only reduces pathogens in the oral cavity, but also inhibits food-borne pathogens. Foods containing viable probiotics may downregulate the transcription of virulence genes in *Listeria* and *Campylobacter*, preventing food contamination and spoilage (Khaneghah *et al.* 2019). Thus, functional food products should favour the use of viable bacteria.

Oral pathogens form biofilms or dental plaques with other oral microbes in the oral cavity, which consist of the extracellular matrix (polysaccharides and glycoproteins) secreted by oral micro-organisms (Huang *et al.* 2011). The matrix blocks direct contact between antibiotics and pathogens, significantly reducing the sensitivity of the antibiotics and causing multiple drug resistance in the oral pathogens (Kouidhi *et al.* 2015). Although mouthwashes containing chlorhexidine, gluconate or cetylpyridinum chloride reduce the growth of oral bacteria, bacteria embedded in biofilms are more resistant to a mouthwash than those in the planktonic state (Masadeh

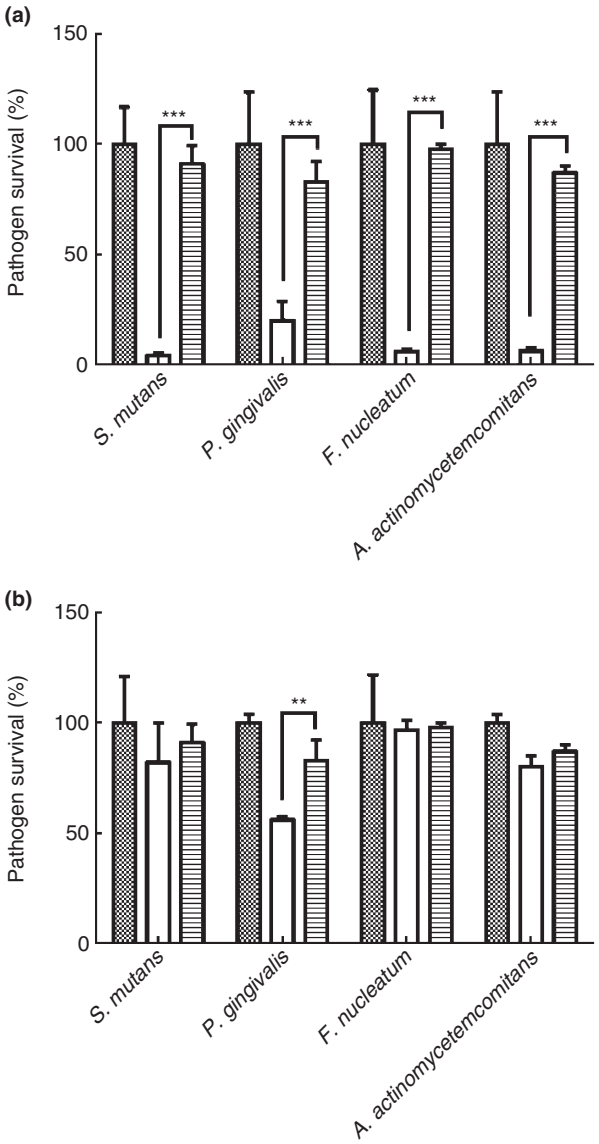

Figure 3 Antibacterial activity of heat-killed lactic acid bacteria (LAB) tablets. LAB tablets containing (a) viable or (b) heat-killed probiotic cells were dissolved in either TSB or BHI medium at a concentration of 0·1 g ml^{-1}. Oral pathogens (10^6 CFU) were co-incubated in the tablet solutions at 37°C for 2 or 3 days. Oral pathogen survival rates were determined using a CFU assay with reference to the media-only control. The bar graph data represent the mean ± SD of three independent experiments, dotted bars = blank, white bar = LAB tablets (viable or heat-killed probiotics), striped bar = vehicle control. Statistical analyses were performed using two-tailed *t*-tests (**$P < 0·01$; ***$P < 0·005$).

et al. 2013). Thus, probiotics are a promising alternative for treating dental biofilms and plaques (Mahasneh and Mahasneh 2017). Nevertheless, further studies are required to investigate the clearance or reduction of oral biofilms by AP-32, ET-66, CT-53 and CP-9. These studies would contribute towards the development of probiotic functional foods for improving oral health.

Overall, our study showed that AP-32, ET-66, CT-53 and CP-9 are probiotic strains that exhibit great potential for the development of functional foods for oral health. Probiotics may produce antibacterial molecules such as short-chain fatty acids and antimicrobial peptides to suppress the growth of oral pathogens. Short-chain fatty acids cause growth inhibition of pathogens by altering transmembrane pH gradients (Sun and O'Riordan 2013). Antimicrobial peptides get inserted into the membrane of pathogens, leading to their lysis (Mallapragada *et al.* 2017). Although these molecules are heat-stable and are active in a wide pH range, heat-inactivated probiotics fail to colonize the oral cavity and constantly produce antimicrobial molecules. Thus, these functional food products should favour the use of viable bacteria, since active probiotics can provide strong and long-term protection.

Materials and methods

Bacterial strains and cultivation

Active and dry *L. salivarius* (AP-32), *L. reuteri* (GL-104), *L. rhamnosus* (CT-53, F-1), *L. paracasei* (ET-66, GL-156), *L. helveticus* (RE-78), *L. acidophilus* (TYCA02) and *B. lactis* (BB-115, CP-9) were obtained from Glac Biotech Co. Ltd (Tainan city, Taiwan). The *Lactobacillus* sp. and *Bifidobacterium* sp. were cultured in MRS (De Man, Rogosa and Sharpe) broth (BD, San Jose, CA) with or without 0·05% cysteine respectively. The LAB were incubated under anaerobic conditions at 37°C for 20 h. The probiotics were collected and diluted to the indicated concentrations.

Oral pathogens and incubation

The oral pathogenic bacteria *S. mutans* (Bioresource Collection and Research Center; BCRC no: 10793T, Hsinchu, Taiwan), *P. gingivalis* (BCRC no: 17689 and 17688 Hsinchu, Taiwan), *F. nucleatum* subsp. *polymorphum* (BCRC no: 17679 Hsinchu, Taiwan) and *A. actinomycetemcomitans* (BCRC no: 14405 Hsinchu, Taiwan) were purchased from the BCRC, Hsinchu City, Taiwan. *S. mutans* was cultured in tryptic soy broth (TSB; BD) (Baktash *et al.* 2018), *P. gingivalis* and *F. nucleatum* subsp. *polymorphum* were cultured in TSB supplemented with 5% sheep's blood and *A. actinomycetemcomitans* was cultured in brain–heart infusion (BHI) broth (BD). All the pathogens were incubated at 37°C for 48 h, and then collected for subsequent experiments.

Analysis of the bacteriostatic activities of viable probiotics

A modified agar overlay method was used to study the antimicrobial activities of the probiotics against oral pathogenic bacteria (Strus *et al.* 2005). Briefly, the selected probiotics (10^9 per ml) were streaked over MRS agar plates with a cotton swab and cultured under semi-anaerobic conditions at 37°C for 48 h, to produce a 2-cm-wide probiotic growth zone. Next, 45°C TSB (*S. mutans, P. gingivalis* and *F. nucleatum* subsp. *polymorphum*) or 45°C BHI (*A. actinomycetemcomitans*) was added to the agar plates. After the agar had solidified, oral pathogenic bacteria were individually inoculated on the plate surface and further incubated for 48 h at 37°C. Bacteriostatic activity was determined by measuring the width of the zones of inhibition using a semi-quantitative scoring system (− to +++).

Analysis of bacteriostatic activities of heat-killed probiotics

To evaluate the antimicrobial activity of the heat-killed LAB, the strains were cultured in MRS or M77 media at 37°C for 20 h and then killed by incubating at 100°C for 1 h. A total of 10^9 heat-killed probiotic cells per ml were co-cultured with the oral pathogens (multiplicity of infection, MOI = 0·01) at 37°C for 48 h. The survival rates of the oral pathogens were determined by comparing their colony formation units (CFUs) to the media-only control. The rates of oral pathogen inhibition were calculated as follows: $(CFU_{media\ control} - CFU_{experimental\ group})/CFU_{media\ control}$ (%).

Antibacterial activity of the LAB tablets

Lactic acid bacteria tablets (1 g) containing viable (AP-32, CP-9 and ET-66; $>10^9$ CFU) or heat-killed (AP-32 and ET-66, $>10^{10}$ CFU) probiotic strains were produced by Glac Biotech Co. Ltd. The LAB tablets were dissolved in either TSB or BHI medium at a concentration of 0·1 g ml^{-1} and then, oral pathogens (10^6 CFU) were introduced into the tablet solutions and co-incubated at 37°C for 2 (*S. mutans*) or 3 (*P. gingivalis*, *F. nucleatum* subsp. *polymorphum* and *A. actinomycetemcomitans*) days. The survival rates of the oral pathogens were determined using a CFU assay compared to the media control.

Determination of H_2O_2 production

Lactic acid bacteria were passaged at least three times in MRS media to stabilize their growth rates, transferred into piperazine-N,N′-bis solution (PIPES) and incubated at 37°C for 5 h with shaking (220 rev min^{-1}). The LAB were then centrifuged and the supernatant was collected after 0, 1, 3 and 5 h. The H_2O_2 levels in the supernatants were detected using H_2O_2 test strips (cat. no. 118789; Merck Millipore, Burlington, MA).

Statistical analyses

All statistical analyses were performed using Microsoft Excel or the GraphPad software. Data represent the mean or mean ± SD of at least two or three independent experiments. Differences were analysed using two-tailed *t*-tests. *P* values of <0·05 (*) were considered significant and those <0·01 or 0·005 (**, ***) were considered highly significant.

Acknowledgements

We thank Glac Biotech Co., Ltd, Tainan City, Taiwan for the provision of probiotic strains and production of oral tablets. The work was funded by Glac Biotech Co., Ltd, Tainan City, Taiwan.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bacteriostatic activity of probiotic strains.