ORIGINAL ARTICLE

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Efficacy of *Lactiplantibacillus plantarum 299* and *299*v against nosocomial oropharyngeal pathogens in vitro and as an oral prophylactic treatment in a randomized, controlled clinical trial

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Abstract

Background: Disturbance in the oropharyngeal microbiota is common in hospitalized patients and contributes to the development of nosocomial pneumonia. *Lactiplantibacillus plantarum 299 and 299v* (Lp299 and Lp299v) are probiotic bacteria with beneficial effects on the human microbiome.

Aim: To investigate how Lp299 and Lp299v affect the growth of nosocomial oropharyngeal pathogens in vitro and to evaluate the efficacy in vivo when these probiotics are administered prophylactically in hospitalized patients.

Methods: The in vitro effect of Lp299 and Lp299v on nosocomial respiratory tract pathogens was evaluated using two methods, the co-culture and agar overlay. In the clinical study, patients were randomized to orally receive either probiotics or placebo twice daily during their hospital stay. Oropharyngeal swabs were analyzed at inclusion and every fourth day throughout hospitalization.

Findings: All tested pathogens were completely inhibited by both Lp299 and Lp299v using the agar-overlay method. In the co-culture experiment, Lp299 and Lp299v significantly ($p < 0.05$) reduced the growth of all pathogens except for *Enterococcus faecalis* co-incubated with Lp299. In the clinical study, daily oral treatment with Lp299 and Lp299v did not influence the development of disturbed oropharyngeal microbiota or nosocomial infection. Proton pump inhibitors, antibiotics, and steroid treatment were identified as risk factors for developing disturbed oropharyngeal microbiota.

Conclusions: Lp299 and Lp299v inhibited pathogen growth in vitro but did not affect the oropharyngeal microbiota in vivo. The ClinicalTrials.gov Identifier for this study is NCT02303301.

KEYWORDS
hospitalization, nosocomial infection, nosocomial pathogens, oropharyngeal microbiota, probiotics

1 | INTRODUCTION

Probiotics are defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Different probiotics display a variety of antimicrobial properties, for example, the production of ammonia, lactic acid, free fatty chains, hydrogen peroxide, and bacteriocins (de Vrese & Schrezenmeir, 2008). *Lactiplantibacillus plantarum 299* and *299v* (Lp299 and Lp299v) are probiotic bacteria within the lactic acid bacteria group. *L. plantarum* can secrete bacteriocins, also called plantaricins (Adebayo et al., 2014; Prabhurajeshwar & Chandrakanth, 2017; Seddik et al., 2017), with inhibitory effects on, for example, oral *Streptococcus mutans* (Hasslöf et al., 2010). A recent review (Simons et al., 2020) presents the possible role of bacteriocins as a part of future antibiotic treatment.

A disturbance in the microbiome of the oropharynx, defined as an overgrowth of normally existing species and/or establishment of new potential pathogens, has been shown to indicate the degree of sickness in the host, and to be associated with increased mortality in the intensive care unit (ICU) and non-ICU patients (Dickson et al., 2020; Johanson et al., 1969). The microbiome of the oropharynx and that of the lower respiratory tract resemble each other, probably due to the microaspiration of oropharyngeal microbiota (Bassis et al., 2015). Microaspiration of disturbed oropharyngeal microbiota has been shown to play a part in the complex pathogenesis behind the development of pneumonia (Bahrani-Mougeot et al., 2007; Huffnagle et al., 2016) Therefore, a large number of studies have investigated the effects of decontamination of the oropharynx (using chlorhexidine or local antibiotics) or of administration of probiotics, with varying results (Bo et al., 2020; Gu et al., 2012; Karacaer et al., 2017; Klarin et al., 2008; Morrow et al., 2010; Wang et al., 2013; Weng et al., 2017). These studies have been carried out in ICU settings or pediatric populations, diminishing the occurrence of respiratory disease as well as antibiotic-associated diarrhea (Hatakka et al., 2001; Ling et al., 2019; Niveen et al., 2016). Because of the heterogeneity in the populations studied, generalized conclusions and recommendations about probiotic benefits are difficult to present.

In this study, we investigated whether Lp299 and Lp299v can reduce or inhibit the growth of nosocomial pathogens in vitro and in vivo. Seven pathogens were selected for the in vitro study, due to their frequent appearance in oropharyngeal cultures in ICU and non-ICU patients according to previous studies (Klarin et al., 2018; Tranberg et al., 2018). The randomized controlled trial aimed to study whether oral administration with lactobacilli could prevent or delay the occurrence of disturbed oropharyngeal microbiota in non-ICU hospitalized patients.

2 | MATERIALS AND METHODS

2.1 | In vitro study

2.1.1 | Bacterial strains

Lp299 and Lp299v were provided by Probi AB, Lund, Sweden. Reference strains of the pathogens *Escherichia coli* (CCUG 24), *Staphylococcus aureus* (CCUG 1800), *Enterococcus faecalis* (CCUG 19916) and *E. faecium* (CCUG 542), *Klebsiella pneumoniae* (CCUG 225), *Pseudomonas aeruginosa* (CCUG 551) and *Enterobacter cloacae* (CCUG 6323) were purchased from Culture Collection, University of Gothenburg, Sweden. Clinical isolates came from the Department of Clinical Microbiology at Skåne University Hospital, Sweden.

2.1.2 | Growth conditions

Lp299 and Lp299v were grown in De Man-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany) and on MRS agar. *E. faecalis*, *E. faecium*, and *S. aureus* were cultured in Todd Hewitt (TH) broth (Becton Dickinson) and agar, whereas *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae* were grown in lysogeny broth (LB) (Sigma-Aldrich, St. Louis, MO, USA) and agar. All strains were cultured at 37°C under aerobic conditions (21% oxygen, 5% CO_2).

2.1.3 | Agar overlay

Overnight cultures of Lp299 and Lp299v were washed in Phosphate-buffered saline (PBS) and adjusted to final concentrations of approximately 2×10^9 colony-forming units (CFU)/ml. Varying amounts (4×10^4, 4×10^5, and 4 × 106 CFU, respectively) of Lp299 or Lp299v were added to 8 ml of warm (42–45°C) MRS agar and poured into Petri dishes. Control plates contained no lactobacilli. After solidification, this bottom agar was incubated at 37°C overnight. The second layer of agar (24 ml), suited for the pathogen, was then cast on top of the MRS agar. Overnight cultures of the pathogens were diluted 1:1000, 1:10,000, and 1:100,000 in PBS, and 10 μl drops of the dilutions were seeded on the top agar. After overnight incubation at 37°C, the growth of the pathogen was assessed. Experiments were repeated twice using reference strains and once with clinical isolates of the pathogen.

2.1.4 | Inhibitory activity of Lp299 and Lp229v

Co-cultures of lactobacilli and pathogens were grown in a mixed broth consisting of 25% (v/v) MRS and 75% (v/v) TH or LB broth.

These proportions provide good growth conditions for both lactobacilli and pathogens. Overnight cultures of Lp299, Lp299v, and pathogen strains were washed and adjusted to bacterial suspensions of 2×10^9 CFU/ml, and 50 µl of the pathogen and 500 µl of Lp229 or Lp299v were added to 10 ml of mixed broth. As a control, 50 µl of the pathogen suspension was grown in mixed broth in the absence of lactobacilli. The co-cultures were incubated for 5 hours at 37°C. Before and after incubation, a small aliquot of each sample was diluted in PBS and plated on 15–20 ml agar suitable for the pathogen. After incubation overnight at 37°C, colonies of the pathogens were counted, and the growth of lactobacilli was ensured. Experiments were performed in triplicate.

2.1.5 | Antibacterial activity of Lp299 and Lp299v supernatants

Overnight cultures of Lp229 or Lp299v were pelleted by centrifugation, and the supernatants were sterile filtered through a 0.22 µm Millex®- GP, Millipore Express® PES Membrane Filter (Merck Millipore Ltd). The cell-free supernatants were then either pH-neutralized with 1 M NaOH to a pH of 5.4 (corresponding to the natural pH of MRS broth); heat-treated at 99°C for 5 minutes, or incubated with pepsin (Sigma-Aldrich), proteinase K (Thermo Scientific), or trypsin (Sigma-Aldrich) at a final concentration of 1 mg/ml for 2 hours at 37°C. After that, the samples were heated to 99°C for 5 minutes to eliminate the protease activity. The inhibitory effect of the supernatants was tested against *E. cloacae* and *S. aureus*. These two were chosen as they were inhibited by Lp299 and 299v, and they differ in Gram staining and natural habitat. 2.5 ml of pH-neutralized, heat-treated, or protease-treated supernatant was added to 7.5 ml of TH or LB to obtain a mixed broth. Untreated supernatant, sterile MRS broth, and MRS adjusted to pH 4.2 with acetic acid were included for comparison. Pathogens were washed and diluted as described in the co-culture experiment, and 50 µl of pathogen solution was added to the mixed broths. As a control, pathogens were incubated with Lp299 or Lp299v in a mixed broth with sterile MRS. Samples were plated on agar before and after incubation for 5 hours as described above. After incubation overnight at 37°C, colonies of the pathogens were counted, and the growth of lactobacilli was ensured when relevant. Experiments were performed in triplicate.

2.2 | Randomized controlled trial

2.2.1 | Study population

Patients were enrolled in the study between 2014 and 2019 at the University Hospital in Lund using the following inclusion criteria: age ≥18 years, obtaining the first oropharyngeal swabs (OPS) within 24 hours of hospital admission, and an expected length of stay of more than 72 hours. Exclusion criteria were respiratory infection and prior

hospitalization within two weeks. The patients were enrolled by the investigators, research nurses, or medical students. The patients were admitted to medical, surgical, or orthopedic wards. A standardized case report form was used to record patient data.

We based our approximative power calculation on a study from 1969, where oropharyngeal cultures had been analyzed throughout the hospitalization in ward patients (Johanson et al., 1969). From that study, we estimated that a sample size of 75 patients in each group would be sufficient to show a significant difference between the groups in the occurrence of disturbed microbiota in the oropharynx. The patients were younger but much sicker in the study from 1969, and patients that would now be admitted to the ICU were treated in general wards. The study from 1969 was observational, and the above factors put together made it difficult to make an exact power calculation.

2.2.2 | Randomization

The randomization was performed directly after inclusion via sealed envelopes at a 1:1 ratio. The randomization was blinded to recruiters, staff, and patients.

2.2.3 | Intervention

Patients received either a combination of 10^{10} CFU Lp299 and 10^{10} CFU Lp299v with 3 grams of maltodextrin or a placebo consisting of only 3 grams of maltodextrin. Both lactobacilli and placebo were manufactured and generously provided by Probi AB, Lund, Sweden, and delivered in identical freeze-dried sachets labeled "A" and "B," respectively. The sachets were kept in a −80°C freezer until use. In the ward, the sachets were kept at 4°C for a maximum of five days. Viability controls of the lactobacilli in the sachets were performed yearly throughout the study period, analyzing sachets stored at both −80°C and 4°C. Before administration to the patient, the contents of the sachets were resuspended in 15 ml of sterile water, allowing the revival of the potential lactobacilli for 20–40 minutes before administering the mixture to the patient. Patients received the assigned mixture twice daily throughout the hospital stay, with instructions to gargle the mixture as long as possible and then swallow. OPSs were taken at inclusion (day 1), on day 3, and after that approximately every fourth day. In all other respects, the patients received standard care.

2.2.4 | Microbiological procedures and definitions

The OPSs were processed by extended microbiological procedures at the Department of Clinical Microbiology, Skane University Hospital in Lund. The laboratory is accredited by the accreditation body (SWEDAC) designated by the Swedish government and is formally recognized as competent according to European and international standards.

For bacteria cultivation, sampling media were inoculated on five types of agar plates (three selective, one differentiating, and one nonselective). All plates were produced in-house, sometimes using commercially available media components (5% horse blood, hematin agar, and UriSelect 4 agar), as listed below:

1. Agar with 5% horse blood (LabM, Heywood) supplemented with 10 mg/L colistin and 15 mg/L nalidixic acid with an optochin disk (selective);
2. Agar with 5% horse blood supplemented with 2 mg/L gentamicin and 25 mg/L nalidixic acid for Gram-positive cocci including *Streptococcus pneumoniae* (selective);
3. Hematin agar (Oxoid™, Thermo Science) supplemented with 300 mg/L bacitracin for fastidious Gram-negative rods including *Haemophilus influenzae* (selective)
4. UriSelect 4 agar (Bio-Rad Laboratories) supplemented with 10 mg/L vancomycin for non-fastidious Gram-negative rods (differentiating)
5. Hematin agar with a colistin disk (nonselective).

The plates were inspected for growth after 16 and 40 hours of aerobic, anaerobic, or CO_2 incubation at 35–37°C. If an inspection result was ambiguous at 40 hours, the plate was incubated for an additional 24 hours to obtain a more definite result. Species identification of bacteria was performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MALDI Biotyper Microbial Identification System, Bruker), using software FlexControl 3.4 and MALDI Biotyper (MBT) Compass 4.1, with MBT Compass Library, DB-7854 MSP (Bruker, Bremen, Germany).

Cultivation and differentiation of *Candida spp.* were based on colony appearance on CHROM Candida agar (CHROMagar, Hägersten, Sweden) after 48 hours of incubation at 35°C.

For a sample to be considered representative of "oropharyngeal microbiota," bacterial species normally found in the oropharynx were required to grow on the nonselective hematin plate as determined by visual inspection by an experienced senior microbiologist and following standard practice (Retchless et al., 2020). Samples were classified as disturbed oropharyngeal microbiota when there was a growth of species not normally found in the oral cavity and/or overgrowth of normal oropharyngeal microbiota on selective and differentiating plates. Samples with disturbed oropharyngeal microbiota were divided into three subclasses (see Figure 4): gut pathogens, respiratory tract pathogens, and yeast.

2.2.5 | Statistical analyses

Inhibitory effects in vitro were analyzed using Student's paired t-test. In the randomized controlled trial, continuous variables were presented as median, minimum, and maximum values. Dichotomous variables were presented as numbers and as a percentage of the total number. For subjects with a normal oropharyngeal microbiota

at inclusion, a univariate Poisson regression was used to analyze the association between the patients' characteristics (predicting variables) and the intervention they were randomized to (dependent variable). Thereafter, a multivariate Poisson regression model using the two strongest predicting variables from the univariate analysis was constructed, in which one additional potential explanatory variable was added to determine whether the model improved or did not improve by including a third variable. A Kaplan–Meyer analysis was performed to test for differences between the placebo and the lactobacilli group regarding "time to first disturbed oropharyngeal swab." Fisher's exact test was used to assess the relationship between the intervention group and nosocomial infection rate.

Statistical analyses were performed using IBM SPSS Statistics 26 for Windows (IBM Corp., Armonk, NY, USA). Odds ratios (ORs) are presented with a 95% confidence interval. $p < 0.05$ was considered significant, and all statistical tests were two-tailed.
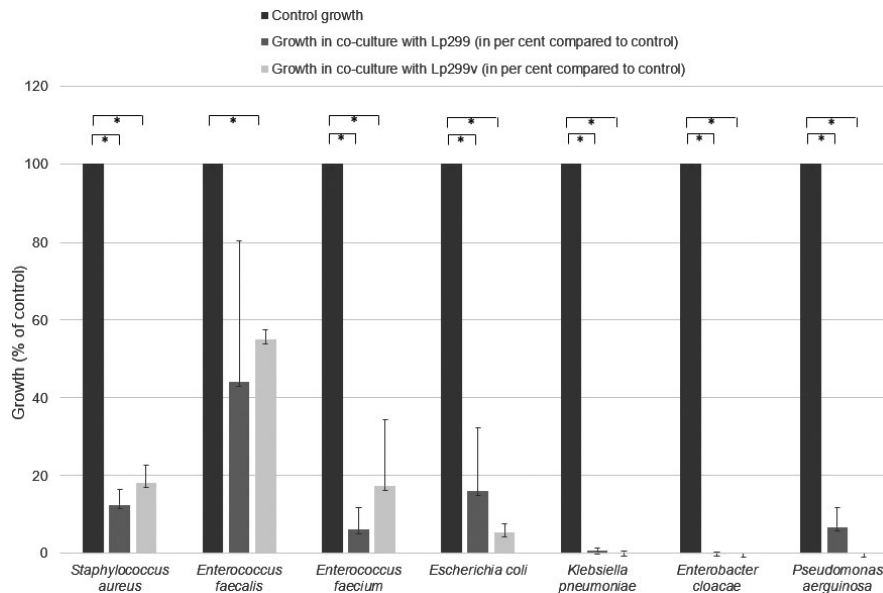
3 | RESULTS

3.1 | Lp299 and Lp299v inhibit the growth of bacterial pathogens in vitro

The inhibitory effect of Lp299 and Lp299v on other bacteria was first tested in an agar-overlay assay, where varying concentrations of lactobacilli were grown in a bottom MRS agar, and the pathogens were seeded on a top agar. Under these conditions, all experiments showed a complete absence of pathogen growth compared to control plates without lactobacilli. Both clinical isolates and the corresponding reference strains were tested and gave the same clear results. See Table A1.

Next, pathogens and lactobacilli were co-cultured in broth to further characterize the inhibitory effect. In this experimental set-up, both Lp299 and Lp299v significantly inhibited the growth of *S. aureus*, *E. cloacae*, *K. pneumoniae*, *E. coli*, *E. faecium*, and *P. aeruginosa*. For two pathogens, *E. cloacae* and *K. pneumoniae*, almost complete eradication of the pathogens was seen, as the number of colonies was close to zero after incubation. *E. faecalis* was significantly inhibited by Lp299v, but in co-culture, with Lp299 the inhibition did not reach statistical significance ($p = 0.12$). See Figure 1.

3.2 | The antibacterial activity of Lp299 AND Lp299v IS pH-dependent

To investigate the mechanism behind the growth-inhibitory effects of lactobacilli, *S. aureus* was incubated in a mixed broth containing cell-free supernatants from overnight cultures of Lp299 and Lp299v. The pH of the supernatants was 4.1 and 4.0, respectively. Figure 2 shows that the supernatants significantly inhibited the growth of *S. aureus* to the same extent as co-incubation with live bacteria. When the pH of the supernatants was elevated to 5.4, corresponding to the pH of MRS broth, the inhibitory effect

FIGURE 1 Co-culture of different pathogens with Lp299 or Lp299v resulted in significant growth inhibition for all pathogens except *Enterococcus faecalis* co-incubated with Lp299. The growth of the pathogen alone in the absence of lactobacilli served as control. *p < 0.05
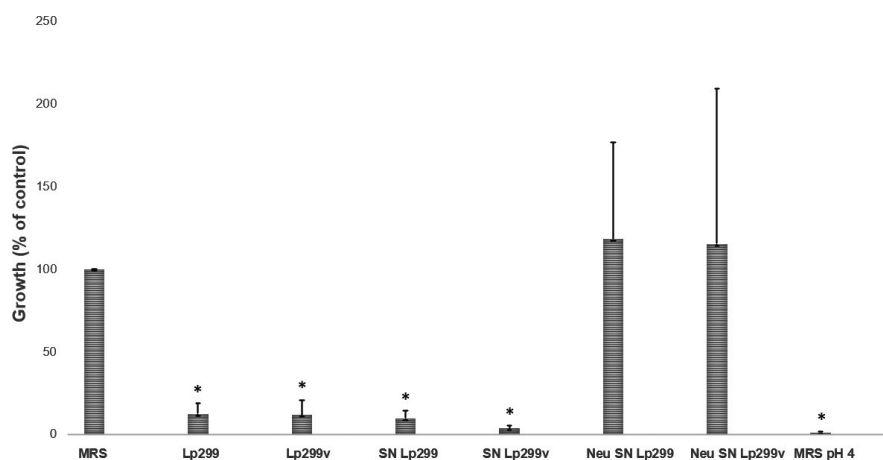
FIGURE 2 The inhibitory effect of Lp299 and Lp299v on *S. aureus* is pH-dependent. *S. aureus* was incubated with Lp299, Lp299v, or cell-free supernatants from overnight cultures of the lactobacilli. To explore the role of pH, *S. aureus* was also incubated with pH-neutralized supernatants and with acidified MRS broth. The bars with an asterisk above them indicate a significant growth inhibition compared to the control with *S. aureus* grown in a mixed broth without lactobacilli. SN =supernatant. Neu =neutralized. *p < 0.05

was abolished, and *S. aureus* grew equally well as in the control. Further, MRS broth made acidic to pH 4.0 (same pH as the supernatants) significantly inhibited *S. aureus* growth to the same extent as the supernatants from both Lp299 and Lp299v (see Figure 2). The same results were obtained when the experiment was performed with *E. cloacae* (see Figure A1).

To examine the possible role of plantaricins secreted by Lp299 and Lp299v, *E. Cloacae* was incubated with Lp299 and Lp299v supernatants that had been heat-treated to denature the protein content, or pre-incubated with the proteinases pepsin, proteinase K or trypsin to digest proteins in the supernatants. All of the pre-treated supernatants showed the same clear growth inhibition as untreated supernatants, whereas the controls incubated with MRS

broth showed expected growth of the pathogen during the incubation time (see Figure A2).

Taken together, the overall conclusion of these experiments is that the inhibitory effect was mainly pH-dependent.

3.3 | Randomized controlled trial

Between the 18th of September 2014 to 1st of May 2019, 135 patients were included and randomized. Eighteen patients were excluded due to non-adherence to protocol. Thus, 117 patients met all inclusion criteria and contributed a total of 337 OPSs. (See Figure 3 for the CONSORT diagram). The median number of OPSs per patient

TABLE 1 Descriptive statistics of patients (n = 117)

Variable	Lactobacilli (n = 58)	Placebo (n = 59)	p
Age, years	76 (22–96)	76 (36–97)	0.926[b]
Gender, male	28 (48%)	26 (44%)	0.712[a]
Body mass index	26 (18–40)	26 (17–40)	0.311[b]
Current or ex-smoker	23 (40%)	24 (41%)	1.000[a]
Diabetes	15 (26%)	8 (14%)	0,108[a]
Alcohol intake >2 times/week	19 (33%)	21 (37%)	1.000[a]
Proton pump inhibitor	12 (21%)	19 (32%)	0.345[a]
Able to walk two flights of stairs	33 (57%)	33 (57%)	0.490[a]
Cortisone medication	3 (5%)	6 (10%)	0.490[a]
Unplanned admission	45 (78%)	42 (71%)	0.526[a]
Antibiotics >24 hours before hospitalization	2 (3%)	1 (1.7%)	0.619[a]
Antibiotics >24 hours during hospitalization	14(24%)	20 (34%)	0.309[a]
Prophylactic antibiotics perioperatively	36 (62%)	31 (53%)	0.352[a]
Oropharyngeal swabs	3 (2–8)	3 (2–6)	0.236[c]
Length of hospital stay (days)	8 (3–32)	7 (3–32)	0.260[c]
Nosocomial infection	4 (6.9%)	10 (17%)	0.153[a]

Data are presented as median (range) or number (percentage).

[a]Fisher's exact test, exact sig. (2-sided).

[b]Independent samples t-test, sig. (2-tailed).

[c]Mann–Whitney U test, exact sig. (2-tailed).

in both groups was 3. All OPSs were representative of oropharyngeal microbiota. The baseline patient characteristics and hospitalization characteristics of the placebo and lactobacilli groups were similar and are presented in Table 1.

Figure 4 presents the microbiological results for the 27 patients showing any type of disturbed oropharyngeal microbiota on any sampling occasion. Each horizontal row corresponds to a patient's observation time, and by following the row from left to right it is possible to see OPS changes over time. Using the color and species key, the figure shows the subclass and pathogen for each disturbed OPS. The upper part of the figure shows the patients receiving placebo and the lower part shows the patients receiving lactobacilli.

In 104 patients (89%), the first OPS at admission was normal. We analyzed results from these patients using univariate and multivariate Poisson analyses. The univariate analyses showed that treatment with lactobacilli yielded an RR of 0.96 (CI 0.36–2.55, p = 0.94) for acquiring disturbed oropharyngeal microbiota during hospitalization.

Both univariate and multivariate analyses showed that treatment with proton pump inhibitor (PPI), cortisone, or antibiotics during hospitalization could be associated with an added risk of developing disturbed microbiota during hospitalization (Table 2).

Kaplan–Meyer analyses were performed to determine whether treatment with Lp299 and Lp299v could delay the development of disturbed microbiota. There was a slight tendency to later development of disturbed microbiota in the treatment group, but the difference was not significant.
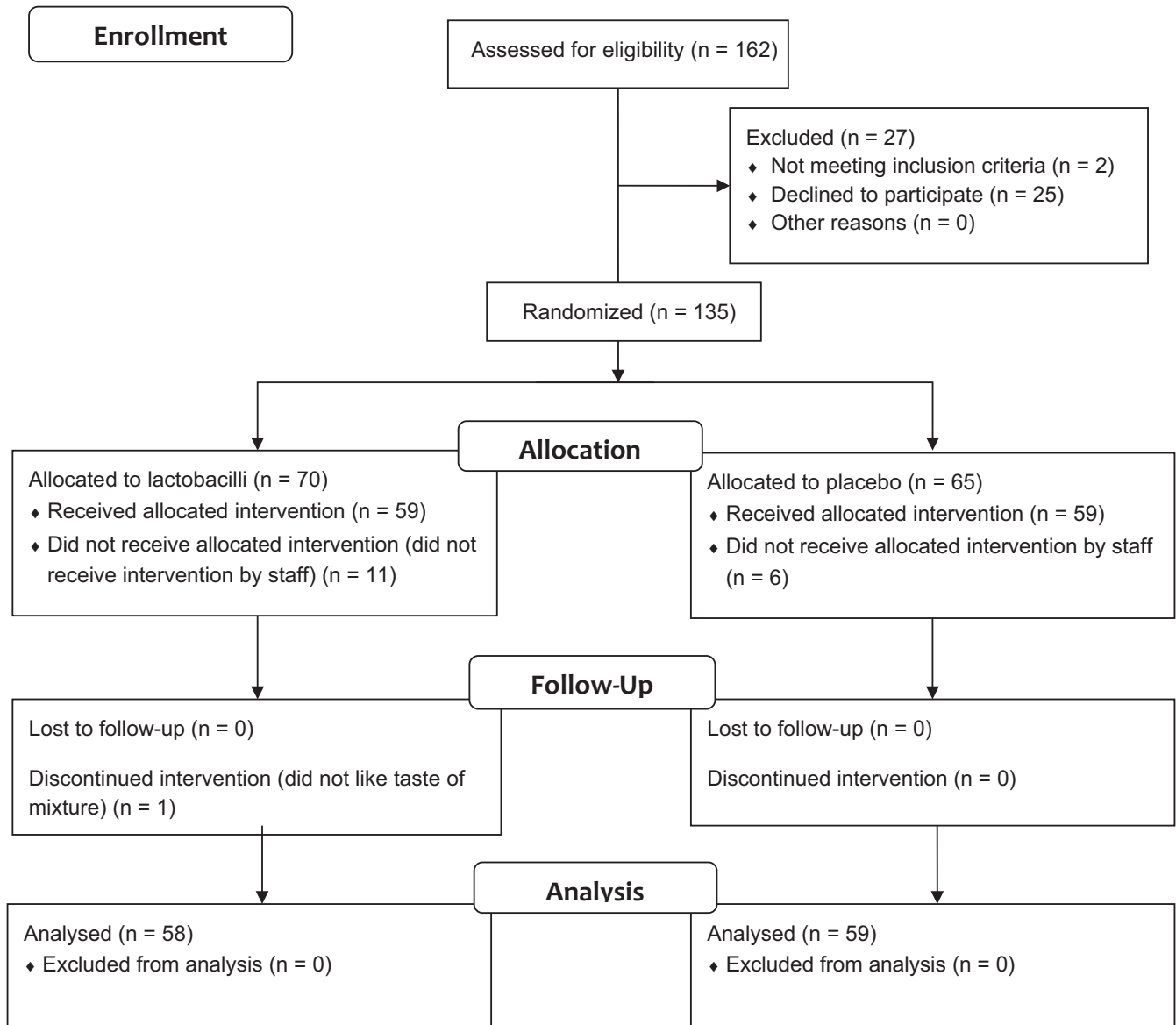
Concerning the risk of developing a nosocomial infection during hospitalization, the difference between the two groups did not reach significance. In the treatment group, 4/58 patients (7%) developed a nosocomial infection, while the incidence in the placebo group was 10/59 (17%, p = 0.153), with no obvious difference in the severity of infection between the groups (Table 1). The causes of nosocomial infection were urinary tract infections, wound infections, and pneumonia.

4 | DISCUSSION

In this combined laboratory and clinical study, we found that Lp299 and Lp299v significantly inhibited in vitro growth of nosocomial pathogens commonly found in the oropharyngeal tract of hospitalized patients. In the randomized controlled trial, no difference between the intervention group and the placebo group could be found regarding changes in the oropharyngeal microbiota or the occurrence of nosocomial infections. The study confirmed the already known risk factors for the development of disturbed oropharyngeal microbiota (Frandah et al., 2013).

The ability of lactobacilli to inhibit pathogen growth has been shown before (Annuk et al., 2003). This has also been specifically shown for Lp299v when Hutt et al. demonstrated its antagonistic effect on _Salmonella enterica_ and _Helicobacter_ pylori (Hutt et al., 2006). Furthermore, a study on oral care in ICU patients showed that Lp299 could be identified in the oropharynx in all patients given the study product (Klarin et al., 2008), indicating that the lactobacilli remain in the oropharynx after oral administration. In this study, we show for the first time that Lp299 and Lp299v inhibit in vitro growth of pathogens known to cause nosocomial respiratory tract infections. Notably, 60% of ICU patients are colonized with at least one of the seven investigated pathogens as early as 24 hours after admission to the ICU (Tranberg et al., 2018). The _L. plantarum_ species have a high production of lactic acid compared to others in the lactic acid bacteria group, and a relatively small production of, for example, hydrogen peroxide and carbon dioxide, which is typical for this group of facultatively heterofermentative lactobacilli (Annuk et al., 2003). In our study, the acidic environment produced by the lactobacilli was essential for inhibiting the in vitro growth of the pathogens under study. However, other factors may also be involved. For example, it has been shown that an acidic pH is necessary for other inhibitory mechanisms to be activated. Several studies on plantaricins show that they are activated at a pH <5 (Lin & Pan, 2019; Song et al., 2014). Although we were unable to demonstrate a plantaricin effect in our

Enrollment

Assessed for eligibility (n = 162)

Excluded (n = 27)
- ◆ Not meeting inclusion criteria (n = 2)
- ◆ Declined to participate (n = 25)
- ◆ Other reasons (n = 0)

Randomized (n = 135)

Allocation

Allocated to lactobacilli (n = 70)
- ◆ Received allocated intervention (n = 59)
- ◆ Did not receive allocated intervention (did not receive intervention by staff) (n = 11)

Allocated to placebo (n = 65)
- ◆ Received allocated intervention (n = 59)
- ◆ Did not receive allocated intervention by staff (n = 6)

Follow-Up

Lost to follow-up (n = 0)

Discontinued intervention (did not like taste of mixture) (n = 1)

Lost to follow-up (n = 0)

Discontinued intervention (n = 0)

Analysis

Analysed (n = 58)
- ◆ Excluded from analysis (n = 0)

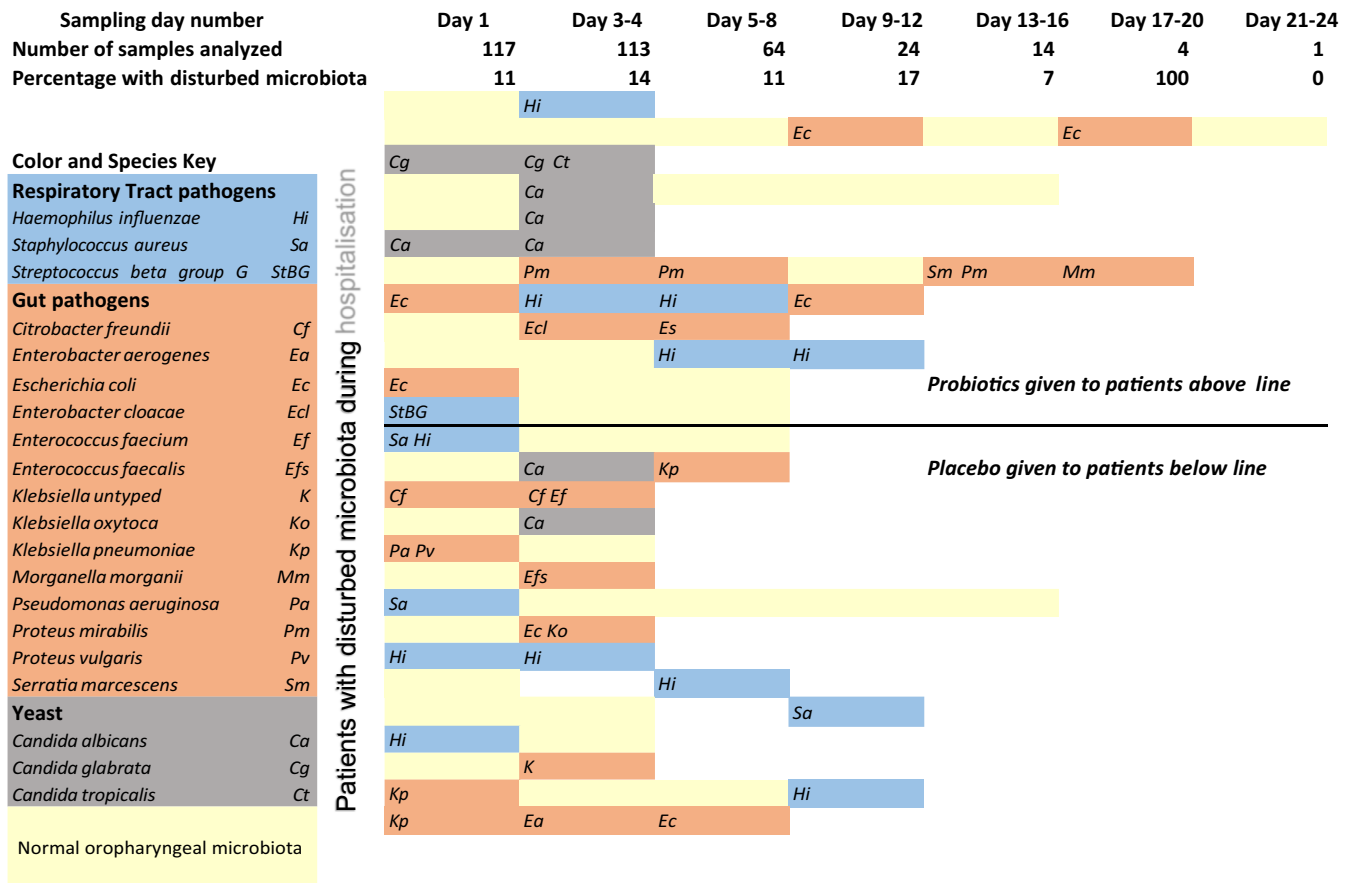Analysed (n = 59)
- ◆ Excluded from analysis (n = 0)

FIGURE 3 The CONSORT flow diagram. CONSORT (Consolidated Standards of Reporting Trials) diagram demonstrating the progress through the phases of the randomized trial of two groups

study, we cannot rule out a possible role of plantaricins in our strains due to the overwhelming effect of acidic pH. Further experiments are required to determine the possible presence and requisites for the activity of bacteriocins in Lp299 and Lp299v.

In the clinical trial, we could not show that the oral administration of Lp299 and Lp299v prevents or delays the occurrence of disturbed oropharyngeal microbiota in non-ICU hospitalized patients compared to placebo. In agreement with earlier findings (Tranberg et al., 2018), we found that 11% of the patients had disturbed oropharyngeal microbiota at admission and that an increasing proportion of the patients developed disturbed oropharyngeal microbiota during their hospitalization (see Figure 3). We also confirmed previously reported findings that treatment with PPI and antibiotics were risk factors for disturbed oropharyngeal microbiota (Frandah et al., 2013; Tranberg et al., 2018). In this study, oral cortisone was strongly

associated with disturbed oropharyngeal microbiota ($p = 0.0025$ in the multivariate Poisson regression analyses shown in Table 2, and $p = 0.0026$ in the univariate Poisson regression), which has not been shown before. The contribution of steroid inhalation treatment to the risk of developing oral candidiasis is well known. In our study, none of the patients who were on cortisone treatment developed disturbed microbiota consisting of Candida species, and only the third was simultaneously on PPI medication.

This study is unique, as it focuses on ward patients. Most previous studies on disturbed oropharyngeal microbiota and its possible contribution to nosocomial pneumonia have focused on intensive care patients. The idea of giving hospitalized patients probiotics is tempting, in many ways. Probiotics are harmless, inexpensive, and may reduce antibiotic use by restoring the patient's microbiota toward being healthier and more normal. When swallowed and

Sampling day number	Day 1	Day 3-4	Day 5-8	Day 9-12	Day 13-16	Day 17-20	Day 21-24
Number of samples analyzed	117	113	64	24	14	4	1
Percentage with disturbed microbiota	11	14	11	17	7	100	0

Color and Species Key

Respiratory Tract pathogens
Haemophilus influenzae	Hi
Staphylococcus aureus	Sa
Streptococcus beta group G	StBG

Gut pathogens
Citrobacter freundii	Cf
Enterobacter aerogenes	Ea
Escherichia coli	Ec
Enterobacter cloacae	Ecl
Enterococcus faecium	Ef
Enterococcus faecalis	Efs
Klebsiella untyped	K
Klebsiella oxytoca	Ko
Klebsiella pneumoniae	Kp
Morganella morganii	Mm
Pseudomonas aeruginosa	Pa
Proteus mirabilis	Pm
Proteus vulgaris	Pv
Serratia marcescens	Sm

Yeast
Candida albicans	Ca
Candida glabrata	Cg
Candida tropicalis	Ct

Normal oropharyngeal microbiota

Patients with disturbed microbiota during hospitalisation

Probiotics given to patients above line

Placebo given to patients below line

FIGURE 4 Oropharyngeal swab (OPS) culture results for the 27 patients who had at least one OPS sample with disturbed microbiota during their hospitalization. Each horizontal bar represents the patient's observation time, and the colored bars indicate an OPS culture result for each sampling time: yellow = normal, blue = respiratory pathogens, terracotta = gut microbiota, gray = yeast species. The top row describes the time frames within which the OPS was obtained. The second and third row shows the number of OPSs collected/analyzed and the percentage of OPSs with disturbed microbiota for the total cohort at each sampling time point. The patients are divided according to whether they received probiotics or placebo during hospitalization

TABLE 2 Poisson regression analysis for developing disturbed oropharyngeal microbiota during hospitalization in 104 subjects with a normal microbiota at admission, (yes n = 14, no n = 90). The number of patients receiving *Lactobacilli* in this analysis was 53, the control group consisted of 51 patients

	Univariate RR (95% CI)	p	Multivariate RR (95% CI)	p
Lactobacilli group	0.96 (0.36–2.55)	0.938		
Lactobacilli group			0.92 (0.33–2.51)	0.864
Diabetes	1.41 (0.49–4.08)	0.528	1.43 (0.48–4.31)	0.523
Lactobacilli			1.10 (0.41–2.96)[a]	0.849[a]
PPI	2.85 (1.10–7.39)	0.031	2.89 (1.08–7.76)[a]	0.035[a]
Lactobacilli			1.13 (0.44–2.90)	0.805
Cortisone	4.22 (1.65–10.8)	0.0026	4.32 (1.68–11.1)	0.0025
Lactobacilli			0.94 (0.36–2.46)	0.893
Antibiotics before hospitalization	2.59 (0.48–13.9)	0.267	2.62 (0.48–14.2)	0.264
Lactobacilli			1.17 (0.44–3.13)	0.753
Antibiotics during hospitalization	3.14 (1.19–8.30)	0.021	3.23 (1.18–8.84)	0.023
Lactobacilli			0.97 (0.37–2.55)	0.952
Unplanned admission	0.83 (0.29–2.43)	0.739	0.83 (0.29–2.41)	0.738

[a]the maximum number of step-halvings was reached but the log-likelihood value cannot be further improved. Output for the last iteration is displayed. RR =risk ratio, PPI =proton pump inhibitor.

digested, the probiotics also influence the intestinal tract immune system. New connections between the composition of the gut microbiota and a wide range of diseases such as irritable bowel syndrome and depression have emerged in the last decade (Didari et al., 2015; Wallace & Milev, 2017).

An important weakness of the clinical trial is that our power calculation was based on older studies with longer hospital stays (Johanson et al., 1969). Consequently, the study was underpowered, and we can therefore unfairly rule out our hypothesis that treatment with lactobacilli can decrease or delay the incidence of disturbed oropharyngeal microbiota during hospitalization. An additional explanation for the lack of effect of probiotic treatment is that changes in the oropharyngeal microbiota take time and a potential contribution to the development of pneumonia even longer. Even if Lp299 and Lp299v showed clear growth inhibition on pathogens in vitro, we might need longer treatment times and longer local exposure to clinically be able to influence the oropharyngeal microbiota. Thus, it cannot be excluded that a study involving a larger patient population and more intense administration of a combination of probiotics would show an effect on a clinically meaningful level.

In conclusion, this study shows that Lp299 and Lp299v inhibit in vitro growth of commonly found nosocomial pathogens in the oropharynx. Oral administration of Lp299 and Lp299v to non-ICU patients did not reduce the risk of disturbed oropharyngeal microbiota or nosocomial infection.

ETHICS STATEMENT

The study protocol was approved by the Regional Ethical Review Board, Lund, Sweden (no. 2013/764). Written informed consent, including permission to collect and publish anonymous data, was obtained from the patients at inclusion.

CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Anna Tranberg: Conceptualization (equal); Data curation (equal); Formal analysis (lead); Funding acquisition (lead); Investigation (lead); Methodology (supporting); Project administration (equal); Visualization (lead); Writing-original draft (lead); Writing-review & editing (equal). **Bengt Klarin:** Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Funding acquisition (supporting); Investigation (supporting); Methodology (supporting); Project administration (equal); Visualization (supporting); Writing-original draft (supporting); Writing-review & editing (equal). **Julia Johansson:** Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Funding acquisition (supporting); Investigation (equal); Methodology (supporting); Project administration (equal); Visualization (supporting); Writing-original draft (supporting); Writing-review & editing (equal). **Lisa I. Påhlman:** Conceptualization (supporting); Investigation (supporting); Methodology (equal); Project administration (equal); Supervision (equal); Visualization (supporting); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the in vitro part of the study are available in the figshare repository at https://doi.org/10.6084/m9.figshare.13312118.v1 and https://doi.org/10.6084/m9.figshare.13311929.v1. The datasets generated and/or analyzed during the RCT part of the study are not publicly available due to patient confidentiality but are available from the corresponding author on reasonable request. The study is registered at ClinicalTrials.gov, with the clinical trial number: NCT02303301: https://clinicaltrials.gov/ct2/show/NCT02303301

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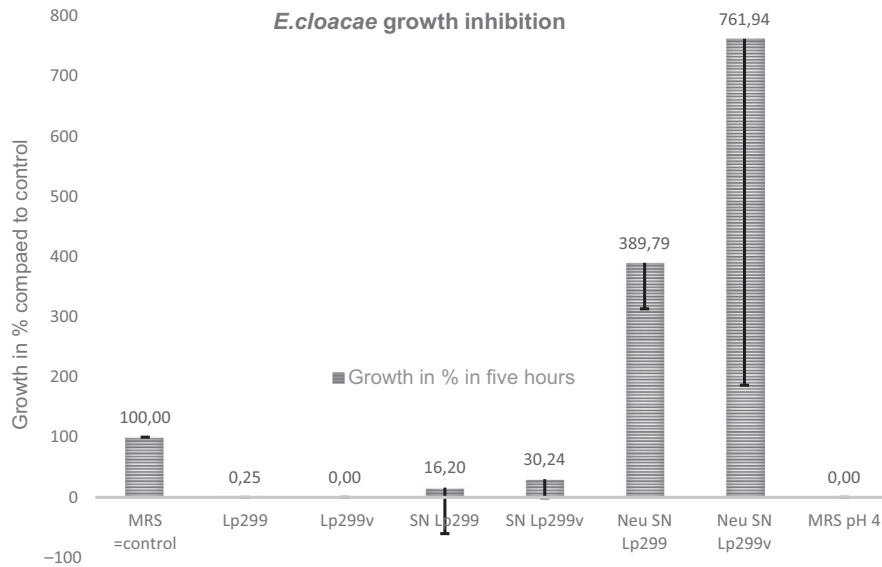
APPENDIX 1

Figure A1 The inhibitory effect of Lp299 and Lp299v on *E. cloacae* is pH-dependent. *E. cloacae* was incubated with Lp299, Lp299v, or cell-free supernatants from overnight cultures of the lactobacilli. To explore the role of pH, *E. cloacae* was also incubated with pH-neutralized supernatants and with acidified MRS broth. There is a significant difference in five-hour growth between the control (*E. cloacae* grown in 25% MRS broth) and all other groups ($p < 0.05$). There is also a significant difference in the five-hour growth between neutralized supernatants and all other groups ($p < 0.05$). SN =supernatant. Neu =neutralized
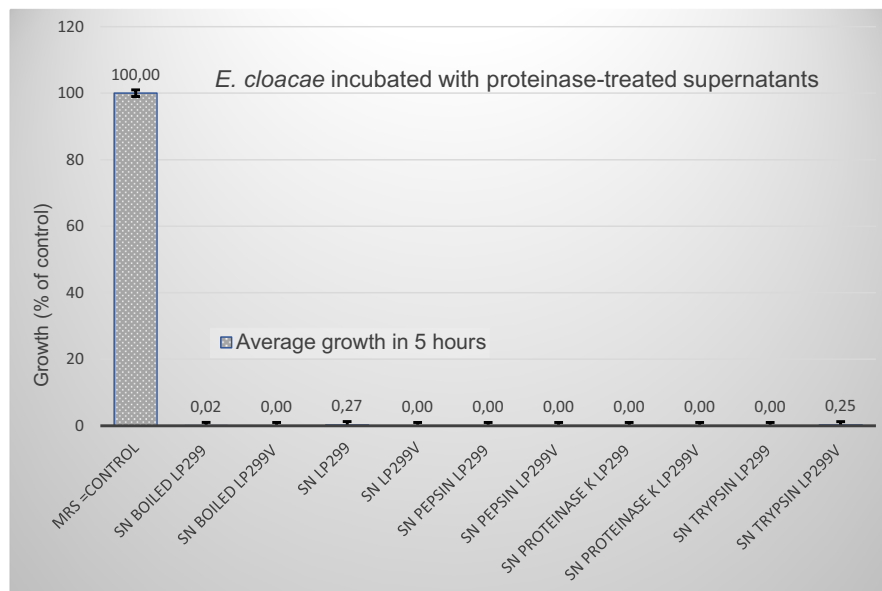

Figure A2 Overnight cultures of *E. cloacae* were allowed to incubate with pure supernatant (SN); boiled SN; and SN treated with the proteinases pepsin, proteinase K, and trypsin. Both Lp299 and Lp299v SN were included in the experiment. The results show that all of the pre-treated supernatants showed the same clear growth inhibition as untreated supernatants, whereas the control (*E. cloacae* incubated with MRS broth) showed expected growth of the pathogen during the incubation time

	Lp299			Lp299v		
	4×10^4 CFU	4×10^5 CFU	4×10^6 CFU	4×10^4 CFU	4×10^5 CFU	4×10^6 CFU
S. aureus (RS)	0	0	0	0	0	0
S. aureus (CI)	0	-	-	0	-	-
E. faecalis (RS)	0	0	0	0	0	0
E. faecalis (CI)	0	-	-	0	-	-
E. faecium (RS)	0	0	0	0	0	0
E. faecium (CI)	0	-	-	0	-	-
E. coli (RS)	0	0	0	0	0	0
E. coli (CI)	0	-	-	0	-	-
K. pneumoniae (RS)	0	0	0	0	0	0
K. pneumoniae (CI)	0	-	-	0	-	-
E. cloacae (RS)	0	0	0	0	0	0
P. aeruginosa (RS)	0	0	0	0	0	0
P. aeruginosa (CI)	0	-	-	0	-	-

TABLE A1 Results from the agar overlay experiment

Abbreviations: CI, Clinical isolate; RS, Reference strain.

Growth described as 0 = no growth, or 1= growth - = experiment not performed.