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Multifactorial inhibition of lactobacilli against the respiratory tract pathogen *Moraxella catarrhalis*

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RESEARCH ARTICLE

Abstract

Probiotics, mainly lactic acid bacteria (LAB), are widely focused on gastrointestinal applications. However, recent microbiome studies indicate that LAB can be endogenous members of other human body sites such as the upper respiratory tract (URT). Interestingly, DNA-based microbiome research suggests an inverse correlation between the presence of LAB and the occurrence of potential pathogens, such as *Moraxella catarrhalis*, an important URT pathogen linked to otitis media, sinusitis and chronic obstructive pulmonary disease. However, a direct interaction between these microbes has not been explored in detail. This study investigated the direct antipathogenic effects of *Lactobacillus* species, including several well-documented probiotic strains, on *M. catarrhalis* using agar-based assays, time course analysis, biofilm assays and minimal inhibitory concentration (MIC) testing. These assays were performed using spent culture supernatans (SCS) at two pHs (4.3 and 7) and D- and/or L-lactic acid at three pHs (2, 4 and 7). In addition, cell line assays for adhesion competition and immunomodulation were used to substantiate the inhibitory effect of lactobacilli against *M. catarrhalis*. A proportion of *Lactobacillus* strains, including the model probiotic *Lactobacillus rhamnosus* GG, showed a strong and direct activity against *M. catarrhalis*. Screening of the activity of the SCS after different treatments demonstrated that lactic acid has an important antimicrobial activity against this pathogen – at least *in vitro* – with mean MIC values for D- and L-lactic acid varying between 0.5 and 27 g/l depending on the pH. Furthermore, *L. rhamnosus* GG also decreased the adhesion of *M. catarrhalis* to human airway epithelial Calu-3 cells with more than 50%, and the expression of mucin MUC5AC, pro-inflammatory cytokines interleukin (IL)-8, IL-1β, and tumor necrosis factor-α at least 1.2 fold. This study suggests that several lactobacilli and their key metabolite lactic acid are possible candidates for probiotic therapeutic interventions against URT infections.

Keywords: probiotics, upper respiratory tract, *Lactobacillus rhamnosus* GG

1. Introduction

Moraxella catarrhalis is a Gram-negative pathogen implicated in many airway diseases, such as 15-20% of acute otitis media (OM) infections, 20% of acute sinusitis episodes in children and 10% of the chronic obstructive pulmonary disease exerbations in adults (Murphy *et al.*, 2009; Parameswaran *et al.*, 2009; Rovers *et al.*, 2004). Interestingly, while the colonisation rate is low in adults (1-5%), *M. catarrhalis* is a common coloniser of the infant nasopharynx (66-100%) (Murphy *et al.*, 2009). To treat upper respiratory tract (URT) infections, antibiotics are still considered as standard therapy, while they have many side-effects including the induction of antibiotic resistance (Goossens *et al.*, 2005). Indeed, a high proportion of *M. catarrhalis* strains are now resistant to beta-lactam antibiotics such as penicillin and amoxicillin (Harrison *et al.*, 2009). Moreover, antibiotics disturb the endogenous

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beneficial microbiota, especially when taken orally. Elimination of key commensal bacteria can facilitate pathogen colonisation in the gastro-intestinal tract and nasopharynx (Tagg and Dierksen, 2003), resulting in unwanted side-effects such as diarrhea and increased susceptibility to subsequent infections. In addition to pathogen restriction, an intact healthy microbiota plays a key role in the education and function of the immune system (Bogaert *et al.*, 2011; Pettigrew *et al.*, 2012; Stearns *et al.*, 2015). For all these reasons, an urgent need exists for alternatives to classical antibiotic therapy for infections with *Moraxella* and other URT pathogens.

In the gastro-intestinal and vaginal tract, probiotic lactobacilli have long been explored as alternatives for antibiotics. Interestingly, several recent microbiome studies have indicated that genera of lactic acid bacteria (LAB) such as *Lactobacillus* and *Lactococcus* can also be normal inhabitants of the URT, in variable relative abundances (Bogaert *et al.*, 2011; Pettigrew *et al.*, 2012; Stearns *et al.*, 2015). In children, a greater abundance of taxa such as *Lactococcus* and *Dolosigranulum* (both LABs) appear to be inversely correlated with *Moraxella* and the risk of developing acute OM (Biesbroek *et al.*, 2014; Pettigrew *et al.*, 2012). Bogaert and colleagues (2011) observed a marked increase in the absolute incidence of *(Brevi)bacillus* and *Lactobacillus* species colonisation in summer (96%) compared to winter (10%) season, the latter of which has the highest prevalence of URT infections. Stearns and colleagues (2015) reported that *Lactobacillus* strains were present in the nasopharyngeal samples of all 51 healthy children tested with a relative abundance varying between 0.0026 and 5.62%, making it the 13th most abundant species in that niche. Among the 19 healthy adults included in their study, lactobacilli were even more prevalent, ranked at the 11th place among commensal species with an abundance varying between 0.0057 and 13.29%. However, these DNA-based microbiome studies have not yet been backed up with mechanistic studies on the direct interactions between LAB and the corresponding URT pathogens. Yet, pilot studies have already shown that the administration of certain LAB could mediate a reduction in the incidence of OM in children (Hatakka *et al.*, 2007; Niittynen *et al.*, 2012; Rautava *et al.*, 2009). However, until now, most of these probiotics were applied orally in liquid formulas for immune boosting effects, with little attention to the possibility of direct inhibition of URT pathogens by the applied LAB.

In this study, the direct antimicrobial activities of various *Lactobacillus* strains, including several well-documented probiotic strains, were explored against *M. catarrhalis*. The analyses were conducted using optimised assays allowing detailed insights in 1:1 competitions between potential probiotic and pathogen.

2. Materials and methods

Bacterial strains and growth conditions

Lactobacillus strains (Table 1) were grown at 37 °C in de Man, Rogosa and Sharpe (MRS) medium (BD Difco, Erembodegem, Belgium). *M. catarrhalis* ATCC25238 was inoculated in Mueller Hinton (MH) (LabM Limited, Heywood, UK) broth and cultured aerobically at 37 °C. Solid media contained 1.5% (w/v) agar. For antimicrobial assays, the agar was enriched with glucose (Sigma Aldrich, Steinheim, Germany) to a final concentration of 5 g/l.

Cell culture

The human human airway epithelial cell line Calu-3 ATCC® HTB-55TM (purchased from ATCC, Molsheim Cedex, France) was cultured in 75 cm^2 flasks containing 20 ml Minimum Essential Medium (MEM) (Life Technologies, Erembodegem, Belgium) supplemented with heat inactivated fetal bovine serum (FBS) (Thermo Fischer, Asse, Belgium) and penicillin-streptomycin (100 U/ml) (Life Technologies) and maintained in a humidified 5% CO_2 incubator at 37 °C. The culture medium was changed every 3-4 days and the cells were passaged weekly at a 1:2 split ratio using a 0.25% trypsin-EDTA solution (Life Technologies). For adhesion and immunomodulation experiments, 0.5 ml of Calu-3 cells were seeded in 24-well culture plates at a density of 1.85×10^6 cells/ml. Approximately a week after seeding, confluent monolayers were obtained.

Preparation of spent culture supernatant of lactobacilli

Lactobacilli were incubated for 19 h (corresponding to a final concentration of $\pm2\times10^9$ cfu/ml) and spent culture supernatant (SCS) was obtained by centrifugation for 15 min at 4,000 rpm at 4 °C. Afterwards, the SCS was filter sterilised (0.20 µm cellulose acetate, VWR, Leuven, Belgium) and the pH (Mettler-Toledo AG, Zaventem, Belgium) and the concentration of D- and L-lactic acid (Roche Yellow Line, R-Biopharm AG, Darmstadt, Germany) were measured. To gain information on the nature of the active antimicrobial molecules produced by the lactobacilli, the SCSs of the different lactobacilli were treated by different methods: (1) heating at 70 °C for 30 min; (2) treatment with proteinase K (50 µg/ml) (Sigma-Aldrich, St. Louis, MO, USA) and incubation for 60 min at 37 °C; and (3) neutralisation to pH 7 by using NaOH.

Growth characteristics of lactobacilli

Growth was measured using Synergy HTX multimode plate reader (BioTek, Winooski VT, USA) by measuring optical density (OD) at 595 nm.

Table 1. Bacterial strains used in this study.

Species	Strain	Relevant genotype or description	Reference and/or source
Lactobacilli			
Lactobacillus casei	ATCC334	Single colony isolate obtained from a stock culture of ATCC334. Originally isolated from emmental cheese	ATCC
L. casei	Shirota	Single colony isolate obtained from a commercially available fermented drink containing *L. casei* Shirota (Yakult®), confirmed by sequencing	Commercial probiotic product
L. casei	DN-114001	Single colony isolate obtained in our lab from a commercially available fermented drink (Actimel®) containing *L. casei* DN-114001, confirmed by sequencing	Lebeer *et al.*, 2007
Lactobacillus parabuchneri	NM63-3	Single colony isolate from spontaneously fermented carrot juice	Lab collection
Lactobacillus paracasei	LMG12586	Single colony isolate obtained from a stock culture of LMG12586. Originally isolated from cheese	BCCM/LMG
Lactobacillus plantarum	LMG1284	Single colony isolate from *L. plantarum* ATCC8014 or LMG1284. Origin unknown	BCCM/LMG
L. plantarum	CMPG5300	Single colony isolate. Originally isolated from the vaginal cavity	Malik *et al.*, 2014
L. plantarum	5057	Single colony isolate of *L. plantarum* 5057. Originally isolated from maize silage	Danielsen, 2002
L. plantarum	WCFS1	Single colony isolate of *L. plantarum* WCFS1. Originally isolated from human saliva	Kleerebezem *et al.*, 2003
Lactobacillus reuteri	RC-14	Single colony isolate from a commercially available probiotic supplement containing *L. reuteri* RC-14, confirmed by sequencing	Commercial probiotic product
Lactobacillus rhamnosus	GR-1	Single colony isolate obtained from a commercially available probiotic supplement containing *L. rhamnosus* GR-1. Originally isolated from the female urethra	Chan *et al.*, 1984, 1985; Reid and Bruce, 2001; Reid and Reid, 1999, ATCC
L. rhamnosus	GG	Single colony isolate Wild type strain, originally isolated from human faeces	Kankainen *et al.*, 2009
Pathogens/control bacteria			
Haemophilus influenzae	ATCC49247	Single colony isolated from expectorated sputum from a 76 year-old male with pneumonia	ATCC
Moraxella catarrhalis	ATCC25238	Type strain, originally isolated from the nasal cavity	ATCC
Streptococcus pneumoniae	ATCC49619	Single colony isolated from sputum of 75-year-old male	ATCC
Escherichia coli	LMG2093	Single colony isolate obtained from a stock culture of LMG2093. Origin unknown	BCCM/LMG

Spot antimicrobial assays with live lactobacilli

The antimicrobial activity of live lactobacilli against the *M. catarrhalis* was explored by standard antimicrobial tests. The antimicrobial activity of *Lactobacillus* cultures was explored by modified spot assays (Schillinger and Lücke, 1989). Briefly, 2 µl of each *Lactobacillus* culture was spotted on a standard agar plate (1.5% w/v) containing medium of the pathogen supplemented to a final concentration of 5 g/l glucose and a spot of another bacterium was added as negative control. These plates were incubated for 48 h at 37 °C. Ca. 10^6 cfu/ml of the overnight pathogen culture were added to 7 ml of soft agar (0.5% w/v) and poured over the plates with *Lactobacillus* spots. The plates were incubated overnight at 37 °C, after which time the inhibition zones were measured.

Radial diffusion antimicrobial assay for SCS of lactobacilli

The antimicrobial activity of SCS was investigated with a modified protocol based on previously described competition assays between lactobacilli and gastro-intestinal pathogens (Lie *et al.*, 1997). Approximately 10^6 cfu/ml of *M. catarrhalis* was added to 60 ml of 1.5% (w/v) agar and poured in a square agar plate. After the plates dried, wholes of 0.4 cm diameter were made in the agar and 30 µl of SCS of lactobacilli was added. The plates were incubated overnight and the inhibition zones were measured. 0.1% hexetidine (Famar Orléans, Orléans, France) and sterile MRS medium brought to pH 4.3 were used as a positive and negative control, respectively.

Time-course analysis of the antimicrobial activity of spent culture supernatans

A time-course analysis of the growth inhibition in suspension was performed as described previously (De Keersmaecker *et al.*, 2006) with minor modifications. Briefly, 190 µl of a diluted overnight culture of *M. catarrhalis* (ca. 10^5 cfu/ml) was added to the wells of a microplate supplemented with 10 µl SCS of lactobacilli to get a total volume of 200 µl. 10 µl 0.1% hexetidine and 10 µl MRS medium at pH 4.3 were used as a positive and negative control, respectively. Bacteria were grown and the OD was measured at 600 nm each 30 min using a Synergy HTX multi-mode reader. Each test was measured at least in triplicate and the average OD was calculated.

Minimal inhibitory concentration assay

The minimal inhibitory concentration (MIC) values were determined based on the procedure described earlier (Nizet *et al.*, 2001). *M. catarrhalis* was grown in Mueller Hinton medium as described above. A 96-well plate was filled to get a total volume of 100 µl with a final concentration of 5×10^5 cfu/ml of the pathogen combined with an adequate concentration of the active molecule. The microplate was incubated overnight at 37 °C and the OD at 600 nm was measured.

Biofilm assays

Biofilm formation of the pathogens in the presence of SCS of the lactobacilli was monitored using static biofilm assays. For *M. catarrhalis*, biofilms were grown as described by Pearson and Hansen (2007) with minor modifications. An overnight culture of *M. catarrhalis* was diluted 1:100 ($\pm10^5$ cfu/ml) in Brain Heart Infusion broth (Difco, BD, Franklin Lakes, NJ, USA). A volume of 190 µl of this suspension was loaded into a 96-well microplate and incubated overnight at 37 °C. To test the antimicrobial activity of the SCS, 10 µl portions of SCS were loaded into the 96-well microplate. 10 µl 0.1% hexetidine and 10 µl MRS medium at pH 4.3 were used as a positive and negative, respectively. After 19 h, the broth was removed from each well and replaced by 200 µl phosphate buffered saline (PBS) plus 10 µl of 0.7% (w/v) crystal violet. After 15 min at room temperature, the wells were emptied and washed three times with deionised water. A 200-µl volume of 95% ethanol was added to each well, and the plate was shaken gently for 15 min. A 150 µl portion of this ethanol solution was then transferred to a new 96-well plate, and the absorbance at 570 nm was measured using a Synergy HTX multi-mode reader.

Adhesion competition assays

The influence of lactobacilli on the adherence of *M. catarrhalis* to human airway epithelial Calu-3 cells was investigated as described previously (Malik *et al.*, 2013) with minor modifications. Competition tests were carried out by adding a volume of 0.5 ml containing *M. catarrhalis* (5×10^7 or 5×10^6 cfu) and *Lactobacillus rhamnosus* GG (5×10^7 cfu) to a tissue culture 24-well plate containing confluent monolayers of epithelial cells, which were allowed to incubate at 37 °C for 1 h to mediate adherence. After incubation, the cells were washed three times with PBS (37 °C) to remove all nonadhering cells and the number of adhering pathogenic cells to the Calu-3 cells was determined by macrodilution method on appropriate agar. Each condition was carried out at least in triplicate.

Induction of cytokine gene expression in Calu-3 epithelial cells

The cytokine response after co-incubation of Calu-3 cells with *M. catarrhalis* and *L. rhamnosus* GG was determined as follows. The bacterial strains were first grown overnight and subsequently centrifuged at 2,000×*g* at 4 °C for 10 min, washed with PBS and resuspended in MEM medium. *M. catarrhalis* (5×10^7 cfu) was added to the Calu-3 epithelial cells, alone or in presence of *L. rhamnosus* GG (5×10^7 cfu). After 4 h of incubation, the cells were rinsed thrice with PBS. Next, total RNA was extracted from the Calu-3 cells with RNeasy isolation kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. A constant amount of 1 µg of total RNA was used for oligo-(dT)-primed cDNA synthesis using ReadyScript® reverse transcriptase (Sigma Aldrich). The cytokine gene expression was measurement by quantitative real time polymerase chain reaction (qRT-PCR).

qRT-PCR

Expression of interleukin (IL)-8, IL-1β, mucin MUC5AC and tumour necrosis factor-α (TNF-α) was analysed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Data were quantified with the ΔΔCt method, relative to the reference genes Cytochrome c1 (CYC1) and ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B). Primer sequences can be found in Table 2. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were strictly followed (Bustin *et al.*, 2009).

Statistics

Data are represented as mean values ± standard deviation. One-way ANOVA and multiple t-tests were used to determine statistical significance in GraphPad Prism (La

Table 2. Primers used for qRT-PCR.

Primer[1]	Oligonucleotide sequence (5'-3')
CYC1 (F)	CATGTCCCAGATAGCCAAGGA
CYC1 (R)	CTTGTGCCGCTTTATGGTGTAG
ATP5B (F)	GCAGGAAAGAATTACCACTACCAAG
ATP5B (R)	TGGTAGCATCCAAATGGGCAA
IL-1β (F)	TTGCTCAAGTGTCTGAAGCAGC
IL-1β (R)	CAAGTCATCCTCATTGCCACTG
IL-8 (F)	TGGCAGCCTTCCTGATTTCT
IL-8 (R)	TTAGCACTCCTTGGCAAAACTG
TNF-α (F)	CCTCTGATGGCACCACCAG
TNF-α (R)	TCTTCTCGAACCCCGAGTGA
MUC5AC (F)	GGGACTTCTCCTACCAAT
MUC5AC (R)	TATATGGTGGATCCTGCAGGGTAG

[1] F = forward; R = reverse.

Jolla, CA, USA). The *P*-value was corrected for multiple comparisons using the Hilm-sidak method in GraphPad Prism. Differences were considered statistically significant at $P<0.05$.

3. Results

A proportion of *Lactobacillus* strains shows antimicrobial activity against *M. catarrhalis*

The direct interaction between living lactobacilli and *M. catarrhalis* was tested via spot assays. In these assays, all lactobacilli tested demonstrated a strong activity against *M. catarrhalis.* Interestingly, several well-known *Lactobacillus* strains, such as *L. rhamnosus* GG, *Lactobacillus casei* Shirota, *L. casei* DN-114001, *Lactobacillus plantarum* LMG1284 and *L. plantarum* WCFS1 were each very active with clear inhibition zones in the range of the positive control 0.1% hexetidine, a common oral antiseptic agent for oropharyngeal infections (Table 3). As a control, non-*Lactobacillus* microbial species such as *Escherichia coli, Haemophilus influenzae* and *Streptococcus pneumoniae,* and the pathogen itself were also included, but expressed no antimicrobial activity against *M. catarrhalis*.

Subsequently, the activities of the secreted metabolites of the *Lactobacillus* strains were explored by testing the antimicrobial activity of their respective SCS in radial diffusion assays. The SCS of almost all *Lactobacillus* strains tested showed a strong activity with inhibition zones larger than 0.5 cm (Table 3).

Table 3. Agar-based antimicrobial screening of the interaction between lactobacilli and *Moraxella catarrhalis*.[1]

Species	Strain	Spot assay[2]	Radial diffusion assay[2]
Lactobacillus casei	ATCC334	+++	++
L. casei	Shirota	+++	+++
L. casei	DN-114001	+++	+++
Lactobacillus parabuchneri	NM63-3	+++	+
Lactobacillus paracasei	LMG12586	+++	++
Lactobacillus plantarum	LMG1284	+++	+++
L. plantarum	CMPG5300	+++	+++
L. plantarum	5057	+++	+++
L. plantarum	WCFS1	+++	+++
Lactobacillus reuteri	RC-14	+++	+++
Lactobacillus rhamnosus	GR-1	+++	+++
L. rhamnosus	GG	+++	+++
Escherichia coli	LMG2093	+	-
Streptococcus pneumoniae	ATCC49619	–	-
Moraxella catarrhalis	ATCC25238	–	-
Haemophilus influenzae	ATCC49247	–	-
0.1% hexetidine		n.a.	+++
Plain MRS[3] pH 4.3		n.a.	–

[1] Inhibition zones of various *Lactobacillus* strains against *M. catarrhalis* tested in two different assays.
[2] – = no inhibition; + = 0-0.2 cm inhibition; ++ = 0.2-0.5 cm inhibition; +++ = >0.5 cm: inhibition; n.a. = not applicable/available.
[3] MRS = de Man, Rogosa and Sharpe medium.

Major antimicrobial compound of *L. rhamnosus* GG against *M. catarrhalis* is pH-dependent, heat-stable, proteinase K-resistant

To explore this antimicrobial activity of the SCS further, we performed inhibition experiments with the SCS of different *Lactobacillus* strains, and monitored the growth of *M. catarrhalis* for 72 h. A prominent strain-specific effect of *Lactobacillus* SCS and its metabolites against *M. catarrhalis* was observed (Figure 1A). Interestingly, addition of the SCS of the model probiotic strain *L. rhamnosus* GG inhibited the growth of *Moraxella* for 72 h to the same extent as the positive control (0.1% hexetidine). This activity was also observed for *L. rhamnosus* GR-1, *L. plantarum* CMPG5300 and *L. casei* ATCC334 but not for slower growing strains exemplified here by *L. parabuchneri* NM 63-3 (Figure 1A). The negative control, i.e. non-inoculated MRS medium brought to pH 4.3, only induced a small delay in growth of *M. catarrhalis*, indicating that the effect is not merely pH related. Subsequent survival and plate counting assays indicated that the effect of SCS on the growth of *M. catarrhalis* was bacteriostatic.

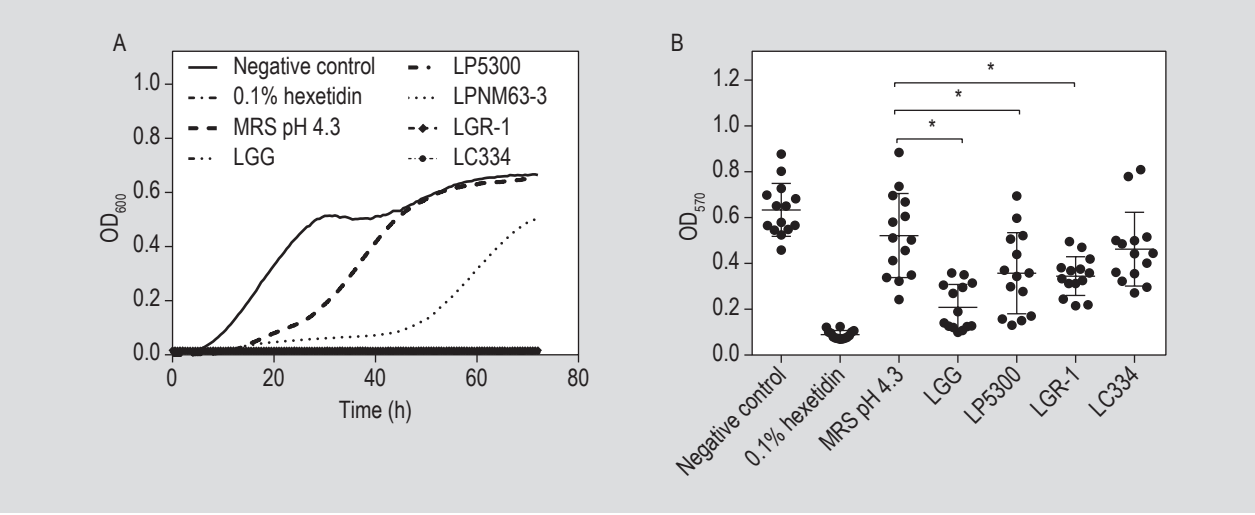

Figure 1. (A) Effect of *Lactobacillus* spent culture supernatant (SCS) against growth of *Moraxella catarrhalis* in suspension. Non-inoculated MRS medium and 0.1% hexetidine were used as negative and positive control, respectively. Graphs of LGG, LP5300, LGR-1 and LC334 are overlapping since they do not show growth. (B) The effect of SCS of different *Lactobacillus* strains on the growth of *M. catarrhalis* as biofilms for 19 h measured by crystal violet staining (OD_{570}). * $P<0.05$.

Since *M. catarrhalis* often resides within biofilms in infected patients (Perez *et al.*, 2014), we explored the capacity of SCS of lactobacilli to prevent biofilm formation. As shown in Figure 1B, SCS of *L. rhamnosus* GG could reduce the biofilm formation of *M. catarrhalis* up to 70% under the tested conditions. Various other *Lactobacillus* strains tested such as *L. rhamnosus* GR-1, *Lactobacillus reuteri* RC-14, *L. casei* DN-114001, *L. casei* Shirota and *L. plantarum* CMPG5300 also showed the potential to reduce biofilm formation, but their activity appeared less reproducible. For *L. casei* ATCC334, we could not observe a significant reduction under the tested conditions (Figure 1B).

We subsequently aimed to identify the active antimicrobial molecules in the SCS of the lactobacilli, by inactivating or removing different classes of active molecules (acids and proteins). We focused on SCS of *L. rhamnosus* GG as our model probiotic strain, because this SCS was most active in the experiments described above. The radial diffusion antimicrobial assay showed the antimicrobial activity of SCS of *L. rhamnosus* GG to be pH-dependent, resistant to proteinase K, and heat-stable, since the inhibition zones only disappeared after neutralising the pH (Figure 2A). In the time-course analysis, the antimicrobial activity disappeared when the SCS was neutralised to pH 7, both when SCS was added in the beginning of the experiment or after overnight incubation of *M. catarrhalis* (20 h). In contrast, SCS treated with heat or proteinase K maintained its antimicrobial activity (Figure 2B). Additionally, the antibiofilm activity of the SCS was clearly decreased when the pH was neutralised. However, merely pH reduction of MRS medium brought to pH 4.3 by HCl did not significantly affect biofilm formation. Heat and proteinase K-treatment also did not significantly affect the antibiofilm activity (Figure 2C).

Lactic acid is an important antimicrobial compound against *M. catarrhalis*

Since the antimicrobial activity of lactic acid against certain gastro-intestinal Gram-negative pathogens has been previously documented to be pH-dependent (De Keersmaecker *et al.*, 2006) and since the above-described experiments clearly point to acids as active effector molecules, we subsequently explored the role of lactic acid in the inhibition of the Gram-negative pathogen *M. catarrhalis*. First, we measured the total amount of lactic acid produced in SCS after overnight growth under standard conditions in MRS medium. This amount differed clearly between the various *Lactobacillus* strains tested as well as the ratio of D- and L-lactic acid. All *L. plantarum* strains tested produced almost similar amounts of D- and L-lactic acid, while the other *Lactobacillus* strains tested appeared to produce almost exclusively L-lactic acid (Figure 3A). A high pH and low concentration of LA were linked to decreased activity against *M. catarrhalis*.

To confirm the antimicrobial activity of D- and L-lactic acid, MIC assays were performed against *M. catarrhalis*. Both D- and L-lactic acid showed a strong activity against *M. catarrhalis* with an MIC of ca. 0.5 g/l (Figure 3B). Interestingly, D- and L-lactic acid were almost 10-fold less active when they were dissolved in a neutral solution, confirming the pH-dependent activity of lactic acid.

Other known more indirect antipathogenic activities of probiotics are competitive exclusion and immuno-modulation. Here, we could show that *L. rhamnosus* GG also inhibited the adhesion of different concentrations of *M. catarrhalis* to Calu-3 cells (Figure 4A). Addition of *L.*

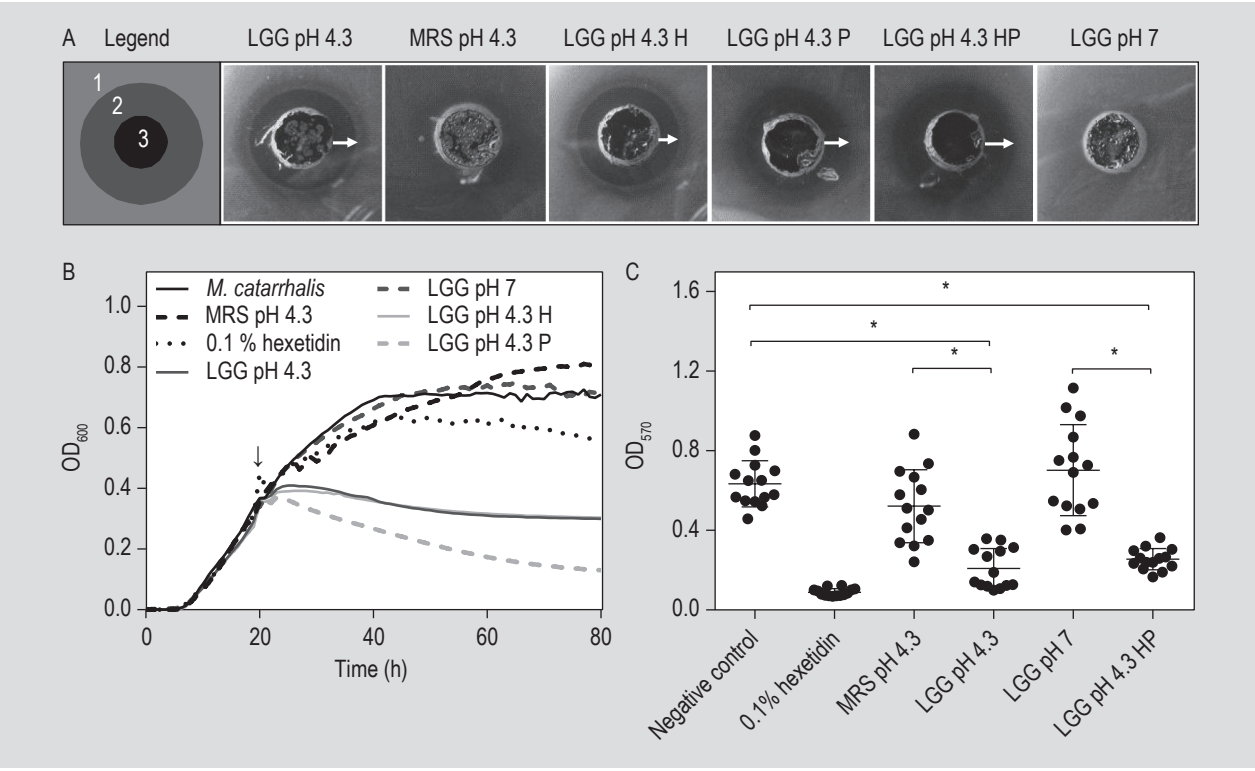

Figure 2. Impact of various treatments on the antimicrobial and antibiofilm effect of spent culture supernatant of *Lactobacillus rhamnosus* GG (LGG) against *Moraxella catarrhalis*. (A) Radial-diffusion assay with spent culture supernatant (SCS). 1 = growth pathogen; 2 = inhibition zone; 3 = well. (B) Time course analysis of *M. catarrhalis*. The arrow indicates the addition of SCS after 20 h incubation of *M. catarrhalis*. Non-inoculated MRS medium brought to pH 4.3 and 0.1% hexetidine served as negative and positive control, respectively. (C) Biofilm formation of *M. catarrhalis*. Treated LGG-SCS was added. H = LGG SCS treated with heat; P = LGG SCS treated with proteinase K; HP = LGG-SCS treated with heat and proteinase K. * *P*<0.05.

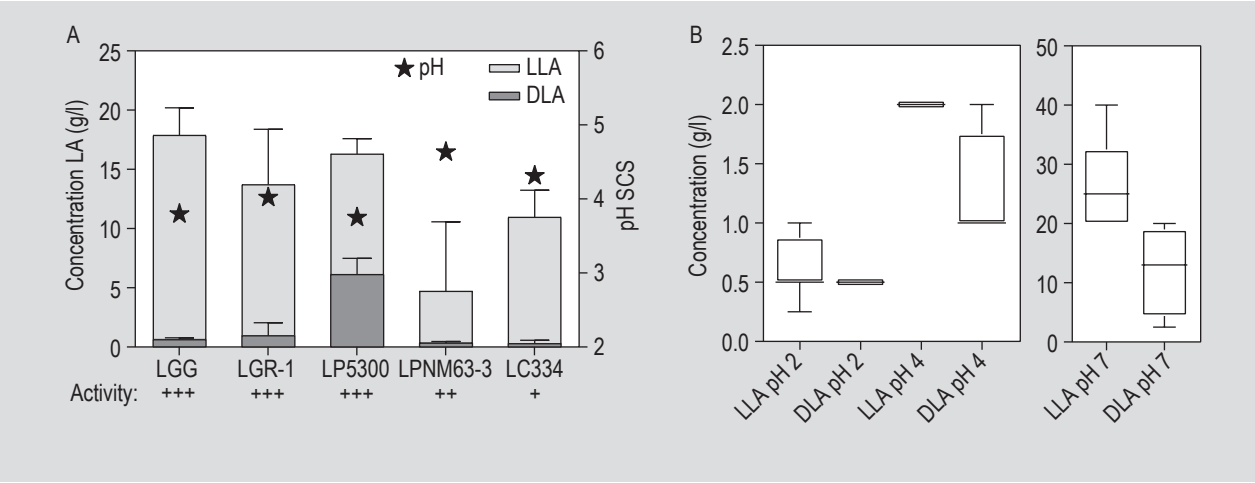

Figure 3. Antimicrobial activity of D- and L-lactic acid (-LA) against *Moraxella catarrhalis*. (A) Concentration of D- and L-LA produced, pH and activity of different *Lactobacillus* strains tested after growth for 19 h in MRS medium. (B) MIC assay results of LA under different pH conditions. The median of the results are given with the upper and lower limit of the data between brackets. Names of the *Lactobacillus* strains can be found in Table 1.

rhamnosus GG in a 1:1 or 10:1 ratio reduced adhesion of *M. catarrhalis* 1.9- and 2.6-fold, respectively (adjusted *P*-values both 0.012). Furthermore, a significant 1.8- (46%), 1.9- (46%), 1.2- (16%) and 1.5-fold (33%) reduction in IL-1β, IL-8, MUC5AC and TNF-α gene expression, respectively, was observed upon 1:1 co-incubation with *L. rhamnosus*

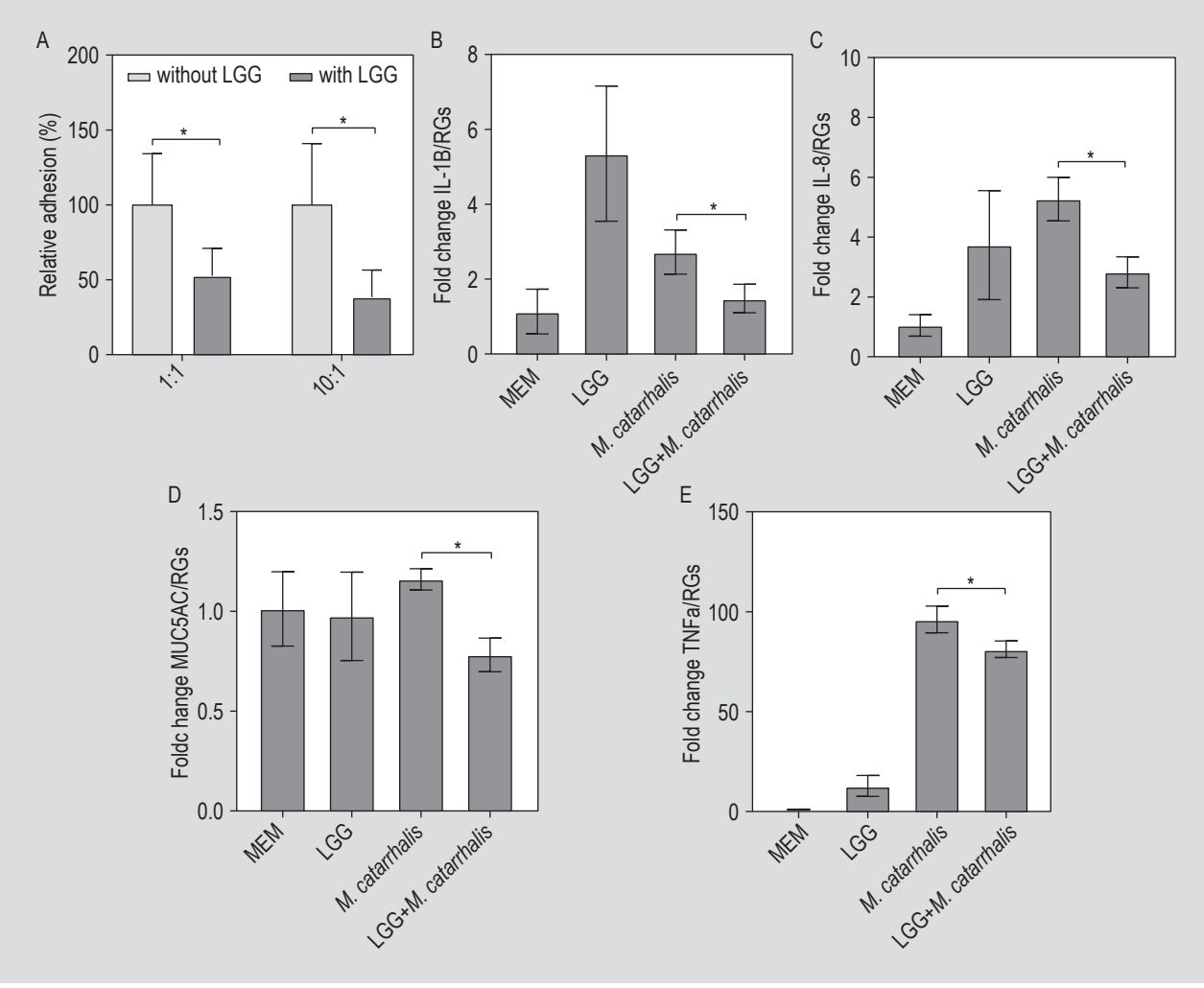

Figure 4. Competition in (A) adhesion and (B-E) immunomodulatory activity of *Lactobacillus rhamnosus* GG (LGG) vs *Moraxella catarrhalis*. mRNA expression of (B) interleukin (IL)-1β, (C) IL-8, (D) MUC5AC and (E) tumour necrosis factor alpha (TNF-α). MEM = only MEM medium. * *P*<0.05.

GG compared to *M. catarrhalis* only in the Calu-3 cell line (Adjusted *P*-values: 0.028, 0.027, 0.030 and 0.011, respectively) (Figure 4B-4E).

4. Discussion

URT infections mediated by *M. catarrhalis* in adults and children have a high incidence and thus form a major health threat. Furthermore, they form a common reason for antibiotic prescription in the clinical practice. Yet, unnecessary prescribing of antibiotics is costly, leads to serious unintended side effects, and increases the risk of developing antibiotic resistance (Boatright *et al.*, 2015). There is thus a clear need for alternatives for antibiotic therapy and prevention, particularly against Gram-negative pathogens (Wenzler *et al.*, 2016). Since pioneering microbial profiling studies have suggested correlations between the occurrence of LAB and reduced risk of URT infections, the use of probiotic lactobacilli in the fight against URT infections holds promise. In this study, we have shown for the first time – to the best of our knowledge – detailed data supporting a direct antimicrobial role for lactobacilli against a main URT pathogen, *M. catarrhalis*.

Our results show a clear potential for several *Lactobacillus* strains to inhibit the growth of *M. catarrhalis* in suspension and as biofilm. Furthermore, *L. rhamnosus* GG was shown to compete with *M. catarrhalis* for adhesion to human airway epithelial Calu-3 cells and decrease the gene expression of IL-1β, IL-8, MUC5AC and TNF-α. Both actively growing lactobacilli and their secreted metabolites in SCS showed a clear direct antimicrobial activity against *M. catarrhalis* in agar-based assays. As to the identification of active compounds, acids appeared to play a major role in the inhibition of the pathogen, since the activity disappeared after neutralising the pH. Furthermore, a link was found between the concentration of lactic acid in the SCS and

the inhibitory activity whichwas also confirmed in broth-based assays.

Most lactobacilli are homofermentative LAB that produce D- and/or L-lactic acid. To investigate the importance of both isomers in the antimicrobial activity of the *Lactobacillus* species, MIC assays were performed. We observed a MIC varying between 0.5 and 27 g/l depending on the pH for L- and D-lactic acid. These MIC values are higher than the MIC of 16 mg/l for amoxicillin, a common used antibiotic for URT infections (Harrison *et al.*, 2009). However, these values should be considered in the context of the human microbiota where live bacteria interact and where applied probiotics could continuously secrete lactic acid as a consequence of their metabolism when alive. Since previous microbiome profiling studies have documented that various *Lactobacillus* OTUs can be present in several individuals in relative amounts up to 13.29% in the URT niche (Abreu *et al.*, 2012; Bogaert *et al.*, 2011; Laufer *et al.*, 2011; Stearns *et al.*, 2015), a local production of lactic acid, or antimicrobial compounds in general, could be sufficient to compete in a direct way with the associated pathogens. In this way, a bacteriostatic activity could preserve the microbial balance in this niche and prevent overgrowth of unwanted pathogens. It is important to note that the MIC values of lactic acid against *M. catarrhalis* were found here to significantly increase with increasing pH. As previously ascertained for the pathogens *E. coli*, *Pseudomonas aeruginosa* and *Salmonella enterica* serovar Typhimurium (Alakomi *et al.*, 2005), the undissociated form of lactic acid appears to be most active also against the Gram-negative pathogen *M. catarrhalis*.

Although we could clearly substantiate an antimicrobial role for lactic acid against *M. catarrhalis* in our present work, possible activity of additional molecules can not be ruled out. These other molecules seem heat-resistant and pH-dependent based on the agar-based antimicrobial and biofilm assays and could have synergistic effects with lactic acid. However keeping in mind that the agar-based assays used in this study favour the identification of small active molecules, due to differences in diffusion velocities as described by Bonev *et al.* (2008), the involvement of other molecules cannot be ruled out. For example, Niku-Paavola *et al.* (1999) observed an inhibitory substance, the cyclic molecule mevalonolactone, in the SCS of *L. plantarum* VTT E-78076 which activity increased from 15 to 60% inhibition in the presence of 1% lactic acid (activity 40%). Such synergistic effects are likely secondary to the possible permeabilisation of the membrane by lactic acid. In addition, we could recently demonstrate an antibiofilm activity of lectin-like proteins of *L. rhamnosus* GG against other Gram-negative pathogens (Petrova *et al.*, 2016) pointing to additional antimicrobial mechanisms of lactobacilli.

More indirect activities such as competition for adhesion sites and nutrients (competitive exclusion) and modulations of the immune system are also postulated to be key factors in the antimicrobial potential of probiotics (Lebeer *et al.*, 2008). In this study, in addition to its direct antimicrobial effect, the effect of *L. rhamnosus* GG on adhesion and pro-inflammatory gene expression in Calu-3 cells by *M. catarrhalis* was therefore investigated. *L. rhamnosus* GG significantly dampened the gene expression of IL-1β, IL-8 and TNF-α after incubation with *Moraxella*. In addition, the gene expression of MUC5AC, a mucin commonly correlated with OM (Kerschner *et al.*, 2010), was significantly reduced. Especially in the URT, mucin production can be problematic and correlated with pathogens as they benefit from the increased nutrient availability and adherence possibility. Also Abreu *et al.* (2012) demonstrated in a mouse model that another URT pathogen (*Corynebacterium tuberculostearicum*) significantly increased the mucin secretion in the host, which could be attenuated in the presence of *Lactobacillus sakei.* The importance and exact molecular signalling events in the activity of lactobacilli against mucin hypersection remains however to be further explored.

To conclude, we have documented in this study that several *Lactobacillus* species show antimicrobial activities against *M. catarrhalis*. Local application of probiotic bacteria could thus have potential as a therapeutic or preventive strategy against URT infections in which this pathogen is involved. However, before such therapies will be feasible, clinical studies in humans will have to confirm our findings.

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