Suppression of opportunistic infectious bacteria by lactic acid bacteria and antibacterial drugs

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Purpose: The objective of this study was to investigate inhibitory effect of lactic acid bacteria and antibiotics on the growth of *Streptococcus pneumoniae* ATCC 33400.
Materials and Methods: Antibiotic activities of five oral probiotic candidate bacteria including two strains of *Lactobacillus crispatus*, *Lactobacillus fermentum*, *Lactobacillus gasseri* and *Streptococcus mitis* and several antibiotics against *S. pneumoniae* were investigated using radial diffusion assay and competition assay. The biofilm formation of *S. pneumoniae* in the presence of 50% saturated ammonium sulfate precipitation fraction from *L. gasseri* and several concentrations of gentamicin were monitored by real time cell analyzer, xCELLigence.
Results: The growth of *S. pneumoniae* was inhibited by two strains of *Lactobacillus crispatus*, *Lactobacillus fermentum* and *Lactobacillus gasseri*. The saturated ammonium sulfate precipitation fraction (0-30% and 30-50%) from *L. gasseri* effectively inhibited growth of *S. pneumoniae*. The biofilm formation of *S. pneumoniae* monitored by real time cell analyzer (xCELLigence) was suppressed by gentamicin in dose dependent manner. Antibacterial substance from *L. gasseri* against *S. pneumoniae* was suggested to be small molecular weight substance.
Conclusion: *L. gasseri* inhibited the growth of *S. pneumoniae* suggesting that *L. gasseri* will be a candidate of probiotics.

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Key Words: antibiotics, lactic acid bacteria, opportunistic infection, probiotics, *Streptococcus pneumoniae*

Introduction

Opportunistic infections in the perioperative phase have a major impact on the patient's life prognosis and postoperative healing processes [1-3]. In addition, treatment and prevention of opportunistic infections at perioperative period are not easy due to the appearance of multidrug resistant bacteria and biofilm formation of opportunistic bacteria [4]. Antimicrobial drugs are used to treat opportunistic infections at the perioperative period, and even if their blood levels increase, sufficient antimicrobials do not migrate to saliva [5-7].

In recent years, studies on the control of opportunistic infectious bacteria by probiotics have been conducted [8,9], and it has been reported that the lactic acid bacteria (LAB) has antibacterial activity against opportunistic infectious bacteria. [10]. Terai et al. [11] screened probiotic candidates against periodontopathic bacteria from human oral cavity and finally selected several candidate bacteria including *Lactobacillus crispatus, Lactobacillus fermentum, Lactobacillus gasseri* and *Streptococcus mitis*. Of these bacteria, strains of *Lactobacillus crispatus* revealed antibacterial activities against *Haemophilus influenzae* (*H. influenzae*) [12] and *Streptococcus pneumoniae* (*S. pneumoniae*) [13]. *H. influenzae*, especially type b form (Hib) is one of causative bacteria involved in meningitis, epiglottitis, and severe sepsis [14,15]. *S. pneumoniae* is one of pathogens of upper respiratory tract infections in humans [16-18].

Therefore, oral care is regarded as important, and control of pathogens such as opportunistic infectious bacteria as well as cariogenic bacteria and periodontopathic bacteria in the oral cavity by probiotics is expected. This study was designed to investigate the inhibitory effect of combined use of antibiotics and probiotics on the

growth of *S. pneumoniae*.

Materials and Methods

Bacterial strains and culture conditions

The five oral probiotic candidate bacteria (*Lactobacillus crispatus* YIT 12319 (*L.c* 1), *Lactobacillus crispatus* YIT 12945 (*L.c* 2), *Lactobacillus gasseri* YIT 12321 (*L.g*), *Lactobacillus fermentum* YIT 12320 (*L.f*) and *Streptococcus mitis* YIT 12322 (*S.m*)) were clinical isolates maintained at Microbiological Research Department, Yakult Central Institute, Yakult Honsha Co., Ltd. and Department of Translational Research, School of Dental Medicine, Tsurumi University. All lactobacillus species were grown in Difco Lactobacilli MRS (MRS) Broth (Becton, Dickinson and Company, Sparks, MD, USA) and *S. mitis* in Bacto Tryptic Soy (TS) Broth medium (Becton, Dickinson and Company). These bacteria were grown under anaerobic condition of 80% N_2, 10% CO_2, and 10% H_2 at 37˚C for 18-24 hours. The culture was centrifuged at 1,710×*g* for 20 minutes to collect cell free culture supernatants (CFCSs) to evaluate antibacterial activities.

S. pneumoniae ATCC 33400, *S. pneumoniae* ATCC 49619 and *H. influenzae* ATCC 9795 were purchased from American Type Culture Collection (Manassas, VA, USA). *S. pneumoniae* GTC 261 and *H. influenzae* GTC 15014 were purchased from GTC Collection (Gifu University Graduate School of Medicine, Gifu, Japan). All strains of *S. pneumoniae* were grown under anaerobic condition of 80% N_2, 10% CO_2, and 10% H_2 at 37˚C for 18-24 hours. Two strains of *H. influenzae* were anaerobically incubated in Muller Hinton (MH) Broth medium (Becton, Dickinson and Company) 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.

Minimal inhibitory concentration (MIC)

An antibiotic-containing liquid medium (200 μL) was placed in the wells on the left end row of 96-well plate, and 100 μL of the same medium was placed in all other wells. One hundred μL from the first row on the left end was transferred to the second row with eight multiple micropipettes and mixed 5 times, then 100 μL of medium were transferred to right one. This handling was repeated to right end row to make a 1/2 serial dilution series of antibiotics. The bacterial suspension (10 μL) was inoculated to all wells and incubated under anaerobic condition for 24 to 48 hours. Turbidity of wells was measured at 540 nm using Micro Plate Reader to confirm bacterial growth after culturing.

Radial diffusion assay (RDA)

A series of tests were performed by employing RDA [19] to determine the antibacterial activities of antibiotics against five candidate probiotic oral bacteria, *S. pneumoniae*, and *H. influenzae* using two layers of agarose gels. The RDA was also used to investigate the antibacterial activities of the five candidate probiotic oral bacteria against *S. pneumonia.*

In the case of *L. crispatus* YIT 12319, *L. crispatus* YIT 12945, *L. gasseri* YIT 12321, and *L. fermentum* YIT 12320, A-broth containing 0.055 g of MRS medium, 0.15 g of agar, and 10 mL of distilled water (DW) was autoclaved at 121˚C for 15 minutes. B-broth containing 1.1 g of MRS medium, 0.15 g of agar, and 10 mL of DW was also autoclaved at 121˚C for 15 minutes. In the case of *S. mitis* YIT 12322 and *S. pneumoniae* ATCC 33400, A-broth containing 0.03 g of TS medium, 0.15 g of agar and 10 mL of DW, and B-broth containing 0.6 g of TS medium, 0.15 g of agar and 10 mL of DW were autoclaved at 121˚C for 15 minutes. A-broth and B-broth

were autoclaved at 105˚C for 1 minute, and kept warm at 48˚C in water bath. In the case of *H. influenzae*, A-broth contained 21 mg of MH medium, 1.5% agarose and 10 mL of DW supplemented with NAD^+, and B-broth contained 42 mg of MH medium, 1.5% agarose and 10 mL of DW supplemented with NAD^+ [12].

The pre-cultured bacterial suspension (300-500 μL) was added to A-broth. After dispersing the bacteria by gentle mixing, the mixture was poured into 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 μL of each sample solution was pipetted into a well. Positive control (bacitracin; 20 Unit/mL) and negative control (phosphate buffered saline, PBS) were included on each plate. The plates were placed inside the clean bench for 5 minutes at room temperature to allow the experimental solutions to diffuse. B-broth was poured over the base layer and allowed to solidify inside a clean bench. The plates were then turned over and cultured under anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2) at 37˚C for 24 hours. The diameters of clear zone (bacterial growth inhibition) around the wells were measured with a slide caliper [12,20,21].

Competition assay

Competition assays were performed to objective the growth suppression effects of four candidate oral probiotic bacteria (*L. crispatus* YIT 12319, *L. crispatus* YIT 12945, *L. gasseri* YIT 12321, and *L. fermentum* YIT 12320) on three strains of *S.pneumoniae* ATCC 33400, *S.pneumoniae* GTC 261, and *S.pneumoniae* ATCC 49619 using agar plates containing a mixture of MRS and TS mediums at a ratio of 1:4. 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 mL of overnight cultures after adjusting the concentrations to an OD_{540nm} = 0.25, as mentioned above. The plates were incubated at 37˚C anaerobically for 24 hours before bacterial growth was inspected and scored.

Co-culture of *L. gasseri* and *S. pneumoniae*

The culture of *L. gasseri* YIT 12321 and *S. pneumoniae* ATCC 33400 were centrifuged at 1,710×*g* for 20 minutes, and the cells were suspended in PBS and adjusted to OD_{540nm} = 0.25. A bacterial cell suspension was mixed at a ratio of 1:1 in the mixture of TS medium and MRS medium at a ratio of 4:1 and cultured at 37˚C for 0, 30, 60, and 120 minutes, (and 6 hours in another case) in the presence or absence of an antibacterial agent under anaerobic condition (80% N_2, 10% CO_2, and 10% H_2). An aliquot of bacterial culture was inoculated on the agar medium composed of TS and MRS medium at a ratio of 4:1 using a spiral plating instrument (Eddy Jet, IUL, Barcelona, Spain) and cultured under anaerobic condition at 37˚C for 48 hours to calculate colony forming unit (CFU).

Real-time monitoring of biofilm formation of *S. pneumoniae*

A real-time cell analyzer (RTCA), xCELLigence (ACEA Bioscience Inc., San Diego, CA, USA) is an instrument to quantify cell proliferation, morphology change and cell-substrate attachment in a real-time manner. A bottom surface of microtiter plate was covered with gold electrode. The xCELLigence was used to monitor biofilm formation of several bacteria [22]. Biofilm formation of *S. pneumoniae* was monitored by xCELLigence in this study. The xCELLigence was set in the incubator. Bacterial broth medium in the presence of gentamicin (0-25 μg/mL), 30% saturated ammonium sulfate fraction from *L. gasseri* and bacterial suspension of *S. pneumoniae* were added to each well of E-plate for xCELLigence and cultured at 37˚C for 40 hours.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Tricine-SDS PAGE was performed with 15% acrylamide gel (e-PAGEL, ATTO Corp, Tokyo, Japan). The molecular mass standards and sample solutions were applied on the gel. After electrophoresis, resulting gel was

cut into two parts. One was stained with EzStain (AE-1340 EzStain Aqua, ATTO Corp.), and the other was incubated with 1% Triton X-100 for 30 minutes, followed by three times rinsing with sterile water for 30 minutes, and finally rinsed with PBS for 30 minutes. The gel was overlaid on the TS agar plate including *S. pneumoniae*, and cultured under anaerobic condition at 37˚C for 24 hours.

Results

MIC of antibiotics against oral probiotic candidates and opportunistic bacteria

Results of MIC of antibiotics against oral probiotic candidate bacteria are shown in Table 1. The five probiotic candidate bacteria were relatively resistant to antibiotics used. *L.c* 1 and *L.c* 2 were resistant to gentamicin and ofloxacin. *L. f* and *L.g* were resistant to many antibiotics except for ampicillin and erythromycin, respectively. *S. m* was sensitive to all antibiotics used. The MIC of antibiotics against opportunistic infectious bacteria was relatively low (Table 2). The two strains of *H. influenzae* were sensitive to five kinds of antibiotics except for vancomycin. The two kinds of *S. pneumoniae* were sensitive to all antibiotics used and especially sensitive to ampicillin.

Table 1 MIC of antibiotics against oral probiotic candidate bacteria

Oral probiotic bacteria		*L. crispatus* (*L. c* 1)	*L. crispatus* (*L. c* 2)	*L. fermentum* (*L. f*)	*L. gasseri* (*L. g*)	*S. mitis* (*S. m*)
Antibiotic (μg/mL)	Ampicillin	0.78	1.56	0.39	200≦	0.0125
	Erythromycin	3.13	1.56	200≦	3.13	0.39
	Gentamicin	200≦	200≦	200≦	200≦	50
	Ofloxacin	200≦	200≦	200≦	200≦	6.25
	Tetracycline	100	25	200≦	200≦	25
	Vancomycin	1.56	12.5	200≦	200≦	1.56

Table 2 MIC of antibiotics against opportunistic infectious bacteria

Opportunistic infection bacteria		*H. influenzae* ATCC 9795	*H. influenzae* GTC 15014	*S. pneumoniae* ATCC 49619	*S. pneumoniae* ATCC 33400
Antibiotic (μg/mL)	Ampicillin	0.2	0.2	0.0125	0.025
	Erythromycin	1.56	1.56	0.1	0.05
	Gentamicin	3.13	3.13	6.25	12.5
	Ofloxacin	0.025	0.025	0.78	1.56
	Tetracycline	0.39	0.39	0.78	0.2
	Vancomycin	200≦	200≦	0.78	0.78

Minimum concentration of antibiotics to reveal clear zone on agar medium

The antibacterial activities of antibacterial substances were often determined by RDA [19]. Therefore, minimum concentration of antibiotics to reveal clear zone on the agar medium against probiotic candidate bacteria was examined by RDA. The results were shown in Table 3. Four lactobacilli were resistant to ofloxacin. Other antibiotics showed variety of antibacterial activity against LAB used. Generally minimum concentration of antibiotics to reveal clear zone assessed by RDA were higher than that of MIC in Table 1. The minimum concentrations of antibiotics to reveal clear zone assessed by RDA against opportunistic infectious bacteria were shown in Table 4. The minimum concentration of antibiotics assessed by RDA was higher than that of MIC in Table 2.

Table 3 Minimum concentration of antibiotics to reveal clear zone assessed by RDA against probiotic candidate bacteria

Oral probiotic bacteria		*L. c* 1	*L. c* 2	*L. f*	*L. g*	*S. m*
Antibiotic (μg/mL)	Ampicillin	50	25	6.25	12.5	1.56
	Erythromycin	3.13	6.25	6.25	3.13	0.78
	Gentamicin	200	200	25	25	200
	Ofloxacin	800≦	800≦	800≦	800≦	25
	Tetracycline	50	50	200	100	12.5
	Vancomycin	800≦	25	800≦	25	1.56

Table 4 Minimum concentration of antibiotics to reveal clear zone assessed by RDA against opportunistic infectious bacteria

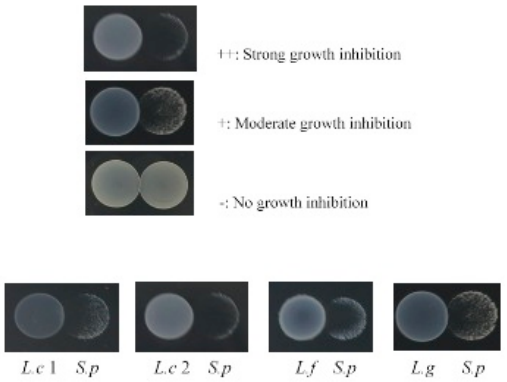
Opportunic infection bacteria		*H. influenzae* ATCC 9795	*S. pneumoniae* ATCC 33400
Antibiotic (μg/mL)	Ampicillin	6.25	1.56
	Erythromycin	50	0.78
	Gentamicin	12.5	25
	Ofloxacin	0.2	50
	Tetracycline	0.39	3.13
	Vancomycin	400	6.25

Competition assay between *L. gasseri* and *S. pneumoniae*

The competition assay between probiotic candidate Lactobacilli and *S. pneumoniae* were performed (Fig.1). *L.c* 2 showed strong growth inhibition against *S. pneumoniae*, and *L.c* 1, *L.f* and *L.g* showed moderate growth inhibition. *L.c* 1 was reported to inhibit *H. influenzae* [12], and *L.c* 2 was demonstrated to inhibit *S. pneumoniae* [13]. In this study, therefore, the growth inhibition of *S. pneumoniae* by *L.g* was investigated in detail.

RDA of culture supernatant of *L. gasseri*

The antibacterial activities of culture supernatant of *L. gasseri* and the fraction obtained with ammonium sulfate precipitation against *S. pneumoniae* were measured by RDA (Fig. 2). The supernatant showed obviously positive antibacterial activity and 0-30% saturated ammonium sulfate fraction and 30-50% fraction revealed stronger activities. A 50-80% saturated ammonium sulfate fraction did not show antibacterial activity (data not shown).



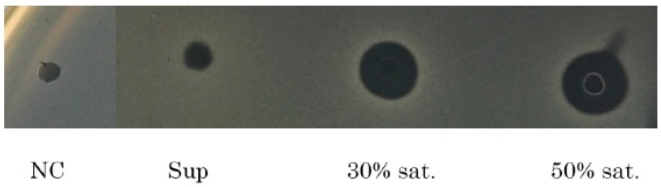
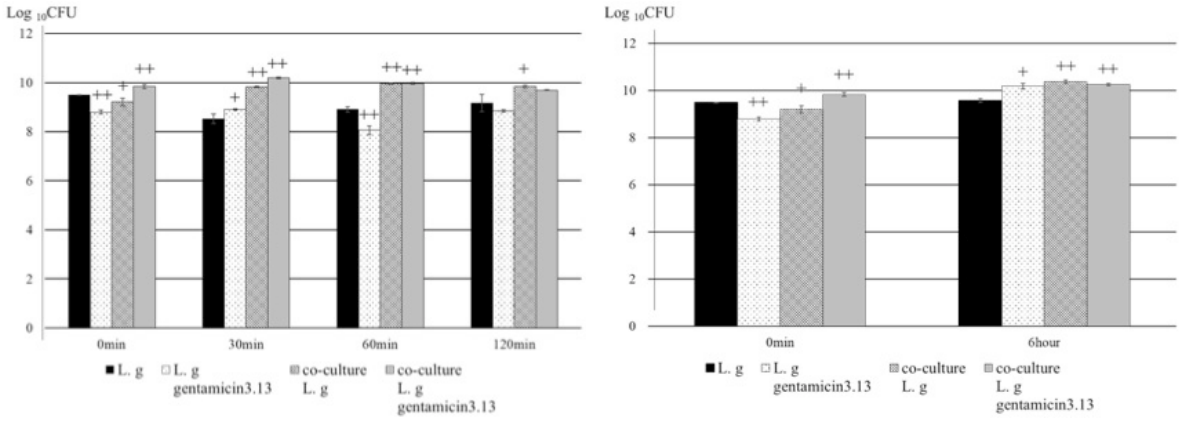


Fig. 1 (left) Competition assay between Lactobacilli and *S. pneumoniae* ATCC 33400
Fig. 2 (right) RDA of culture supernatant of *L. gasseri*. NC: negative control (PBS), Sup: culture supernatant of modified MRS, 30% sat.: 0-30% saturated ammonium sulfate fraction, 50% sat.: 30-50% saturated ammonium sulfate fraction

Co-culture of *L. gasseri* and *S. pneumoniae*

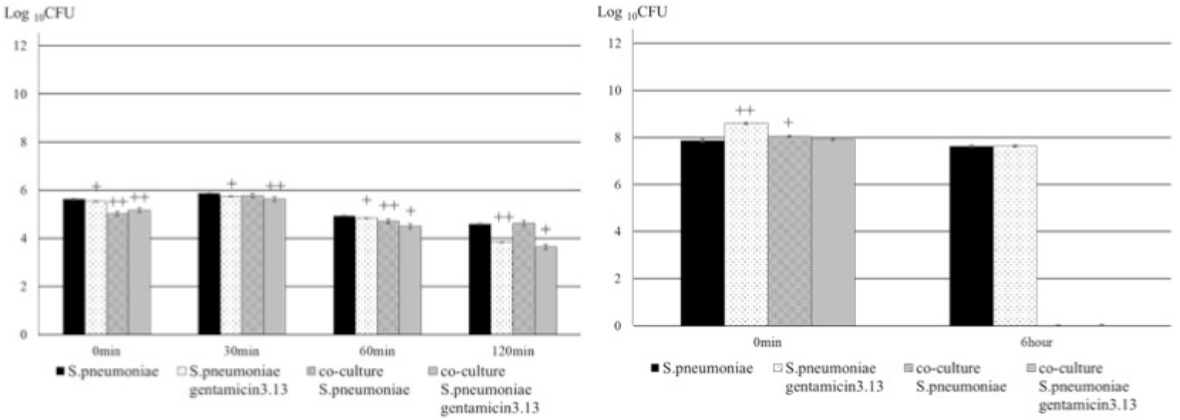
The co-culture of *L. gasseri* and *S. pneumoniae* ATCC 33400 was performed in the presence or absence of gentamicin (3.13 μg/mL). As shown in Figs. 3a and 3b, the cell number of *L. gasseri* was not changed up to 6

hours culture. On the other hand, the cell number of *S. pneumoniae* decreased after 2 hours culture, and disappeared after 6 hours culture (Figs 4a and 4b).



t-test; + $p < 0.05$, ++ $p < 0.01$

Fig. 3a (left) CFU of *L. g* in co-culture of *L. g* and *S. p* during 2 hours in the presence of GM 3.13 μg/mL ($n = 3$)
Fig. 3b (right) CFU of *L. g* in co-culture of *L. g* and *S. p* for 6 hours in the presence of GM 3.13 μg/mL ($n = 3$)



t-test; + $p < 0.05$, ++ $p < 0.01$

Fig. 4a (left) CFU of *S. p* in co-culture of *L. g* and *S. p* during 2 hours in the presence of GM 3.13 μg/mL ($n = 3$).
Fig. 4b (right) CFU of *S. p* in co-culture of *L. g* and *S. p* for 6 hours in the presence of GM 3.13 μg/mL ($n = 3$).

Real-time monitor of *S. pneumoniae* biofilm formation by xCELLigence

The biofilm formation of *S. pneumoniae* was monitored by xCELLigence in real-time manner in the presence of 50% saturated ammonium sulfate fraction from *L. gasseri* and various concentrations of gentamicin (GM) (Fig. 5). As shown in Table 2, the MIC of gentamicin against *S. pneumoniae* was 12.5 μg/mL. The biofilm formation curve in the presence of 6.25 μg/mL gentamicin was slightly inhibited compared to that of control. The biofilm formation curves in the presence of more than MIC (12.5 μg/mL) of gentamicin were inhibited in dose dependent manner.

SDS-PAGE of 50% saturated ammonium sulfate fraction

Fig. 6 shows the results of SDS-PAGE and subsequent staining with EzStain (left) and for antibacterial activity (right; clear zone on the gel shown with arrow). Antibacterial activity was observed around molecular mass of 2-3KD.

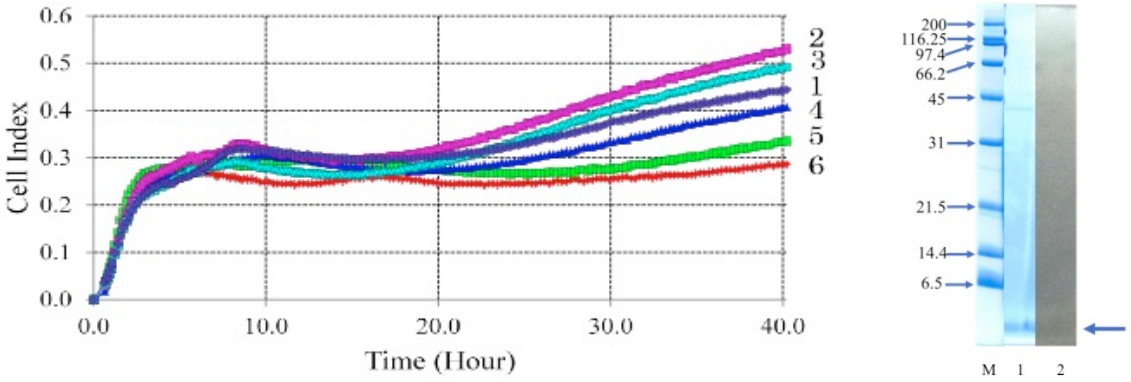


Fig. 5 (left) Real-time monitoring of biofilm formation of *S. pneumoniae* by xCELLigence in the presence of 50% saturated ammonium sulfate fraction from *L. gasseri* and various concentrations of gentamicin (GM).
1, GM (0); 2, GM (1.56), 3: GM (3.13), 4: GM (6.25), 5: GM (12.5), 6: GM (25) (μg/mL)
Fig. 6 (right) SDS-PAGE analysis of 50% saturated ammonium sulfate fraction of *L. gasseri*
Lane M: molecular mass standards; 200, 116.25, 97.4, 66.2, 45, 31, 21.5, 14.4, and 6.5 kDa, Lane 1: 50% saturated ammonium sulfate fraction stained with EzStain, Lane 2: gel overlaid on agar medium containing *S. pneumoniae*

Discussion

The MIC of six kinds of antibiotics against five strains of probiotic candidate bacteria were relatively high except for *S. mitis* (Table 1), and that against opportunistic bacteria such as *H. influenzae* and *S. pneumoniae* were relatively low (Table 2). These results showed that four strains of *Lactobacillus* were relatively resistant to antibiotics used and opportunistic bacteria were relatively sensitive to antibiotics. This difference was utilized in this study to examine the effect of co-culture of *L. gasseri* and *S. pneumoniae* in the presence of antibiotics. The minimum concentration of antibiotics to reveal clear zone on the agar medium against probiotic candidate bacteria and opportunistic bacteria was examined by RDA. Generally minimum concentration of antibiotics to reveal clear zone assessed by RDA was higher than that of MIC. Existence of agar and difference of collision rate between antibiotics and bacteria would influence on the results.

All strains of *Lactobacillus* used showed antibacterial activity against *S. pneumoniae* in competition assay (Fig. 1). In this study, the effect of *L. gasseri* on the growth of *S. pneumoniae* ATCC 33400 was investigated in detail. The antibacterial activities of *L. gasseri* against *S. pneumoniae* were observed in culture supernatant, 0-30% and 30-50% saturated ammonium sulfate precipitation fractions assessed by RDA (Fig. 2). The antibacterial activity of *Lactobacillus crispatus* LBS 17-11 (same as YIT 12945) against *S. pneumoniae* ATCC 33400 were reported in 35-80% saturated ammonium sulfate precipitation fraction [13]. They reported that the antibacterial activity was ascribed to the substance with molecular mass of approximately 7 kDa. In this study, the antibacterial substance in 30-50% saturated ammonium sulfate precipitation fractions was examined by SDS-PAGE, and antibacterial activity against *S. pneumoniae* was observed at a band with molecular mass of 2-3 kDa (Fig. 6). *L. gasseri* is known to produce some kinds of gassericin such as gassericin A [23], E [24], and T [25]. Molecular mass of these gassericin are around 5 kDa. The similarity between these gassericin and our antibacterial substance is unclear. The entity of antibacterial substance in this study is unidentified, so that we need further investigation in the future.

In co-culture experiment, the growth of *S.pneumoniae* was inhibited by sub-dose of MIC of gentamicin in the presence of *L. gasseri* (Figs. 4a and 4b). Combined effect of *L. gasseri* and antibiotics would be suggested to suppress the growth of *S. pneumoniae*. Because limited amounts of gentamicin in blood is secreted into saliva, sufficient concentration of gentamicin will not be expected in saliva [7]. The help by probiotics will promote better condition of oral cavity.

A real-time cell analyzer (RTCA), xCELLigence is an instrument to quantify cell proliferation, morphology change and cell-substrate attachment in a real-time manner. xCELLigence is used in wide fields including cell biology and bacteriology [26]. In fact, biofilm formation of some bacteria including *S. aureus* was investigated by using xCELLigence [27]. In this study, xCELLigence was employed to monitor real-time biofilm formation of *S. pneumoniae* in the presence of 50% saturated ammonium sulfate precipitation fraction and various concentrations of gentamicin, and the biofilm formation was inhibited by gentamicin (same as MIC level and more) in dose dependent manner (Fig. 5). The probiotics will help to maintain healthy oral circumstances and *L. gasseri* might be candidate of oral probiotics for healthy individuals and for especially patients in the prognosis and postoperative healing processes.

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Conflict of Interest
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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