

Assessing the drug resistance profiles of oral probiotic lozenges

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ABSTRACT

Background: Probiotic lozenges have been developed to harvest the benefits of probiotics for oral health, but their long-term consumption may encourage the transfer of resistance genes from probiotics to commensals, and eventually to disease-causing bacteria.

Aim: To screen commercial probiotic lozenges for resistance to antibiotics, characterize the resistance determinants, and examine their transferability *in vitro*.

Results: Probiotics of all lozenges were resistant to glycopeptide, sulfonamide, and penicillin antibiotics, while some were resistant to aminoglycosides and cephalosporins. High minimum inhibitory concentrations (MICs) were detected for streptomycin (>128 µg/mL) and chloramphenicol (> 512 µg/mL) for all probiotics but only one was resistant to piperacillin (MIC = 32 µg/mL). PCR analysis detected erythromycin (*erm(T)*, *ermB* or *mefA*) and fluoroquinolone (*parC* or *gyr(A)*) resistance genes in some lozenges although there were no resistant phenotypes. The *dfpD*, *cat-TC*, *vatE*, *aadE*, *vanX*, and *aph(3'')-III* or *ant(2'')-I* genes conferring resistance to trimethoprim, chloramphenicol, quinupristin/dalfopristin, vancomycin, and streptomycin, respectively, were detected in resistant probiotics. The rifampicin resistance gene *rpoB* was also present. We found no conjugal transfer of streptomycin resistance genes in our co-incubation experiments.

Conclusion: Our study represents the first antibiotic resistance profiling of probiotics from oral lozenges, thus highlighting the health risk especially in the prevailing threat of drug resistance globally.

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

Probiotics; antibiotic resistance; probiotic supplements; oral probiotics; probiotic lozenges; dental probiotics; *Lactobacillus*; horizontal gene transfer

Introduction


Oral lozenges containing a formulation of probiotic bacteria have been recently developed to harvest the benefits of probiotics for oral health. It is long known that probiotics can confer various health benefits such as improving the host immune system [1,2], preventing cancer and virus-inflammatory lung damage [3,4], decreasing cholesterol and preventing cardiovascular diseases [5], improving blood glucose and lipid profiles [6,7], preventing diabetes [8], and enhancing cognitive function and mental health [9]. As the health claims of probiotics continue to emerge, the number of foods containing probiotics also increases [10,11]. This is exemplified by the overwhelmingly popular probiotic health or dietary supplements targeted for improving intestinal health with beneficial claims ranging from regulating the gut microbiota [12], and reducing lactose intolerance [13], increasing bioavailability of nutrients [14], preventing gastrointestinal infections [15,16], and treating gastroenteritis and antibiotic-associated diarrhea [17]. In recent

years, probiotics have also been proposed as alternative to or as adjuvant for antibiotic treatments [18–22]. For instance, evidence *in vitro* showed that lactobacilli exhibit anti-carbapenem resistant enterococci while evidence in mouse models showed that *Lactobacillus paracasei* CNCM I-3689 reduces the drug resistant enterococci amount in the feces [23,24]. Moreover, recent clinical trials also showed that the colonization of multi-drug resistant pathogens in the human gut, can be counteracted by treatment with a mixture of probiotics during antibiotic therapies [25,26].

The benefits of probiotics in oral health have been consistently reported for the treatment of caries [27,28], periodontal disease [29–32], fungal infection [33], and halitosis [34,35], through synergistic mechanisms that include the inhibition of bacteria commonly associated with oral diseases such as *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Candida albicans* by antimicrobial compounds, enhancement of local or systemic immune responses, and out-competing disease-causing bacteria for

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 Supplemental data for this article can be accessed [here](#)

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adhesion [36]. Collectively, probiotics lead to direct antagonistic effects against pathogens and/or reduction of inflammation and tissue destruction [37,38]. Intestinal swallowable probiotic supplements in the form of capsules or tablets do not remain in the oral cavity long enough for probiotics to be retained in the mouth. As such, probiotic oral lozenges were developed to deliver probiotics directly to the mouth and enable them to adhere, form biofilms, and colonize oral cavity surfaces [39,40].

Much like probiotics for gut health, probiotic strains for oral health are predominantly *Lactobacillus* and *Streptococcus* strains, which are 'Generally Recognized as Safe (GRAS)' according to the European Food Safety Authority (EFSA) [41]. They were also granted the 'Qualified Presumption of Safety (QPS)' status by the U.S. Food and Drug Administration (FDA) [42] on the condition that they do not harbor known genes conferring resistance to clinically and veterinary important drugs [43]. However, recent studies have reported that probiotics in food supplements are resistant to multiple antibiotics [44,45]. This is in addition to the numerous reports of drug resistance in probiotics from other foods [46–50]. Since probiotic supplements contain much higher bacteria per serving compared to other foods, any adverse health effects would therefore be more pronounced [51,52]. One such health concern is the risk of transmitting resistance determinants. The long-term consumption of oral probiotic lozenges may encourage the transfer of resistance genes from probiotics to over 700 bacterial species in the oral cavity [53]. Over time, the oral microbiota may act as a reservoir for antibiotic resistance genes which could then be transferred to bacteria commonly associated with oral diseases such as *S. mutans* and *P. gingivalis* [54,55], thus rendering antibiotic treatments ineffective.

This health concern has been raised for probiotic foods harboring antibiotic resistance genes [46,51,52,56–68], and the same applies to the oral cavity because much like the gut, it contains a complex and rich diversity of microbiota [69]. Moreover, the nutrient-rich environment, suitable and stable temperature of around 37°C, a stable pH range of 6.5–7.0 considered ideal for most bacteria, as well as the moist and large surface areas in the oral cavity, further encourage the trafficking of resistance determinants [70]. While drug resistance of gut probiotic supplements has been reported, the resistance profiles of oral probiotic lozenges have not been examined. Thus, this study aims to analyze the resistance profiles of probiotics from oral lozenges and the transferability of resistance determinants.

Materials and methods

Probiotic lozenges, probiotic drinks, and antibiotics

Six popular brands of probiotic oral lozenges were purchased. Only probiotic supplements in the form of lozenges intended for dental applications or oral health were selected. Those in the form of capsules or tablets intended for gut health were eliminated from our selection. Products that contain heterogeneous populations of *Lactobacillus* spp. were prioritized. Other selection factors such as the overall reputation of the manufacturers, and the product ratings and reviews, were also considered. The probiotic oral lozenges are subsequently designated as A, B, C, D, E, F, G, and H, the product information such as the bacteria strains and amounts, and the country of manufacture are listed in Table 1. Commercially available *Lactobacillus* containing probiotic drinks such as probiotic milk, yogurt, and juice were purchased from local food markets in China.

Powdered or crystallized antibiotics: chloramphenicol, doxycycline, erythromycin, piperacillin, and streptomycin, were purchased from Sigma, USA. The antibiotics were dissolved in sterile Milli-Q water or ethanol to 10 mg/mL stock concentrations and stored at –20°C. Prior to the broth microdilution experiments, antibiotic stocks were diluted to 1 mg/mL and 0.1 mg/mL working concentrations as required.

Bacteria recovery and enumeration

To recover probiotic bacteria, oral probiotic lozenges were ground and dissolved in phosphate buffered saline (PBS) and immediately spread on De Man, Rogosa and Sharpe (MRS) agar which is selective for growing lactobacilli. The dissolved samples were cultured overnight in MRS broth at 37°C in an orbital shaker at 250 rpm for the enrichment of probiotic bacteria. To culture *S. mutans*, *Streptococcus gordonii*, *Streptococcus sanguinis*, and *Enterococcus faecalis*, the Brain Heart Infusion (BHI) medium was used. Glycerol stocks (50%, v/v) were prepared as required and stored at –80°C.

The drop plate method was used to enumerate probiotic bacteria. One hundred milligrams of probiotic lozenges were dissolved in 1 mL PBS and a series of dilutions from 10⁻¹ to 10⁻⁷ were prepared. Five microliters of each diluted sample were dropped onto MRS agar using aseptic methods and incubated for 48 h at 37°C. MRS plates with the appropriate dilutions that produce discernable single colonies of bacteria, were photographed, and enumerated using ImageJ. The enumerated viable bacteria were compared with the claims of the

Table 1. Oral probiotic lozenges product information.

Product label	Probiotic bacteria	Bacteria amount	Country of manufacture
A	<i>Lactobacillus paracasei</i>	1–3 billion	USA
	<i>Lactobacillus reuteri</i>	CFU	
	<i>Lactobacillus sakei</i>	per	
	<i>Lactobacillus salivarius</i>	lozenge	
B	<i>Streptococcus salivarius</i> M18	1 billion	USA
	<i>Lactobacillus plantarum</i> (heat treated)	per lozenge	
C	<i>Lactobacillus reuteri</i> DSM 17938	0.2 billion	Belgium
	<i>Lactobacillus reuteri</i> ATCC PTA 5289	CFU per lozenge	
D	<i>Lactobacillus acidophilus</i> HA-122	1–2 billion	USA
	<i>Lactobacillus casei</i> HA-108	CFU per lozenge	
	<i>Bifidobacterium bifidum</i> HA-132		
	<i>Lactobacillus rhamnosus</i> HA-111		
	<i>Lactobacillus salivarius</i> HA-188		
	<i>Lactobacillus brevis</i> CECT 7480	1–5 billion	
E	<i>Lactobacillus plantarum</i> CECT 7481	CFU per lozenge	USA
	<i>Pediococcus acidilactici</i> CECT 8633		
	<i>Lactobacillus acidophilus</i>	2–6 billion	
	<i>Lactobacillus reuteri</i>	CFU	
F	<i>Lactobacillus paracasei</i>	per	Canada
	<i>Lactobacillus salivarius</i>	lozenge	
	<i>Streptococcus thermophilus</i>		
	<i>Streptococcus salivarius</i> K12		
	<i>Streptococcus salivarius</i> M18		
	<i>Lactobacillus paracasei</i>	1–3 billion	
G	<i>Lactobacillus reuteri</i>	CFU per lozenge	USA
H	<i>Lactobacillus reuteri</i> DSM 17938	0.2 billion	Belgium
		CFU per 5 drops	

manufacturers on the product label or information sheet (Table 1). Only contributions from *Lactobacillus* strains were considered. Bacteria enumeration was conducted in biological triplicates with each containing nine droplets of the dissolved samples.

Identification of probiotic isolates by 16S rRNA sequencing

The bacterial genomic DNA was extracted with lysozyme treatment following a Gram-positive bacteria lysis protocol using the QIAprep Miniprep Kit (Qiagen, MD) according to the manufacturer's instructions. The concentration and purity of extracted DNA were determined on a NanoDropOne spectrophotometer (ND-ONE-W, ThermoFischer, WI) before being sent to Genewiz, Inc. (Suzhou, China) for PCR detection and

sequencing of the 16S rRNA using the universal primers 27 F: 50-AGAGTTTGTATCCTGGCTCAG-30, and 1492 R: 50-GGTTACCTTGTTACGACTT-30. Resulting sequences of approximately 1,500 bp were compared to the NCBI GenBank database using the blastn tool [71,72].

Antibiotic susceptibility test

The disc diffusion method was used to screen for antibiotic susceptibility against a wide range of antibiotics. Commercial antibiotic discs of different classes were purchased from HiMedia, India. Probiotic lozenges dissolved in PBS were incubated overnight in MRS broth at 37°C and adjusted to 7×10^6 CFU/mL ($OD_{600} = 0.6$) prior to the disc diffusion and broth microdilution assays. In the diffusion test, one hundred microliters of bacteria culture were spread evenly onto MRS agar before carefully placing the antibiotic ring on the bacteria lawn. One antibiotic ring contained 12 antibiotic discs and a total of 4 different antibiotic rings: Dodeca G-I-Plus (DE002), Dodeca G-II-Plus (DE009), Dodeca G-III-Plus (DE018), and Dodeca G-IV-Plus (DE023), containing a total of 31 unique antibiotics with different mode of actions, were used. After 48 h of incubation at 37°C, the plates were photographed and the diameter of inhibition (clear) zones forming around the antibiotic discs were measured by ImageJ. Antibiotic susceptibility tests were conducted at least twice, and each antibiotic was tested at least four times on the same sample. Bacteria are determined to be resistant to an antibiotic if the inhibition zones had diameters less than $2 \times$ the diameter of the antibiotic disc i.e. <12 mm, while they were determined to be partially resistant if the inhibition zones had diameters between 12 and 15 mm. The minimum inhibitory concentrations (MICs) were determined for the representative antibiotics: chloramphenicol, doxycycline, erythromycin, piperacillin, and streptomycin, representing different classes of antibiotics, on 96-well plates using the broth dilution method. The MICs were determined from the dose response curves and compared to the cut-off values for resistance as determined in the guidelines provided for *Lactobacillus* spp. by the European Food Safety Authority (EFSA) [43]. In this study, a conservative approach was adopted in assigning resistance to probiotics. For the disc diffusion assay, the quality control data in the manufacturer's technical guide which followed the performance standards for antimicrobial disk susceptibility tests of the Clinical and Laboratory Standards Institute (CLSI) [73], suggested that inhibition zones with diameters less than three times the diameters of the antibiotic discs (<18 mm) were considered resistant for most antibiotics when tested on representative Gram positive (*Staphylococcus aureus* ATCC 259230) and Gram negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) bacteria but, we assigned resistance only if the

inhibition zones were less than two times the diameter of the antibiotic discs (<12 mm). Likewise, for the broth microdilution assays, resistance was only assigned if the MIC values exceeded the thresholds of all *Lactobacillus* spp. listed by the EFSA [43].

Detection of antibiotic resistance genes by PCR

Genomic DNA from the probiotics of oral lozenges were extracted using the QIAprep Miniprep Kit (Qiagen, MD). The concentration and purity of extracted DNA was determined on a NanoDropOne spectrophotometer (ND-ONE-W, ThermoFischer, WI). Gene-specific primers for known antibiotic resistance genes, such as gentamicin, streptomycin, kanamycin, neomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, vancomycin, quinupristin/dalfopristin, linezolid, trimethoprim, rifampicin, ciprofloxacin, and others, were purchased from Sangon Biotech, Shanghai, China. The respective annealing temperatures and amplicon sizes were determined from the literature (Table 2). The amplification program was as follows: initial denaturation step at 94°C for 5 min; 30 cycles of: 94°C for 45 s, annealing temperature for 45 s and 72°C for 45 s; and a final extension of 10 min at 72°C. The amplicons were analyzed on 1% (w/v) agarose gel to confirm the DNA fragment size.

Conjugative transfer of resistance genes from probiotics to bacteria implicated in diseases

To examine the transmissibility of resistance genes from probiotics of oral lozenges to bacteria commonly associated with diseases during co-incubation *in vitro*, the liquid culture and filter mating technique were employed. Representative probiotics from one oral lozenge which was determined to be resistant to streptomycin, were mixed with the recipients i.e. *S. mutans*, *S. gordonii*, *S. sanguinis*, or *E. faecalis* that were susceptible to streptomycin at a probiotic-to-recipient ratio of 1:1 and 10:1, respectively. The mixed cultures were incubated for 24 h at 37°C and then spread on MRS agar which is selective for lactobacilli with and without 100 µg/mL of streptomycin, and on Mannitol Salt Agar (MSA) which is selective for *Streptococcus* strains and *E. faecalis* with and without 100 µg/mL of streptomycin, respectively. As controls, monocultures of probiotics and recipients were grown and spread on plates alongside the co-cultures.

Another 2 mL of bacteria mixtures were filtered through a sterile nitrocellulose MCE membrane filter MF-Millipore (2.5 cm diameter, 0.45 µm pore size, Merck Millipore, Ireland) using a Millipore pump with a negative pressure of -50 kPa. The membrane filters were then carefully placed onto BHI agar, in

which both donor and recipient are culturable. As controls, 2 mL of pure probiotics and recipient cultures were also passed through the membrane filters and grown on BHI agar plates respectively. After 72 h of incubation at 37°C, the membrane filters were placed in 1 mL PBS in a sterile microcentrifuge tube and vortexed to free the bacteria. Another 1 mL PBS was used to wash the plates and the washings were placed in the sterile tube. Serial dilutions were made and 50 µL of each diluted sample were spread onto selective agar plates.

The liquid culture and filter-mating experiments were repeated at least three times in duplicates. The colonies on the selective agar were observed after 2 days of culture. If there was a transfer of resistance gene from the streptomycin resistant probiotic to the recipient, the colonies of the recipient bacteria would be detected on the streptomycin containing MSA agar.

Results and discussion

Recovery and enumeration of probiotics from oral lozenges

Probiotic bacteria, predominantly *Lactobacillus* spp. from eight commercial probiotic lozenges labelled A, B, C, D, E, F, G, and H (Table 1), were recovered on MRS agar, and enumerated using the drop plate method. Since MRS is selective for *Lactobacillus*, only contributions from these strains of bacteria were taken into consideration in our analysis. Except for product B, all probiotic lozenges contained live bacteria. Product B contained heat-killed probiotic bacteria, and thus were unable to be recovered in the laboratory. Although previous studies have shown that heat-inactivated probiotics could still inhibit oral bacteria such as *S. mutans*, *P. gingivalis*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans*, it was suggested that long-term protection against these presumed pathogens would require active probiotics [90]. Based on the boxplot in Figure 1, all products except product A contained live bacteria that were about 0.2–10% fewer than that claimed by the manufacturers. Product A contained live bacteria that was comparable to the claims of the manufacturer. It must be noted that the oral lozenges dissolved in PBS were immediately spread onto MRS agar to avoid exposure to conditions that might affect bacteria viability *in vitro*.

Left panel shows a box and whisker plot of enumerated bacteria amounts for probiotics recovered on agar plates (dark boxes) from commercially available oral lozenges labelled A, B, C, D, E, F, G, and H, respectively, compared to the amounts claimed by the manufacturers (light boxes). Product B contained heat-killed probiotic

Table 2. Primer sequences and PCR conditions for the detection of antibiotic resistance genes (ARG).

Antibiotic	ARG	Primers 5'-3'		Annealing T. (°C)	Amplicon size (bp)	Reference
		Forward, top;	Reverse, bottom)			
Gentamicin	<i>aac(6')-aph(2'')</i>	CCAAGAGCAATAAGGGCATA	CACTATCATAACCACTACCG	60	220	[128]
	<i>aac(6')leaph(2'')la</i>	CAGAGCCTTGGGAAGATGAAG	CCTCGTGAATTCATGTTCTGGC	58	348	[74]
Streptomycin	<i>strA</i>	CTTGGTGATAACGGCAATTC	CCAATCGCAGATAGAAGGC	55	548	[129]
	<i>strB</i>	ATCGTCAAGGATTGAAACC	GGATCGTAGAACATATTGGC	56	509	[129]
	<i>aadA</i>	ATCCTTCGGCGCGATTTTG	GCAGCGCAATGACATTCTTG	56	282	[129]
	<i>aadE</i>	ATGGAATTATCCCACCTGA	TAAAAACCCCTATTAAGCC	50	565	[129]
	<i>ant(6)</i>	ACTGGCTTAATCAATTTGGG	GCCTTCCGCGCCTCACCG	53	597	[124]
Kanamycin	<i>aph(3'')-III</i>	GCCGATGTGGATTGCGAAAA	GCTTGATCCCCAGTAAGTCA	52	292	[129]
	<i>ant(2'')-I</i>	GGGCGCGTCATGGAGGAGTT	TATCGCGACCTGAAAGCGGC	67	329	[129]
Neomycin	<i>aph(3'')-I</i>	AACGTCTTGCTCGAGGCCGCG	GGCAAGATCCTGGTATCGGTCTGCG	68	670	[129]
	<i>aph(3'')-III</i>	GCCGATGTGGATTGCGAAAA	GCTTGATCCCCAGTAAGTCA	52	292	[129]
Tetracycline	<i>tet(M)</i>	GGTGAACATCATAGACACGC	CTTGTTTCGAGTCCCAATGC	55	401	[75]
	<i>tet(K)</i>	TCGATAGGAACAGCAGTA	CAGCAGATCCTACTCCTT	55	169	[135]
	<i>tet(W)</i>	GAGAGCCTGTATATGCCAGC	GGGCGTATCCACAATGTTAAC	64	168	[76]
	<i>tet(L)</i>	GTMGTTGCGCGCTATATTCC	GTGAAMGRWAGCCACCTAA	55	696	[76]
	<i>tet(O)</i>	AATGAAGATCCGACAATTT	CTCATGCGTTGTAGTATTCCA	55	781	[135]
	<i>tet(S)</i>	ATCAAGATATTAAGGAC	TTCTCTATGGGTAATC	55	573	[135]
Erythromycin	<i>erm(A)</i>	AAGCGGTAAACCCCTCTGAG	TCAAAGCCTGTCGGAAATGG	55	441	[77]
	<i>erm(B)</i>	GAAAAGGTACTCAACCAATA	AGTAACGGTACTTAAATTGTTTAC	54	639	[135]
	<i>erm(B)-1</i>	CATTTAACGACGAAACTGGC	GGAACATCTGTGGTATGGCG	54	405	[75]
	<i>erm(C)</i>	TCAAACATAATATAGATAAA	GCTAATATTGTTTAAATCGTCAAT	50	642	[135]
	<i>erm(T)</i>	TATTATTGAGATTGGTTCAGGG	GGATGAAAGTATTCTAGGGATTT	55	395	[78]
	<i>mefA</i>	AGTATCATTAACTACTAGTGC	TTCTTCTGGTACTAAAAGTGG	55	348	[79]
Clindamycin	<i>lnu(A)</i>	GGTGGCTGGGGGTAGATGTATTAAGTGG	GCTTCTTTTGAATAATCATGGTATTTTCGATC	55	323	[76]
	<i>lnu(B)</i>	CCTACCTATTGTTGTGGAA	ATAACGTTACTCTCCTATTTTC	54	925	[80]
Chloramphenicol	<i>catA</i>	GGATATGAAATTTATCCCTC	CAATCATCTACCCATGAAT	50	486	[80]
	<i>cat</i>	TTAGGTTATTGGGATAAGTTA	GCATGRTAACCATCACAWAC	48	300	[81]
	<i>cat-TC</i>	CAATAGCGACGGAGAGTTAGG	AATCCTGCATGATAACCATCAC	55	384	[82]
Ampicillin	<i>blaZ</i>	ACTTCAACACCTGCTGCTTTC	TAGGTTCTAGATTGGCCCTTAG	58	240	[135]
	<i>bla</i>	CATARTTCCGATAAATASMGCC	CGTSTTTAACTAAGTATSGY	51	297	[81]
	<i>mecA</i>	GGGATCATAGCGTCATTATTC	AGTTCTGCAGTACCGGATTTGC	58	1429	[135]
Vancomycin	<i>vanA</i>	ATGAATAGAATAAAAGTTGCAATAC	CCCCTTTAACGCTAATACGAT	62	1028	[83]
	<i>vanB</i>	CCCGAATTTCAAATGATTGAAAA	CGCCATCCTCCTGCAAAA	59	457	[83]
	<i>vanC</i>	GCTGAAATATGAAGTAATGACCA	CGGCATGGTGTGATTTGTT	58	832	[83]
	<i>vanE</i>	TGTGGTATCGGAGCTGCAG	GTCGATTCTCGCTAATCC	52	513	[76]
	<i>vanX</i>	TCGCGGTAGTCCCACCATTCGTT	AAATCATCGTTGACCTGCGTTAT	55	454	[80]
Quinupristin/ Dalfopristin	<i>vatC</i>	GAAATGGTTGGGAGAAGCATACC	CAGCAATCGGCCCGTTTG	64	392	[76]
	<i>vatE</i>	CTATACCTGACGCAAATGC	GGTTCAAATCTTGGTCCG	52	490	[76]

(Continued)

Table 2. (Continued).

Antibiotic	ARG	Primers 5'-3' (Forward, top; Reverse, bottom)	Annealing T. (°C)	Amplicon size (bp)	Reference
Linezolid	<i>csr</i>	TGAAGTATAAAGCAGGTTGGGAGTCA ACCATATAATTGACCACAAGCAGC	55	746	[84]
Trimethoprim	<i>dfrA</i>	CTTTTCTACGCACTAAATGTAAG CATTATCAATAATTGTCGCTCAC	50	474	[80]
	<i>dfrD</i>	GGAAGGGCTTTACCTGACAGAAG CGACATAAGGCAAGAACATAACATA	50	175	[80]
Rifampicin	<i>rpoB</i>	TAACCGTGGTCTTGGCTDGAATWYGAAAC ATCAAACCAATGTTAGNCTTCWGGDGTTC	59	1100	[85]
Ciprofloxacin	<i>gyrA</i>	GAYTATGCWATGTCAGTTATTGT GGAATRTTRGAYGTCATACCAAC	45	286	[129]
	<i>parC</i>	TATTCTYAAATAYATCATTARGA GCYTCNGTATAACGCATMGCCG	50	286	[81]
	<i>int</i>	GCGTGATTGTATCTCACT GACGCTCCTGTTGCTTCT	50	1028	[86]
Macrolide antibiotics	<i>msrA/B</i>	GCAAATGGTGATGTAAGACAAC ATCATGTGATGTAACAAAAT	52	399	[87]
	<i>msrA</i>	GGCACAATAAGAGTGTAAAAGG AAGTTATATCATGAATAGATTGCCTGTT	40	939	[88]
	<i>msrC</i>	AAGGAATCCTTCTCTCCG GTAACAAAATCGTCCCG	55	343	[89]
	<i>Tn554</i>	AAGCGTAAACCCCTCTGAG TCAAAGCCTGTCGGAATTGG	55	440	[77]

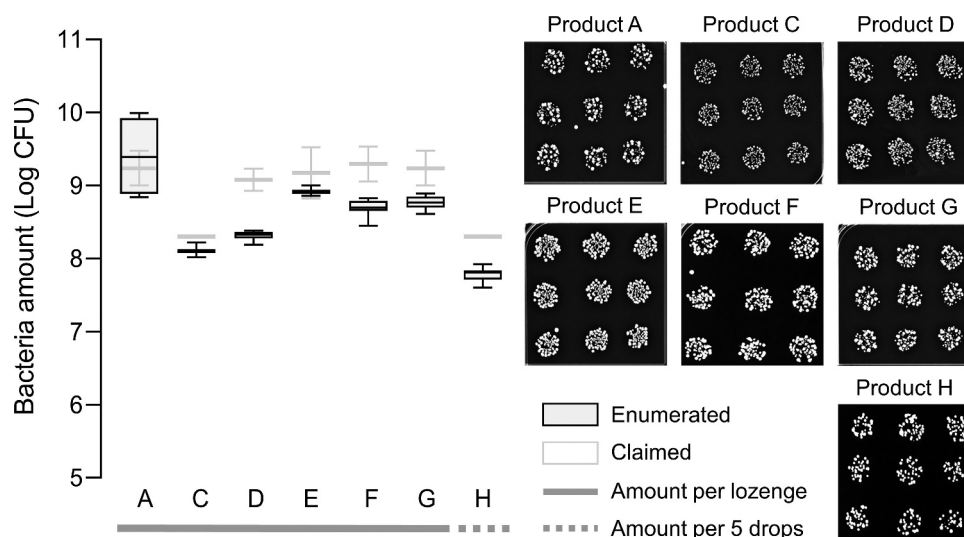


Figure 1. Enumeration of probiotic bacteria from oral lozenges.

bacteria and were thus not viable. Oral lozenges were dissolved in PBS and immediately dropped onto MRS agar plates and incubated for 24–48 h at 37°C. Agar plates were then photographed for processing on ImageJ. Representative images of recovered probiotic bacteria at the appropriate dilutions that gave rise to single bacteria colonies, that are shown on the right panel. All experiments were done in biological duplicates where each contained nine drops of the respective PBS-dissolved oral lozenge. Note: Only *Lactobacillus* strains were recovered and considered in our enumeration.

Underestimation of bacteria amounts in probiotic food products is not uncommon as previous studies have also reported discrepancies in bacteria amounts for yogurt and fermented milk [91–94], and importantly also for intestinal probiotic supplements [44,45] and other commercially available oral lozenges [95]. However, the enumerated

amounts are above the threshold of 10^6 colony-forming units (CFU) per serving regarded to be sufficient to confer health benefits [96], or the recommended daily consumption of 10^9 – 10^{10} CFU [97]. Other studies have also reported poor tolerance of probiotic bacteria to stomach pH and bile salts [98–102]. While encapsulation technologies and other additives have to some extent improved the viability of probiotics transiting through the gastrointestinal tract [103–106], their stability under oral conditions such as antimicrobial proteins in the saliva and the inhibitory effects exerted by the native oral microbiota, remain uncertain [107–109]. Since probiotic lozenges are designed to dissolve gradually in the mouth, it is therefore critical that probiotic bacteria tolerate those conditions long enough for them to adhere on the surfaces of, and colonize, the oral cavity [39,40].

Screening for antibiotic resistance in probiotics from oral lozenges

The antibiotic susceptibility of *Lactobacillus* probiotic strains from oral lozenges A, C, D, E, F, and G, was analyzed by the disc diffusion method. Resistance to more than 30 antibiotics representing different modes of actions i.e. acting on the cell wall, ribosomal subunits 30S and 50S, or DNA, were examined. The antibiotic disc had a diameter of 6 mm. Probiotics were determined to be resistant to an antibiotic if the inhibition zones had diameters less than 2 × the diameter of the antibiotic disc i.e. <12 mm, while they were determined to be partially resistant if the inhibition zones had diameters between 12 and 15 mm. Antibiotic resistance profiles of probiotics of all products are summarized as a heatmap in Figure 2, and the corresponding bar graphs of their antibiograms are shown in Supplemental Figure S1.

Left panel illustrates the workflow of the antibiotic susceptibility test by disc diffusion. PBS-dissolved oral lozenges were spread onto MRS agar in square petri dishes and a ring of antibiotic discs was carefully placed onto the bacteria lawn. Agar plates were incubated for 24–48 h at 37°C and then photographed for processing on ImageJ. The right panel shows the antibiotics used in

this study and their modes of action, as well as a heatmap of the resistant profiles of *Lactobacillus* probiotic bacteria from oral lozenges labelled A, C, D, E, F, and G, respectively. The diameter of the antibiotic disc was 6 mm. Probiotics were determined to be resistant to an antibiotic if the inhibition zones had diameters less than 2 × the diameter of the antibiotic disc i.e. <12 mm (R), while they were determined to be partially resistant if the inhibition zones had diameters between 12 and 15 mm (PR). Bar graphs of all antibiograms were shown in Supplemental Figure 1. All experiments were done in biological duplicates. Note: Antibiotic resistance profiles belong only to *Lactobacillus* strains of the respective oral lozenge.

Probiotics from all oral lozenges were resistant to vancomycin, teicoplanin, and co-trimoxazole (Figure 2). Resistance to vancomycin and teicoplanin was expected as *Lactobacillus* harbors chromosomally encoded D-Ala-D-lactate in the peptidoglycan instead of the D-Ala-D-Ala dipeptide, which prevents the binding of these antibiotics [110]. Since it is an intrinsic property of *Lactobacillus* which is not transferable, it is therefore not a clinical concern even though vancomycin is used intravenously and orally to treat various bacterial infections, including *Clostridium difficile* and methicillin-resistant *S. aureus* (MRSA) [111]. However, previous studies

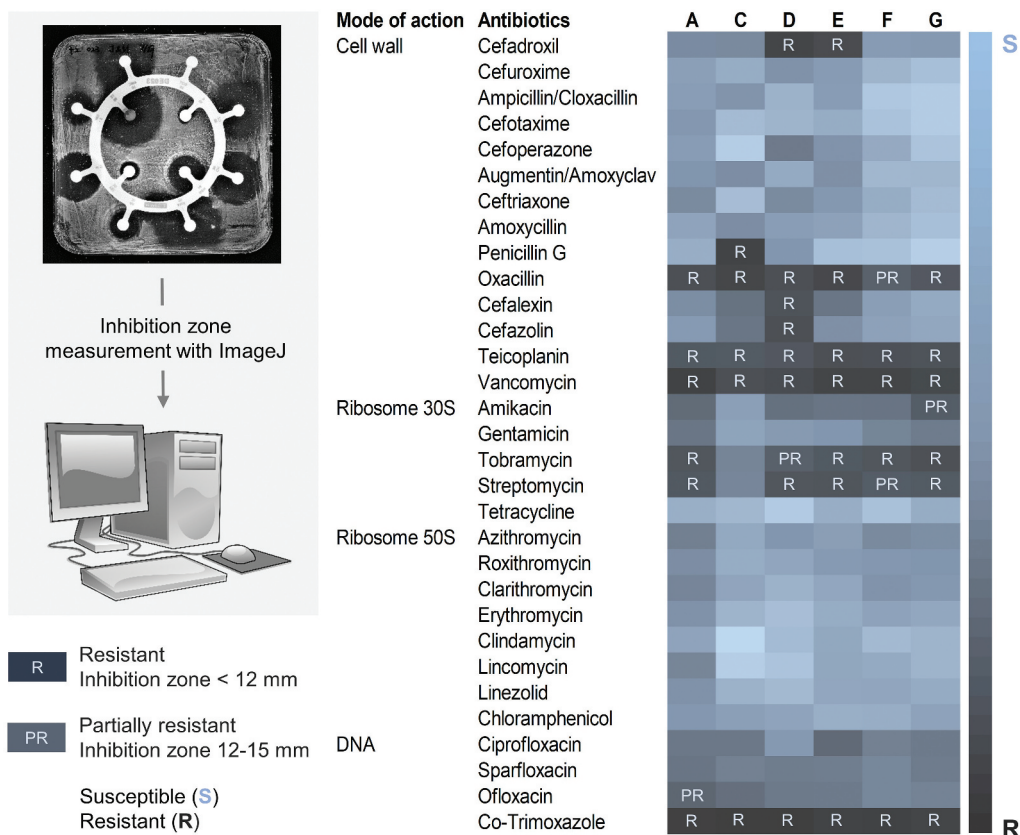


Figure 2. Antibiotic resistance profiles of probiotics from oral lozenges.

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in mice colonized with human microbiota have demonstrated the conjugative transfer of *vanA* gene clusters among *E. faecium* strains, and importantly also, from *Enterococcus faecium* strains to *L. acidophilus* [112,113]. This has raised clinical concerns especially on the treatment of *Lactobacillus* bacteremia in patients with existing conditions such as ulcerative colitis [114–116]. More recently, *vanA* plasmids transmission between different *Enterococcus* spp. was reported to be prevalent in hospital settings [117].

Co-trimoxazole, which is a combination of sulfamethoxazole and trimethoprim, is commonly used to treat a broad range of bacterial infections including those caused by MRSA [118]. Since it acts on the biosynthetic pathway of the vitamin folic acid that is lacking in most lactobacilli, resistance to co-trimoxazole is considered intrinsic [119]. However, it was speculated that the high exposure to co-trimoxazole especially in developing countries [120] could lead to a high resistance of lactobacilli especially given the fact that mobile resistance determinants of trimethoprim have been identified and are becoming increasingly prevalent [121–123].

Except for product C, resistance to streptomycin and tobramycin was detected in probiotics from all lozenges (Figure 2). Consistently, our broth microdilution assays also showed MIC for streptomycin of 128 µg/mL or higher for probiotics from all lozenges except for product C, which had a MIC of 8 µg/mL (Figure 3). Aminoglycoside antibiotics bind irreversibly to the bacterial 30S ribosomal subunit and interfere with the initiation complex of mRNA and the 30S subunit during protein synthesis. High resistance to streptomycin has also been reported in *Lactobacillus* from human samples and commercial probiotic foods including starter cultures, curd, yoghurt, and dairy products [56,124–127]. Since lactobacilli lack the cytochrome-mediated electron transport that enables the uptake of aminoglycoside drugs, their resistance is thought to be intrinsic [46,128]. However, genes encoding for aminoglycoside-modifying enzymes which are localized on transposons or plasmids, such as acetyltransferases, nucleotidyltransferases and phosphotransferases, have been identified in lactobacilli [128–133]. Moreover, there is high frequency of mutation affording high resistance to streptomycin and kanamycin in lactobacilli which has compounded the impact of resistance gene acquisition along the food chain as aminoglycoside resistance genes such as nucleotidyltransferase *ant(6)* from lactobacilli are highly identical to those from pathogens and commensals in animals [46,48,128].

Lactobacillus probiotic strains from oral lozenges labelled A, C, D, E, F, and G, were respectively grown on MRS broth containing a gradient of antibiotics (0–512 µg/mL) in quadruplicates on 96-well plates. The

plates were incubated for 24 h at 37°C with shaking, and the absorbance at OD₆₀₀ was measured. Bacteria growth of wells containing antibiotics were expressed as percentage (%) of the no-antibiotic wells. Representative antibiotics of different classes: penicillins (piperacillin), aminoglycosides (streptomycin), macrolides (erythromycin), tetracyclines (doxycycline) and chloramphenicol (chloramphenicol), were used in this study. The MIC values of the respective antibiotics were determined from the dose-response curves and summarized in the table at the bottom panel. Resistance to the respective antibiotics was determined based on the microbiological breakpoints for *Lactobacillus* strains as determined by the European Food Safety Authority (EFSA) [43]. According to EFSA, the highest cut-off values for *Lactobacillus* strains are 64 µg/mL for streptomycin, 8 µg/mL for chloramphenicol, 4 µg/mL for ampicillin (used as reference for piperacillin), 32 µg/mL for tetracycline (used as reference for doxycycline), and 1 µg/mL for erythromycin. Note: Antibiotic resistance profiles belong only to *Lactobacillus* strains of the respective oral lozenge.

Probiotics of all lozenges were resistant to oxacillin which is a beta-lactam antibiotic that binds to penicillin-binding proteins at the cell wall but only probiotics of lozenge C showed resistance to penicillin G (Figure 2), which belongs to the same antibiotic class as oxacillin. Although usually sensitive to penicillin, *Lactobacillus* resistant to oxacillin has been reported in human milk, curd, and raw milk cheeses [126,134]. They could be conferred by the plasmid-encoded resistance genes *blaZ* and *mecA* found in lactobacilli from raw and processed pork and chicken meat products, as well as staphylococci from humans and animals [135–138]. Beta-lactamases encoded by *blaZ* or the alternative penicillin binding protein, PBP2a encoded by *mecA*, could also confer protection against other beta-lactam antibiotics such as cephalosporins: cefadroxil, cefalexin, and cefazolin, which were ineffective against probiotics of oral lozenge D and E (Figure 2). *Lactobacillus* resistance to cephalosporins have been reported in curd, fermented table olives, commercial dairy products and fermented plant materials, turkeys, and human milk samples [126,139–141]. The resistance could be conferred by plasmid-born genes such as the extended-spectrum β-lactamases *bla_{TEM}* and *bla_{SHV}*, which have been shown through whole-genome sequencing, to be disseminated in *E. coli* from farm animals and humans [142]. Intermediate resistance to ofloxacin and amikacin was also detected in probiotics from products A and G, respectively (Figure 2). In the case of ofloxacin, mutations in the *GyrA* or *ParC* genes have been associated with quinolone resistance in *Lactobacillus* [143].

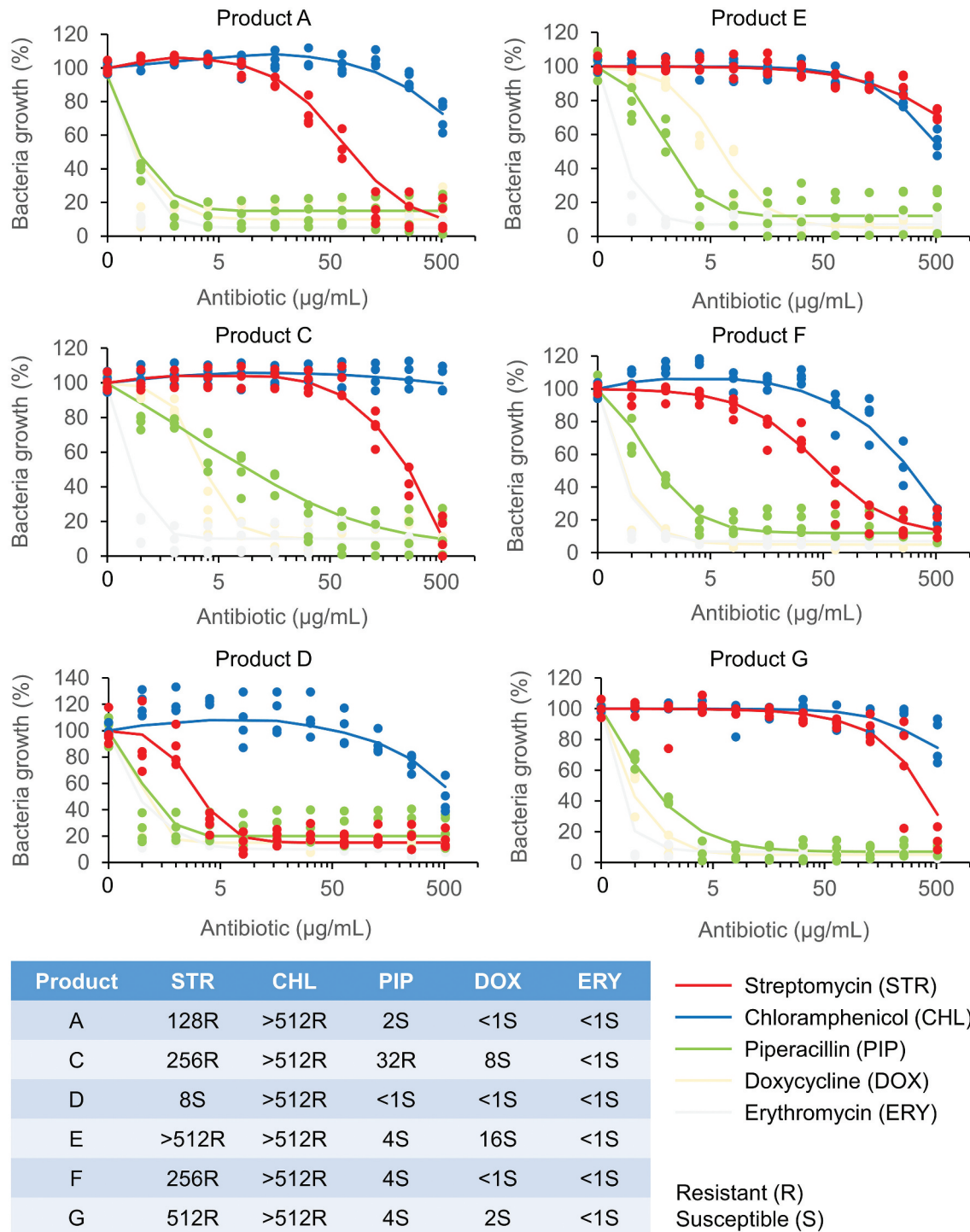


Figure 3. Dose-response curves and minimum inhibitory concentration (MIC) values of probiotics of oral lozenges.

In agreement with the disc diffusion experiments, the broth microdilution assays showed that probiotics from all lozenges were susceptible to erythromycin with MICs <1 µg/mL (Figure 3), although erythromycin resistance is commonly reported in lactobacilli from starter cultures, dairy, plant and poultry products, fermented foods, swine meat, and human samples. Their corresponding resistance genes such as *erm(B)*, *erm(C)*, *mefA*, and *lnuA*, have also been characterized [125,129,130,135,139,140,144]. Doxycycline which

has a mode of action similar to tetracycline, was effective against probiotics from all products but has higher MICs for resistant lactobacilli also commonly reported in probiotics from products C, E, and G, respectively (Figure 3). Consistently, no resistance to tetracycline was detected in the disc diffusion experiments (Figure 2). Tetracycline starter cultures, dairy, fermented sausages, plant and poultry products, swine meat, and human samples and their corresponding resistance genes *tet(W)*, *tet(L)*, *tet(K)*, *tet(S)*, and *tet(M)* have also been

characterized [56,124,125,129,130,135,140,144,145]. Piperacillin, which has a mode of action similar to penicillin, was effective against probiotics of all products except for oral lozenge C which had a MIC of 32 µg/mL (Figure 3). Consistently, in the disc diffusion experiments, probiotics of product C also showed resistance to penicillin G (Figure 2). In contrast to the disc diffusion experiments where all probiotics were susceptible to chloramphenicol (Figure 2), the broth microdilution studies showed high MICs (>512 µg/mL) for probiotics of all products (Figure 3). The chloramphenicol acetyltransferase *cat-TC* gene was previously reported in *Lactobacillus* from dairy products such as raw milk, cream, yogurt, cheese, and human samples such as mouth, feces, and vagina [144]. Since the *cat* genes are plasmid-borne [146], they could be transmitted to other lactobacilli more effectively in broth cultures than on agar plates.

Detection of antibiotic resistance genes by PCR

We attempted to characterize the antibiotic resistance genes by PCR using gene-specific primers (Table 2). The trimethoprim resistance gene *dfpD* which is a plasmid-encoded dihydrofolate reductase, was detected in probiotics from lozenges A, C, D, and E (Table 3). It could account for their resistant phenotypes and pose a risk of intra- and inter-species acquisitions (Figure 2) [147].

Although the erythromycin resistant phenotype was not observed in the disc-diffusion and broth microdilution studies, the plasmid-encoded erythromycin resistance gene *erm(T)* was detected in probiotics of lozenge A and C while *erm(B)* was detected in probiotics from products D and F, respectively. Additionally, the macrolide resistance gene *mefA* that encodes for efflux channels, was also detected in probiotics from product D (Table 3). The plasmid-encoded chloramphenicol acetyltransferase *cat-TC* gene was detected in probiotics from lozenge D and G, which could account for the resistant phenotype observed in the broth microdilution studies (Table 3) (Figure 3).

The aminoglycoside resistance gene *aadE* detected in probiotics from oral lozenge G could account for the resistant phenotype. The aminoglycoside 3'-phosphotransferase *aph(3'')-III* and aminoglycoside-2''-O-nucleotidyltransferase *ant(2'')-I* genes which are known to confer resistance to kanamycin, were detected in probiotics of the respective products D, G, and F (Table 3). They could confer cross-protection to streptomycin which has a similar mode of action.

The DNA topoisomerase and DNA gyrase genes *parC* and/or *gyr(A)* were detected in probiotics of

Table 3. PCR detection of antibiotic resistance genes in probiotic lozenges.

Product	<i>Lactobacillus</i> strain	Antibiotic resistance genes
A	<i>Lactobacillus paracasei</i> <i>Lactobacillus reuteri</i> <i>Lactobacillus sakei</i> <i>Lactobacillus salivarius</i>	<i>erm(T)</i> , <i>rpoB</i> , <i>gyr(A)</i> , <i>parC</i> , <i>dfpD</i>
C	<i>Lactobacillus reuteri</i> DSM 17938 <i>Lactobacillus reuteri</i> ATCC PTA 5289	<i>erm(T)</i> , <i>rpoB</i> , <i>gyr(A)</i> , <i>parC</i> , <i>dfpD</i>
D	<i>Lactobacillus acidophilus</i> HA-122 <i>Lactobacillus casei</i> HA-108 <i>Lactobacillus rhamnosus</i> HA-111 <i>Lactobacillus salivarius</i> HA-188	<i>aph(3'')-III</i> , <i>erm(B)</i> , <i>mefA</i> , <i>cat-TC</i> , <i>rpoB</i> , <i>gyr(A)</i> , <i>parC</i> , <i>dfpD</i>
E	<i>Lactobacillus brevis</i> CECT 7480 <i>Lactobacillus plantarum</i> CECT 7481	<i>gyr(A)</i> , <i>dfpD</i>
F	<i>Lactobacillus acidophilus</i> <i>Lactobacillus reuteri</i> <i>Lactobacillus paracasei</i> <i>Lactobacillus salivarius</i>	<i>ant(2'')-I</i> , <i>erm(B)</i> , <i>vanX</i> , <i>vate</i>
G	<i>Lactobacillus paracasei</i> <i>Lactobacillus reuteri</i>	<i>aadE</i> , <i>aph(3'')-III</i> , <i>cat-TC</i> , <i>vanX</i> , <i>rpoB</i> , <i>gyr(A)</i>

Note: Detected resistance genes were reflective of *Lactobacillus* in the oral lozenge and not specific to individual strains.

oral lozenge A, C, D, and E, but there were no resistant phenotypes to fluoroquinolones: ciprofloxacin and ofloxacin, except for probiotics of oral lozenge A which were only partially resistant to ofloxacin (Table 3) (Figure 2). On the other hand, while resistance to oxacillin was detected in probiotics of all lozenges, and also to penicillin G in the case of product C (Figure 2), the corresponding transmissible genes *blaZ* and *mecA* were not detected. The RNA polymerase B subunit *rpoB* gene that confers resistance to rifampicin, was detected in probiotics from products A, C, D and G. Oral lozenges F and G harbored the vancomycin resistance gene *vanX* while only product F harbored the quinupristin/dalfopristin resistance gene *vate* (Table 3).

The resistance determinants detected in our studies were mostly consistent with those of previous studies on lactobacilli from various foods, animals, and human sources [48,56,124,125,130,135,140,141,148]. One notable difference was the promiscuity of tetracycline resistance genes *tet(W)*, *tet(M)*, *tet(S)*, *tet(O)*, *tet(Q)*, *tet(36)*, *tet(Z)*, *tet(W/O)*, *tet(O/W/32/O/W/O)*, *tet(K)*, and *tet(L)* in lactobacilli from poultry and meat products, starter cultures, fermented foods, and the human intestine [125,135,149–151], which were not detected in this study. Notably, previous studies conducted on lactobacilli from intestinal probiotic supplements reported resistance to a broad range of antibiotics including teicoplanin, vancomycin, amikacin, tobramycin, streptomycin, cephalixin, all of which, were also reported in

this study. Also consistent with our data, no resistance to erythromycin, clindamycin, tetracycline, and ampicillin was reported from the previous studies [42,45].

Single strain analysis of antibiotic resistance

To examine the contribution of individual strains to the resistance profile of the probiotic lozenges, we selected as representatives, single bacteria colonies from products C and D, extracted their genomic DNA, and resolved their identities by 16S rRNA sequencing. All the four strains *L. acidophilus*, *L. casei*, *L. salivarius* and *L. rhamnosus* listed on the label of product D, were represented in our 16S rRNA sequencing analysis showing >98% identities to known sequences deposited in the NCBI GenBank [71]. From the disc diffusion assay, apart from the expected intrinsic resistance of *Lactobacillus*, we observed that none of the single isolates from product D was resistant to cephalosporin antibiotics such as cefadroxil, cefalexin, and cefazolin although resistance to these drugs was detected in the heterogenous populations of bacteria of product D (Figure 4). This may be due to the absence of plasmids carrying cephalosporin resistance genes in the pure isolates tested. Oxacillin resistance was also not found in the single strains, and this could be attributed to the absence of the *mecA* gene known to confer resistance to oxacillin although it was detected in the heterogenous populations of product D. It is conceivable that plasmids carrying the resistance determinants are present only in a fraction of bacterial cells in product D, thus affording them resistance to the same antibiotics in the mixed populations. All other resistance genes detected in product D, were present in the single isolates. As expected, the *cat-TC* gene conferring resistance to chloramphenicol was present in all single isolates of product D while the *rpoB* gene responsible for rifampicin resistance was detected in three out of four isolates. The *GyrA* or *ParC* genes

associated with quinolone resistance in *Lactobacillus*, were detected in *L. casei*, *L. salivarius* and *L. rhamnosus*, respectively, while the erythromycin resistance gene *erm(B)*, was detected only in *L. casei*. Although only *L. rhamnosus* was phenotypically partially resistant to streptomycin, the streptomycin resistance gene *aph(3^{''})-III* was only detected in *L. acidophilus*. On the contrary, amikacin resistance which was not detected in the mixed populations of product D, was found to be present in three of the four single isolates of product D. *L. reuteri* of product C was resistant to co-trimoxazole, teicoplanin, vancomycin, penicillin, and oxacillin, which is consistent with the antibiogram of product C, although it also showed additional resistance to cefazolin. In the broth microdilution assay, the MICs for all tested antibiotics except for streptomycin, were generally lower for the single strains than for the mixed populations of product D. Similarly, *L. reuteri* from product C also gave lower MICs than that of product C although it was the only *Lactobacillus* strain present in this product. Except for *erm(T)*, all the resistance genes *parC*, *rpoB*, *gyr(A)*, and *dfrD*, detected in product C, were present in the *L. reuteri* isolate (Figure 4). While single strain resistance profiles enable comparisons with threshold values determined by the European Food Safety Authority (EFSA) [43] antibiograms of the oral lozenges as a whole, is more reflective of the actual diet where heterogenous populations of probiotics are normally consumed. Furthermore, a conservative approach was adopted in this study where resistance was only assumed if the MIC values exceeded the thresholds of all *Lactobacillus* spp. listed by the EFSA.

Top panel shows the antibiotics used in this study and their mode of actions, as well as a heatmap of the resistance profiles of *Lactobacillus* from products C and D. The diameter of the antibiotic disc was 6 mm. Probiotics were determined to be resistant to an antibiotic if the inhibition zones had diameters



Figure 4. Antibiograms of single strains isolated from probiotic lozenges.

less than $2 \times$ the diameter of the antibiotic disc i.e. <12 mm (R), while they were determined to be partially resistant if the inhibition zones had diameters between 12 and 15 mm (PR). All experiments were done in biological duplicates. The bottom panel shows the MIC values of the corresponding isolates. *Lactobacillus* strains were grown on MRS broth containing a gradient of antibiotics (0–512 $\mu\text{g}/\text{mL}$) in quadruplicates on 96-well plates. The plates were incubated for 24 h at 37°C with shaking, and the absorbance at OD_{600} was measured. Bacteria growth of wells containing antibiotics were expressed as percentage (%) of the no-antibiotic wells. Representative antibiotics of different classes: penicillins (piperacillin), aminoglycosides (streptomycin), macrolides (erythromycin), tetracyclines (doxycycline) and chloramphenicol (chloramphenicol) were used. Resistance to the respective antibiotics was determined based on the microbiological breakpoints for *Lactobacillus* strains as determined by the European Food Safety Authority (EFSA) [43]. According to EFSA, the highest cut-off values for *Lactobacillus* strains are 64 $\mu\text{g}/\text{mL}$ for streptomycin, 8 $\mu\text{g}/\text{mL}$ for chloramphenicol, 4 $\mu\text{g}/\text{mL}$ for ampicillin (used as reference for piperacillin), 32 $\mu\text{g}/\text{mL}$ for tetracycline (used as reference for doxycycline), and 1 $\mu\text{g}/\text{mL}$ for erythromycin. Abbreviation of antibiotics: AZM: Azithromycin; AK: Amikacin; GEN: Gentamicin; CIP: Ciprofloxacin; CFR: Cefadroxil; CXM: Cefuroxime; RO: Roxithromycin; AX: Ampicillin/Cloxacillin; CTX: Cefotaxime; CPZ: Cefoperazone; CLR: Clarithromycin; SPX: Sparfloxacin; AMC: Augmentin/Amoxycylav; CTR: Ceftriaxone; AMX: Amoxycillin; E: Erythromycin; CD: Clindamycin; OF: Ofloxacin; L: Lincomycin; TOB: Tobramycin; P: Penicillin G; OX: Oxacillin; LZ: Linezolid; COT: Co-Trimoxazole; VA: Vancomycin; TE: Tetracycline; C: Chloramphenicol; CN: Cefalexin; CZ: Cefazolin; S: Streptomycin; TEI: Teicoplanin.

Despite some variations, the single isolates generally exhibited lower resistance to antibiotics compared to the mixed populations in the oral lozenges as determined through our disc diffusion, broth microdilution and molecular characterization studies. Our data implied synergistic effect or cooperativity operating in the heterogenous populations of probiotic lozenges including mechanisms such as horizontal gene transfer that is strengthened through surface adherence and biofilm formations and extracellular DNA, thus affording resistance to a broader range of antibiotics [60,152]. More recently, it has been shown that even without antibiotic pressure, horizontal gene transfer helps establish low frequency of resistance genes to potentiate adaptation of bacteria to future environmental changes such as when antibiotics are present [153]. As such, a comparative metagenomics analysis conducted in conditions as close as possible

to the oral cavity, would be required to observe the change in the gene pool with and without antibiotics. Since it is conceivable that plasmids carrying the resistance determinants are present only in a fraction of bacterial cells, the long-term consumption of heterogenous populations of probiotics in the form of health supplements such as oral lozenges, could exacerbate the spread of antimicrobial resistance in the oral cavity.

Conjugative transfer of resistance genes from probiotics to bacteria implicated in diseases

The capacity of conjugal transfer of streptomycin resistance genes from probiotics represented by product G to *S. mutans*, *S. sanguinis*, *S. gordonii*, and *E. faecalis*, were examined. An illustration of the liquid culture co-incubation and filter mating conjugative transfer workflow and representative images of transconjugant selections, are shown in Figure 5. Since *aadE* and *aph(3'')-III* genes that confer resistance to streptomycin were detected in probiotics of product G, they were selected as the donor in both liquid co-culture incubation and filter mating conjugative transfer experiments. Previously, co-transfer of plasmid-encoded aminoglycoside and macrolide resistance genes *erm(B)*-Tn5405-like element and *aac(6')-Ie-aph(2'')-Ia* was detected *in vitro* and in the gut of mice [154]. Notably, the transfer of the erythromycin resistance plasmid pAM81 between *S. gordonii* and *E. faecalis* has been observed *ex vivo* using prepared root canals of sterilized teeth [155]. However, we detected no transconjugants on the streptomycin agar plates (Figure 5). Thus, our results indicated that the antibiotic resistance genes were not transferred between the donor and the recipient strains in the current experimental settings.

An illustration of the liquid culture co-incubation and filter mating conjugative transfer workflow is shown in the top panel. Probiotics donor from oral lozenges which are resistant to one antibiotic, and recipient which is susceptible to the same antibiotic, were incubated at 1:1 or 10:1 probiotics-to-recipient ratio in BHI broth for 24 h at 37°C with shaking. The liquid co-cultures were then spread on MSA and MRS agar plates with and without antibiotics, to detect conjugative transfer of resistance determinants from probiotics to the recipient. Lateral transfer of resistance genes was also examined by filter mating where probiotics resistant to one antibiotic and recipient susceptible to the same antibiotic, were localized on a $0.45 \mu\text{m}$ filter at 1:1 and 10:1 probiotics-to-pathogen ratio. The filter was placed on BHI agar and incubated for 24 h at 37°C . Bacteria were released from the filter in PBS and immediately spread onto MSA and MRS agar plates with and without antibiotics. The presence of recipient bacteria colonies on

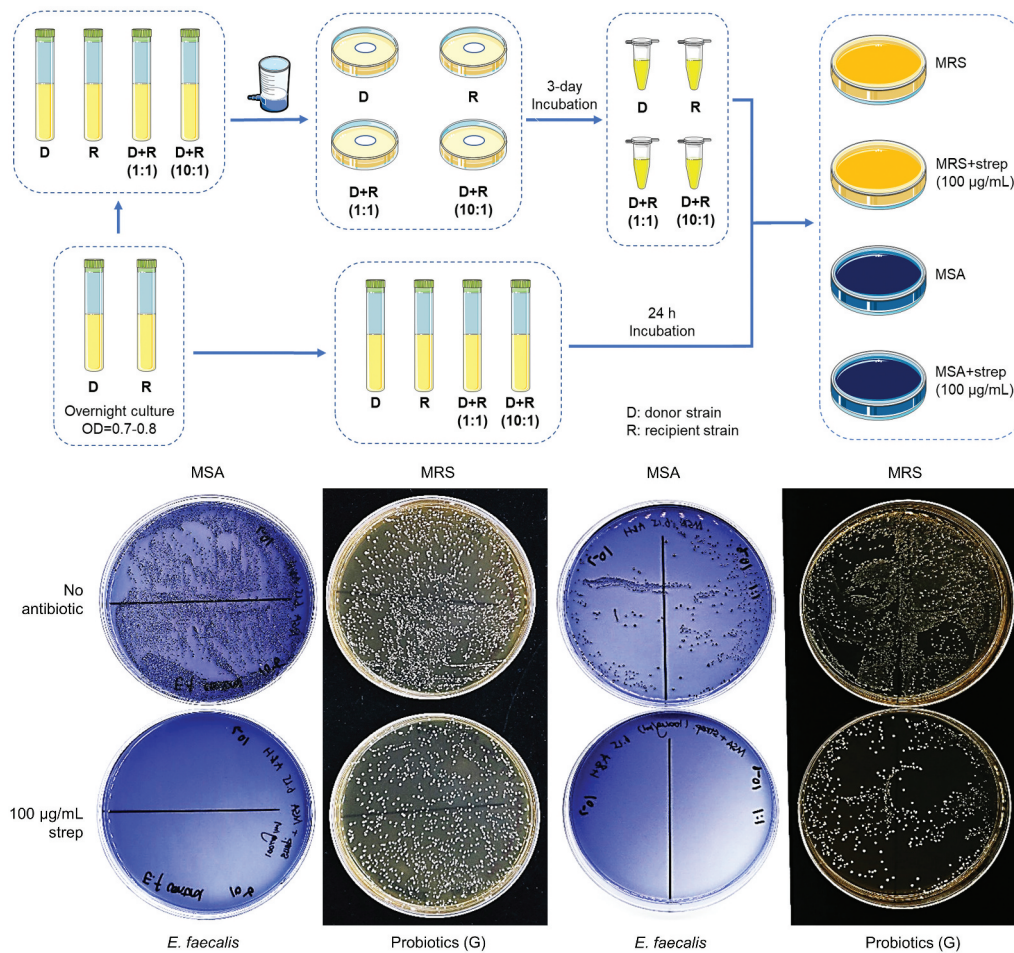


Figure 5. Transmission of resistance genes by conjugative transfer.

MSA agar plates containing antibiotics, would indicate transfer of resistance genes from probiotics. Representative pictures of agar plates from the filter mating experiment are shown in the bottom panel. Strep represents streptomycin; D, donor probiotic from product G; R, recipient *S. mutans*, *S. gordonii*, *S. sanguinis*, or *E. faecalis*. Note: MRS is selective for *Lactobacillus* strains which were unable to grow on MSA agar.

It was previously shown that the erythromycin resistance plasmid *pLFE1* in *L. plantarum* isolated from raw milk cheese could be transferred to another *Lactobacillus* and to the pathogens *Listeria innocua*, *Listeria monocytogenes*, and *E. faecalis*, through filter-mating experiments [156]. Likewise, the tetracycline resistance gene *tet(M)* located on the *Tn916* transposon in *L. paracasei* could also be transferred to *E. faecalis* [150]. Moreover, conjugal transfer of erythromycin and tetracycline resistance genes from *Lactobacillus* to pathogens in the animal gut, *in vitro*, and during food fermentation, were also detected [149]. Yet, there are also studies that detected no conjugal transfer of resistance genes from lactobacilli isolated from fermented milk and sausages to *E. faecalis* and *S. aureus* [145,147]. *L. fermentum* strains isolated from human feces and

commercial dairy products were also unable to transfer their tetracycline and erythromycin resistance genes to pathogens such as *Staphylococcus* and *Listeria* strains by filter mating [140], while rifampicin and fusidic acid resistant lactobacilli from human origins also failed to transfer their resistance determinants to other lactobacilli, and to the pathogens *E. faecium* and *E. faecalis*, respectively [125]. Moreover, the transfer of plasmid encoded pediocin PA-1 like bacteriocin from *L. plantarum* to *E. faecalis* examined *in vitro* by filter mating as well as *in situ* using a soymilk model, were also not detected [157].

As conditions *in vitro* are not representative of the complexity and dynamics of the oral cavity and gut, actual rates of resistance gene transfer were thought to be underestimated [158]. Factors that may affect resistance gene transmission include the presence of commensal and pathogenic bacteria populations in healthy, antibiotic treated, and different genetic backgrounds of subjects, chemical and physical parameters such as bile salts, temperature, oxygen levels, and the requirement of biofilm formation [158–163]. As such, resistance gene trafficking must be examined *in vivo* taking into consideration various health, genetics, and nutritional factors. Notably, a recent metagenomics profiling of mice and human

gastrointestinal tracts reported that probiotics worsen the resistome expansion caused by a prior course of antibiotics, as evidenced by an elevated number of strains carrying antibiotic resistance genes, thus directly linking probiotics with the alteration of antibiotic resistance gene reservoir in the human gut [164].

Comparative analysis of probiotic lozenges antibiograms with probiotic drinks

We also assessed the antibiotic resistance profiles of commonly available probiotic drinks such as probiotic milk, yogurt, and juice. Like many probiotic drinks, the probiotic drinks examined in this study contained only one type of *Lactobacillus*. From our enumeration studies of the recovered probiotic strains, the probiotic drinks contained approximately two to three orders of magnitude fewer bacteria compared to the oral lozenges per weight. Their antibiograms showed mostly intrinsic resistance such as resistance to vancomycin, teicoplanin and co-trimoxazole. However, resistance to tobramycin, streptomycin, and ciprofloxacin, were detected in one or more probiotic drinks. They also generally had lower MICs than the probiotic lozenges (Supplemental Figure S2). The high amounts of heterogenous populations of probiotic bacteria in supplements such as oral lozenges have been previously thought to encourage the spread of antimicrobial resistance [45,54], thus their long-term consumption may pose a higher risk to human health.

Conclusion

In conclusion, we report that probiotics from oral lozenges are resistant to multiple antibiotics belonging to glycopeptides, aminoglycosides, penicillins, and/or cephalosporins. The resistant probiotics display high MICs for streptomycin (>128 µg/mL), chloramphenicol (>512 µg/mL), and piperacillin (32 µg/mL), but are susceptible to doxycycline and erythromycin. Our PCR analysis detected genes conferring resistance to erythromycin, chloramphenicol, fluoroquinolone, aminoglycosides, vancomycin, rifampicin, and quinupristin/dalfopristin, in the probiotic lozenges. Additionally, our analysis of single strains isolated from probiotic lozenges and of probiotic drinks showed generally lower resistance to antibiotics compared to the mixed populations in the oral lozenges. Although we detected no conjugal transfer of antibiotic resistance genes *in vitro*, the presence of plasmid-encoded resistance genes in probiotics of oral lozenges, highlighted the potential of probiotics to acquire resistance genes during food processing or along the food chain from farm to fork [51,57–60,64,66,165–168]. This notion is further strengthened by the fact that the healthy oral microbiome resistome revealed recently through

whole-genome sequencing and real-time quantitative PCR microarray, contain highly prevalent genes conferring resistance to macrolides, lincosamides, streptogramins, and tetracyclines [55].

Taken together, our study represents the first antibiotic resistance profiling of probiotics from oral lozenges which serves not only to inform consumers and medical practitioners on the potential health risk, but also to encourage a more comprehensive study on the mechanisms underlying the transferability of resistance genes, especially in probiotics that do not carry the resistance determinants. Considering the global threat of drug resistance [169] and concomitant with the rising trend of dietary or health supplements [10], our data indicate a potential threat to human health.

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Disclosure statement

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.

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Author contributions

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Data availability statement

The original contributions presented in the study are included in the article or Supplemental Information, further inquiries can be directed to the corresponding authors.

Ethics statement

There are no animal or human experiments involved in this study

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