

Effects of *Lactobacillus crispatus* as a candidate for oral probiotic bacteria on *Haemophilus influenzae*

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Purpose: To verify the effects of five candidates for oral probiotics on *Haemophilus influenzae* and to more characterize the effects of *Lactobacillus crispatus* YIT 12319 (L.c1).

Materials and Methods: Cell free culture supernatants (CFCs) were acquired from cultures of the clinical isolates (oral probiotic candidate bacteria). Two laboratory strains of *H. influenzae* (type b and non-typeable) were freshly cultured in Muller-Hinton broth medium. A paired competition assay was performed using each clinical isolate versus one of the two *H. influenzae* strains on culture plates. Antibacterial effects of all unprocessed CFCs, neutralized CFCs, and an ammonium sulfate precipitate of the CFCs against *H. influenzae* were determined using a radial diffusion assay. The bactericidal effect of L.c1 CFCs was also verified using a colony formation assay. Furthermore, the organic acids produced by L.c1 were analyzed using HPLC.

Results: L.c1 displayed uninterrupted growth by suppressing the growth of both strains of *H. influenzae* equally, while the results for the rest of the clinical isolates were inconsistent. Only unprocessed CFCs of L.c1 clearly inhibited the growth of *H. influenzae* strains and showed potentially strong bactericidal effects. Reasonable amounts of lactic acid, acetic acid, succinic acid, and propionic acid were detected by HPLC.

Conclusion: The effects of L.c1 on *H. influenzae* strains observed in this study will serve as important supporting evidence for the potential use of lactobacillus species as probiotics. In addition, the growth appeared to be inhibited by the organic acids produced by L.c1.

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Key Words: *Haemophilus influenzae*, *Lactobacillus crispatus*, oral probiotics, organic acid

Introduction

Haemophilus influenzae has been frequently detected as a commensal bacterium of the human nasopharynx. For the past few decades, it has been highlighted as a major cause of a variety of respiratory conditions.¹ *H. influenzae* is responsible for a variety of infections in humans including septicemia, bronchitis, pneumonia, and acute otitis media (AOM).² Over the last few years, pneumonia has been the third leading cause of death in Japan, where 98.9/100,000 people die of the disease per year (<http://www.mhlw.go.jp/english/database/db-hw/populate/index.html>), making it a particularly alarming health issue.³ Previously, it was demonstrated that *H. influenzae* and anaerobes are primarily detected in patients aged 40-64 years.⁴ In recent years, it has been revealed that *H. influenzae* plays a role in chronic lower respiratory tract inflammation.

H. influenzae is a gram-negative coccobacillus with a variable shape (pleomorphic) that grows both aerobically and anaerobically.^{5,6} *H. influenzae* is divided into typeable and nontypeable strains based on the presence or absence of a polysaccharide capsule. The typeable strains, which have this capsule, are classified into six serotypes (designated a to f) based on their ability to react with antisera against recognized polysaccharide capsules.^{5,7,8} The type b form of *H. influenzae* (designated as Hib) is the most prominent typeable form; its capsular polysaccharides, polyribosylribitol phosphate (PRP), is considered to be the most virulent form of the bacterium.⁹ Invasive disease caused by Hib mainly affects infants and children, causing potentially life-threatening conditions such as meningitis, epiglottitis, and severe sepsis.^{9,10}

Non-typeable *H. influenzae* (NTHi) lacks the capsule gene and thus does not express the polysaccharide

capsule. NTHi causes upper and lower respiratory-tract infections and is a significant cause of AOM in children. NTHi exacerbates other respiratory diseases and can persist chronically in patients with Chronic Obstructive Pulmonary Disease (COPD).⁵ *H. influenzae* infection is a life-threatening risk factor associated with general anesthesia and pre- or post-operative COPD. COPD is a major health problem; it is the fourth leading cause of mortality worldwide and the third leading cause in Japan.¹¹ By virtue of anatomical positioning, the upper respiratory tract flora and the oral microbiota exist in a very short transitional range. The oral microbiota are among the most complex of the human body, with more than 700 bacterial species having been detected in the oral cavity.^{12,13} A number of research reports have shown that strains of *H. influenzae* have been detected in saliva and oral flora.^{14,15}

Use of probiotics is a potential preventive strategy for lowering the mortality rate caused by COPD or other respiratory infectious diseases in Japan. Probiotics are microorganisms that have been claimed to provide health benefits when consumed.^{16,17} The 2001 World Health Organization definition of probiotics is “live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host”. More specifically, they are believed to modify the flora in the human body by replacing harmful microbes with useful microbes. Based on that concept, efforts have been made to identify oral probiotic bacteria.^{18,19} In a recently published article, it was suggested that at least seven new isolated lactic acid bacterial strains show promising properties for use as probiotics, alone or as a part of a probiotic formula, to improve oral health.²⁰ Similarly, isolates from healthy adults are being preserved and tested in some laboratories in Japan, including ours. These include *Lactobacillus crispatus* YIT 12319 (L.c1), *Lactobacillus crispatus* LBS 17-11 (L.c2), *Lactobacillus fermentum* YIT 12320 (L.f), *Lactobacillus gasseri* YIT 12321 (L.g), and *Streptococcus mitis* YIT 12322 (S.m) as potential candidate probiotic bacteria for the human oral cavity.

However, the effects of these five bacteria on *H. influenzae* remain to be explored. Therefore, this study was designed to examine the growth inhibitory and bactericidal effects of L.c1, which was selected as a leading strain from the five candidate probiotic bacteria based on activity against *H. influenzae* ATCC 9795 (a Hib laboratory strain) and *H. influenzae* GTC 15014 (an NTHi laboratory strain).

Materials and Methods

Bacteria and growth conditions

The five candidate oral probiotic bacteria (L.c1, L.c2, L.f, L.g, and S.m) are clinical isolates from the Department of Translational Research, Tsurumi University School of Dental Medicine. All *Lactobacillus* species were grown in Lactobacilli MRS Broth (Bacto, Becton, Dickinson and Company, Sparks, MD, USA) and S.m was grown in Tryptic Soy (TS) broth medium (Bacto). All bacteria were grown under anaerobic conditions of 80% N₂, 10% CO₂, and 10% H₂ at 37°C for 24-48 hours. The cultures were then centrifuged at 1,710 × g for 20 minutes to collect cell free culture supernatants (CFCSs) to evaluate antibacterial activities.

H. influenzae ATCC 9795 was purchased from American Type Culture Collection (Manassas, VA, USA) and *H. influenzae* GTC 15014 was purchased from GTC Collection (Gifu University Graduate School of Medicine, Gifu, Japan). Both strains of *H. influenzae* were anaerobically incubated in Muller-Hinton broth medium (MH; Bact) supplemented with 150 μL/mL of NAD⁺ (Oriental Yeast Co., Ltd., Tokyo, Japan) (culture medium named as MHN) at 37°C for 24 hours. The cell suspension was prepared with PBS after centrifuging the culture medium at 1,710 × g for 20 minutes.

Competition assay

Competition assays were performed to observe the growth suppression effects of five candidate oral probiotic bacteria (L.c1, L.c2, L.f, L.g, and S.m) on two strains of *H. influenzae* (ATCC 9795 and GTC 15014) using agar plates containing a mixture of MRS and MHN mediums at a ratio of 1:1. Each pair of competitive species were inoculated on the plate at the same time in close proximity (5 mm or 6 mm apart) using 5 μ L of overnight cultures after adjusting the concentrations to an $OD_{540}=0.2$, as mentioned above. The plates were incubated at 37°C anaerobically for 24 hours before bacterial growth was inspected and scored.

Radial diffusion assay (RDA)

A series of tests were performed by employing an RDA²¹ to investigate the antimicrobial activities of the five candidate probiotic oral bacteria (L.c1, L.c2, L.f, L.g, and S.m) on the growth of two strains of *H. influenzae* using two layers of agarose gels.

For the underlay gel, an agarose solution was prepared containing 210 mg MH, 1.5% agarose (UltraPur Agarose and Reagents, Invitrogen, Carlsbad, CA, USA) supplemented with NAD^+ , and 100 mL of distilled water (DW) in a conical flask, which was then boiled in a water bath for 5 minutes. The agarose solution was then transferred to 15 mL culture tubes (10 mL each aliquot) and autoclaved. After removal from the autoclave, the tubes were placed into a water bath set at 50°C to allow the agarose solution to cool to 50°C. *H. influenzae* strains were grown in 4 mL MH broth overnight (as described above). Then, 300 μ L of MH broth containing approximately 6.1×10^6 bacterial cells were added to each 15 mL culture tube containing 10 mL aliquot of agarose solution. After dispersing the micro-organisms by gentle mixing, the mixture was poured into a 10 cm diameter sterile Petri dish and allowed to solidify inside a clean-bench. A series of wells (2 mm in diameter) was punched in the agar, and 5 μ L of each test solution was pipetted into a designated well. A positive control well containing tetracycline (TC; 16 μ g/mL) and two negative control wells containing only sterile phosphate buffered saline (PBS) and MRS broth were included on each plate. The plates were kept inside the clean-bench for 15 minutes at room temperature to allow the experimental solutions to diffuse. For the overlay gel that provides nutrients for microbial growth, an agarose solution was prepared as described above, except that it only contained MH (420 mg in 10 mL DW) and 1.5% agarose (150 mg) supplemented with NAD^+ . This solution was then poured over the base and allowed to solidify inside a clean bench. The plates were then turned over and incubated under anaerobic conditions (80% N_2 , 10% CO_2 , and 10% H_2) at 37°C for 24 hours, the diameters of the clear (bacterial growth inhibition) zones around the wells were measured with a slide caliper.^{22,23}

At this stage, as L.c1 appeared to have stronger effects, further experiments were performed that focused only on this bacterial strain. Culture media samples containing the bacteria were collected at five different growth stages (0, 6, 12, 24, and 48 hours), the OD at 540 nm was measured immediately, the samples were centrifuged to collect the CFCs, the pH was measured, and aliquots were prepared. We then utilized aliquot filter devices (Nippon Genesis Co., Ltd., Tokyo, Japan) with molecular cutoffs of 3K, 5K, and 10K Daltons. As molecules below the molecular cutoff pass through the filter, CFCs containing molecular compounds of sizes less than 3K, 5K, and 10K Daltons were collected separately and used for RDA.

In addition, one set of aliquots was incubated with carboxypeptidase Y (CPY; Oriental Yeast Co., Ltd., Tokyo, Japan) at 37°C for 30 minutes, with the goal of hydrolyzing any available proteins or peptides. The resulting products were used for RDA as described above.

Bactericidal effect analyzed by Colony Forming Units

One-milliliter aliquots of the *H. influenzae* ATCC 9795 cell suspensions were centrifuged ($1,470 \times g$ 15 minutes 4°C), supernatants were discarded, and bacterial pellets were suspended in 1 mL of the L.c1 CFCSs by gentle vortexing. Immediately after 5 minutes incubation, suspensions were centrifuged, the supernatants discarded, and cell pellets were suspended and held in 1 mL of chilled PBS to be analyzed for viability, as described previously.²⁴ Each bacterial cell suspension (500 μL) was serially diluted to a 100 folds final dilution before plating. Treated and control samples were plated in petri dishes containing MHN agar medium (Bacto) with a spiral plating instrument (Eddy Jet, IUL, Barcelona, Spain). After 48 hours incubation under anaerobic conditions at 37°C , the number of CFUs was counted.

Analysis of the organic acids in CFCS

As the unprocessed CFCS of L.c1 consistently showed the strongest growth inhibition effect on both strains of *H. influenzae*, with the pH falling below 5 before 12 hours and to 4 after 24 hours, it was selected for further analysis. Acids were extracted from the L.c1 CFCS collected at the five timepoints mentioned above and were used as separate samples. Briefly, each 0.9 mL sample was added to 0.1 mL of perchloric acid, mixed, and kept at 4°C to settle. After 4 hours, the mixture was filtered and centrifuged ($20,600 \times g$, 5 minutes) and the solution was collected after passing through a $0.45 \mu\text{m}$ membrane filter. Five organic acids were separated from each sample using a column of a Waters 2690 Separations Module HPLC system and the electric conductivity of each organic acid was measured using a Waters 432 Conductive Detector (Waters Corp., Milford, MA, USA).

Results

Competition assay

Results of the competition test are shown in Table 1 and Fig. 1. Only L.c1 could suppress growth of both strains of *H. influenzae* (Hib and NTHi types) at equal score levels, with all test scores showing ++. Among the five candidate probiotic bacteria, L.c1 demonstrated the strongest effects on *H. influenzae* strains. For S.m, mild growth inhibition effect was observed against *H. influenzae* ATCC 9795 only (score: +).

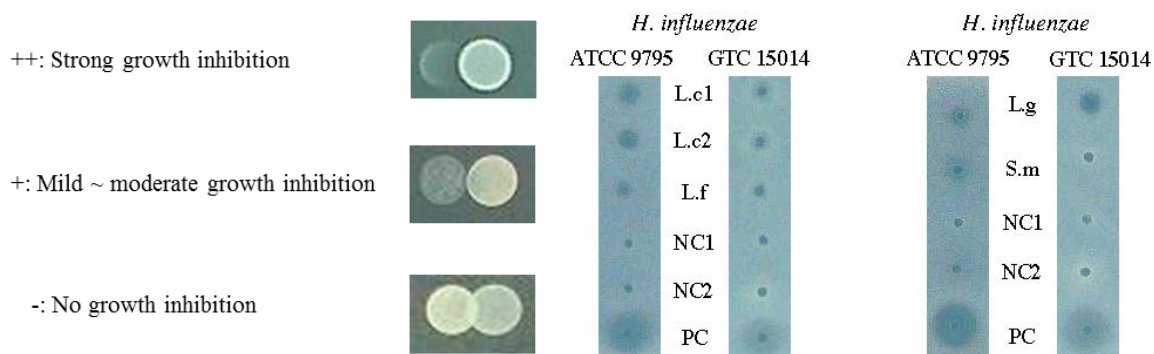


Fig. 1

Fig. 2

Fig. 1 Growth inhibition score of competition assay

Fig. 2 Photographs represent the results of radial diffusion assays ($n=3$ for each experiment). The CFCSs of five candidate oral probiotic bacteria formed clear zones (inhibition zones) indicating the antibacterial activities against two strains of *H. influenzae*. NC1= negative control; PBS and NC2= negative control; medium only. PC= positive control; tetracycline.

Growth inhibition of *H. influenzae* observed on RDA

Similar results were observed on RDA: the CFCS of L.c1 produced the largest inhibition of growth (antibacterial activity) on both strains of *H. influenzae* compared to the other four species, as shown in Table 2 and Fig. 2. Although L.g was a close second, followed by L.f, S.m was not able to produce any detectable clear zones. On the other hand, L.c2 showed a greater effect on *H. influenzae* GTC 15014, but a smaller effect on *H. influenzae* ATCC 9795. Interestingly, the unprocessed CFCS of L.c1 produced the largest clear zone in all experiments (Table 3), while the neutralized CFCS or 80% saturated $(\text{NH}_4)_2\text{SO}_4$ precipitate did not produce any detectable clear zones. The unprocessed CFCS of L.c1 showed a gradual fall in pH, which decreased to below 5 before 12 hours, reached 4 at 24 hours, and further decreased to 3.8 at 48 hours, maintaining a proportional relationship with the growth of bacteria (Fig. 3). It was also found that the CFCSs with molecules less than 3 kD (low molecular mass compounds) were most effective compared to the CFCSs containing larger molecules (Table 3). Moreover, CPY-treated L.c1 CFCS demonstrated the same antibacterial activity on both strains of *H. influenzae* based on the size of the clear zones in RDA. The production of low molecular weight acids appears to correlate with the largest inhibition zones on the agar plates. Actually, lactic acid (0.1-1 M) and acetic acid (0.1-1 M) as well as other organic acids showed antibacterial activity against both strains of *H. influenzae*.

Table 1 Growth competition assay between five candidate oral probiotic bacteria and two strains of *H. influenzae*

Strains of <i>H. influenzae</i>	<i>L. crispatus</i> YIT 12319 (L.c1)	<i>L. crispatus</i> LBS 17-11 (L.c2)	<i>L. fermentum</i> YIT 12320 (L.f)	<i>L. gasseri</i> YIT 12321 (L.g)	<i>S. mitis</i> YIT 12322 (S.m)
ATCC 9795	++	++	++	++	+
GTC 15014	++	+	+	-	-

Growth inhibition score of competition assay is based on the score expressed in Fig. 1 ($n=3$ for each assay).

Table 2 Antibacterial activity of culture supernatants of oral probiotic bacteria against two strains of *H. influenzae*

Culture supernatant of	Antibacterial activity (diameter of clear-zone expressed in 'mm') against	
	<i>H. influenzae</i> ATCC 9795	<i>H. influenzae</i> GTC 15014
<i>L. crispatus</i> YIT 12319 (L.c1)	4.33±0.10	4.56±0.17
<i>L. crispatus</i> LBS 17-11 (L.c2)	3.86±0.25	4.28±0.08
<i>L. fermentum</i> YIT 12320 (L.f)	3.04±0.16	2.91±0.05
<i>L. gasseri</i> YIT 12321 (L.g)	4.04±0.22	4.17±0.13
<i>S. mitis</i> YIT 12322 (S.m)	n.d.	n.d.

$n=3$ for each experiment, n.d.: Not detected

Table 3 Antibacterial activity of *L. crispatus* YIT 12319 (L.c1) CFCS samples against *H. influenzae*

Samples	Antibacterial activity (diameter of clear-zone expressed in mm) against	
	<i>H. influenzae</i> ATCC 9795	<i>H. influenzae</i> GTC 15014
Unprocessed CFCS	4.33±0.10	4.56±0.17
Neutralized culture supernatant	n.d.	n.d.
80 % saturated $(\text{NH}_4)_2\text{SO}_4$ precipitation	n.d.	n.d.
Unprocessed CFCS after		
3 kDa ultrafiltration	3.83±0.07	4.28±0.09
5 kDa ultrafiltration	3.51±0.05	4.03±0.31
10 kDa ultrafiltration	3.09±0.11	3.89±0.16
CFCS treated with CPY	4.07±0.25	4.18±0.13
CFCS treated with CPY after 3 kDa ultrafiltration	3.82±0.54	3.82±0.18
PBS (NC1)	n.d.	n.d.
Culture medium only (NC2)	n.d.	n.d.
Tetracycline (PC)	10.04±0.15	9.79±0.10

Bactericidal effect detected on colony forming units (CFU)

Direct application of the unprocessed L.c1 CFCS to the *H. influenzae* ATCC 9795 cell suspension caused a significant reduction in colony formation (Fig. 4). This effect could not be detected for the other CFCSs, indicating that a strong bactericidal effect was induced by the unprocessed L.c1 CFCS only.

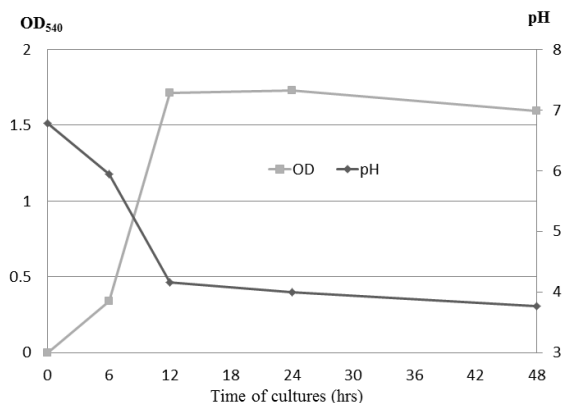


Fig. 3

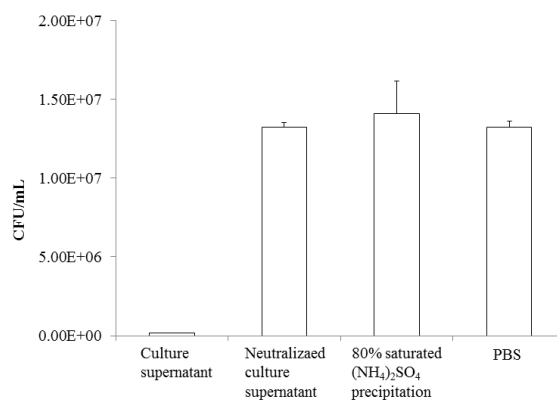


Fig. 4

Fig. 3 Graph displays the optical density (OD) of the broth medium and pH values of CFCSs collected at different time points (0, 6, 12, 24, and 48 hours) from *L. crispatus* YIT 12319 (L.c1) cultures. The OD value took a pick in between 6 to 12 hours with the rapid growth of bacteria which didn't change significantly after that. The pH curve fell linearly with the time of culture and reached to 3.77 at 48 hours.

Fig. 4 Graph shows the revived colony forming units (per mL) of *H. influenzae* ATCC 9795 after application of samples for 5 minutes ($n=3$ for each sample). For unprocessed L.c1 CFCS the revived colony number was remarkably low (only 1.65E+05) which was more than 1.00E+07 for other samples indicating strong bactericidal effect on *H. influenzae* ATCC 9795.

Organic acids detected by HPLC

Four organic acids (lactic acid, acetic acid, succinic acid and propionic acid) were revealed to be present in L.c1 CFCS upon HPLC analysis (Table 4). In particular, the lactic acid concentration increased linearly with the time of culture, reaching 118.22 mM at 48 hours, although the original medium (0 hour CFCS) had a concentration of only 5.35 mM. Acetic acid and succinic acid also increased in concentration considerably at 48 hours, while the increase in propionic acid concentration was minimal, even at 48 hours. Formic acid concentration did not change through the culture period. As expected, lactic acid and acetic acid constituted 55% and 39% of the total organic acid solution, respectively, suggesting that these two acids play a major role in the results acquired above.

Table 4 Organic acid concentration of *L. crispatus* YIT 12319 (L.c1) culture supernatant as analysed by HPLC

CFCSs collected at	Organic acid concentration (mM)								
	Lactic acid	Acetic acid	Succinic acid	Propionic acid	Formic acid	Isobutyric acid	Butyris acid	Isovaleric acid	Valeris acid
0 hour	5.35	63.43	0.56	1.96	0.87	n.d.	n.d.	n.d.	n.d.
6 hours	12.88	66.07	0.65	2.01	0.92	n.d.	n.d.	n.d.	n.d.
12 hours	92.54	66.78	2.05	2.13	0.86	n.d.	n.d.	n.d.	n.d.
24 hours	109.93	73.1	5.15	2.25	0.87	n.d.	n.d.	n.d.	n.d.
48 hours	118.22	82.96	10.86	2.42	0.89	n.d.	n.d.	n.d.	n.d.

n.d.; Not detected

Discussion

In this study, the effects of five candidate oral probiotic bacteria originally isolated from human cavities were investigated for their antibacterial potential against *H. influenzae*. All Lactobacillus strains showed reasonably effective antibacterial activities with variable strength: *L. crispatus* YIT 12319 (L.c1) had the most

promising activity on both strains of *H. influenzae* (ATCC 9795 and GTC 15014), while L.f and L.g had somewhat less consistent results. It seem to be that L.f and L.g also have similar effects on *H. influenzae*, if not equivalent. L.c2 was not so far behind in terms of activity, but S.m didn't show any effects on *H. influenzae* in this study.

Generally, the resistance of bacteria is due to the outer membrane, which is a constituent of the cell envelope in all gram-negative bacteria.²⁵ This membrane acts as an efficient permeability barrier against macromolecules and hydrophobic substances that is largely attributed to the lipopolysaccharide (LPS) layer of the outer membrane. In the present study, CFCSs containing molecules less than 3 kD in size showed greater antibacterial activity. That may include low molecular mass compounds; acids, alcohols, CO₂, diacetyl, hydrogen peroxide, peptides, amines, bacteriocines, and other metabolites.^{25,26} It is now known that symbiotic (probiotic) gut microorganisms release of various soluble low molecular weight molecules of different chemical nature (surface and exogenous proteins, nucleases, serpins, sirtuines, other enzymes, lectins, peptides, amines, bacteriocines, fatty and amino acids, lactones, furanons, miRNA, NO, etc).²⁷ The mechanism(s) underlying the antibacterial activity of probiotic Lactobacillus strains appears to be multifactorial, including lowering of the pH and the production of lactic acid and antibacterial compounds such as bacteriocins and nonbacteriocin.²⁵⁻²⁸ In this study, organic acids secreted into the MRS broth culture medium by lactic acid bacteria during growth appear to be the most effective components, with lactic acid as the most potent component. The results of this study clearly indicated that L.c1 produced lactic acid at high concentrations that linearly increased with the time of culture. They also suggest that lactic acid was the main component in the L.c1 CFCS that inhibited the growth of and killed *H. influenzae*. In addition, acetic acid and succinic acid may provide a considerable supportive role. Interestingly, S.m produced CFCS with a somewhat higher pH value of 4.7 (data not shown) compared to L.c1 and didn't display detectable antibacterial effects on either strain of *H. influenzae*. This may be an indication that acids with higher pH value are not as effective in the suppression of growth of *H. influenzae*, need to be studied further. On the contrary, the involvement of proteins, peptides, or proteolytic enzymes was ruled out by treating the CFCS with CPY and heat-treatment (data not shown) which produced the same results as the untreated CFCS of L.c1. Therefore, the results in the present study suggest that organic acids with low pH (3.8) are responsible for the growth inhibition and killing of *H. influenzae*.

As lactic acid is thought to be one of important factors to cause dental caries, we examined the cariogenicity of L.c1 by using artificial mouth model system and low cariogenicity of L.c1 would be reported elsewhere. There are incidences of the development or aggravation of COPD after general anesthesia during medical or dental procedures resulting from pushing the lung microbiome deep into the lung follicles. At present, there are only few preventive procedures available to reduce that risk. As observed in this study, the probiotic candidate Lactobacilli has the ability to suppress the growth of *H. influenzae*, and may have similar effects on other gram-negative microbes. Therefore, they could play a vital role in reducing the risk of COPD by slowly predominating the flora of the mouth, pharynx, and upper respiratory area, thereby delivering a potential health benefit. It may serve as a supplement with the practice of improving oral hygiene and frequent professional oral health care that reduce the progression or occurrence of respiratory diseases among high-risk elderly adults living in nursing homes and especially those in intensive care units.^{11,20}

However, as bacteria in the oral cavity can protect themselves from even high doses of broad-spectrum antibiotics by forming biofilms,²⁹ the application of probiotics would be milder and could serve as a long-term

prophylactic oral health maintenance tool. This approach wouldn't bring about a sudden change in the oral flora, but could interfere the growth of opportunistically pathogenic bacteria, including *H. influenzae*, similarly as other lactobacilli strains that can reduce the virulence of putative opportunistic oral pathogens, and may provide insights to future therapeutic approaches for the respective diseases.^{18,19,30} The study furthermore emphasizes the need for detailed studies on candidate oral probiotic bacteria, including their effectiveness against other gram-negative pathogens that inhabit the mouth, thorax, nasopharynx, and upper respiratory tract.

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Conflict of interests

N. Hanada and S. Imai received research fund from Yakult Central Institute (Tokyo, Japan). All remaining authors declare no potential conflicts of interest with respect to authorship and publication of this article.

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