IN VITRO AND *IN VIVO* PROBIOTIC POTENTIAL OF *LACTOBACILLUS* SPP. FOR OTITIS MEDIA.

ir. Marianne van den Broek

Marianne.vandenbroek@uantwerpen.be

University of Antwerp

Faculty of Sciences

Department of Bioscience Engineering

Promotor:

Prof. Dr. ir. Sarah Lebeer

Doctoral Jury:

Prof. Apr. Paul Cos, UAntwerp, Belgium

Prof. Surbhi Malhotra, UAntwerp, Belgium

Prof. Dr. ir. Jos Vanderleyden, KU Leuven, Belgium

Dr. Arjen Nauta, FrieslandCampina, The Netherlands

“Success is going from failure to failure without losing your enthusiasm”

- Winston Churchill -

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Maar over jouw liefde, daarvan zing ik niet
Die is te mooi en te edel voor dit lied
Over jouw liefde, daarvan zing ik niet
Die is te rijk en te groot
Te diep, te verheven
Onmogelijk te vangen in mijn vergank'lijk lied

- Marianne -

Antwerpen, 2018

SAMENVATTING

Het menselijk lichaam bevat een groot aantal micro-organismen, die in nauw contact samen leven met hun gastheer en collectief de microbiota worden genoemd. Het menselijk lichaam kan onderverdeeld worden in tenminste vier fundamenteel microbiële niches die elk een verschillende samenstelling hebben en die een belangrijke rol spelen in het leven van het individu. Onder deze niches worden de bovenste luchtwegen, de huid, het genitaal kanaal en het maagdarmkanaal gerekend. De microbiota van de bovenste luchtwegen, waar dit doctoraatsproefschrift op focust, beschermt de gastheer tegen pathogene kolonisatie door betere opname van aanwezige nutriënten dan de pathogenen, door competitie voor aanhechtingsplaatsen en door stimulatie van immuunreacties die de productie van antimicrobiële en anti-inflammatoire componenten bevorderen. Uit onderzoek blijkt dat ook melkzuurbacteriën behoren tot de microbiota van de bovenste luchtwegen en deze zelfs gekoppeld zijn aan een 'gezond' ecosysteem. Deze waarnemingen vormden de basis van ons gevoerde onderzoek in de zoektocht naar probiotica voor de bovenste luchtwegen. Aangezien er nog maar weinig onderzoek is gedaan naar probioticatoepassingen in de nasofarynx, is er dus ook weinig kennis over het potentieel van goed gedocumenteerde lactobacillen in deze niche.

Eén van de meest voorkomende aandoeningen van de bovenste luchtwegen bij kinderen is acute otitis media (AOM). Cijfers geven aan dat tot 50% van de kinderen jonger dan 1 jaar al minstens één episode van AOM heeft doorstaan. Alle types van OM worden veroorzaakt door een functionele stoornis van de buis van Eustachius. Dit kan veroorzaakt worden door fysieke obstructie door gezwollen adenoïden of door een ontsteking van de membranen in de nasofarynx. Bacteriële pathogenen zoals *Streptococcus pneumoniae, Haemophilus influenzae*, en *Moraxella catarrhalis* brengen zo'n ontsteking vaak teweeg. Hoewel antibiotica beschouwd worden als de standaard therapie, brengen ze veel neveneffecten met zich mee zoals het ontstaan van antibioticaresistentie.

In dit doctoraatsproefschrift werden de volgende vragen behandeld omtrent het potentieel van probiotische lactobacillen:

1. Kunnen lactobacillen de drie voornaamste (A)OM pathogenen inhiberen?

2. Wat zijn hierbij belangrijke antimicrobiële componenten?
3. Kunnen lactobacillen ook zorgen voor indirecte mechanismen zoals pathogeenexclusie en inhibitie van ontstekingsmerkers door de (A)OM pathogenen?
4. Kan oraal ingenomen *Lactobacillus rhamnosus* GG zich voortbewegen naar verschillende niches van de bovenste luchtwegen en zo de plaatselijke microbiota beïnvloeden?

Om een antwoord te vinden op deze vragen, ontwikkelden we eerst een screeningsplatform dat bestond uit zowel agar-, planktonische als biofilmtesten, om de antimicrobiële activiteit na te gaan van lactobacillen tegen de drie voornaamste (A)OM pathogenen. Zo goed als alle lactobacillen die werden getest toonden een uitgesproken antimicrobiële activiteit tegen *M. catarrhalis* en een minder duidelijke werking tegen *H. influenzae* en *S. pneumoniae.* Zowel supernatans als levende bacteriën konden de groei van *M. catarrhalis* inhiberen. Zowel planktonische groei als groei in een biofilm werd aangetast door (het supernatans van) de lactobacillen. Vervolgens trachtten we de voornaamste antimicrobiële component te identificeren in het supernatans. Hiervoor werd het supernatans behandeld met proteinase K of hitte of werd het op een neutrale pH gebracht om activiteit van, respectievelijk, eiwitten/peptiden en/of zuren te onderzoeken. Aangezien de activiteit van het supernatans volledig verdween na neutralisatie van de pH, werd melkzuur beschouwd als een belangrijk antimicrobieel metaboliet. Lactobacillen die meer melkzuur produceerden (D- en/of L-) toonden inderdaad een hogere activiteit tegen (A)OM pathogenen. Bovendien zorgde toevoeging van L- of D-melkzuur voor inhibitie van planktonische groei (MIC waarden tussen 0.5 en 25 g/L afhankelijk van de pH) en biofilmvorming (bij toevoeging 0.5 g/L D-melkzuur). Ondanks deze resultaten is melkzuur waarschijnlijk niet het enige actieve molecule, aangezien verschillende resultaten ook de aanwezigheid van andere effectormoleculen, waarschijnlijk kleine antimicrobiële peptiden zoals bacteriocines, suggereerden. Onderzoek naar dergelijke peptiden is echter complexer en we zijn er niet in geslaagd om hun productie te induceren. Maar niet enkel de productie van antimicrobiële componenten is belangrijk tegen (A)OM pathogenen. Onderzoek in andere niches zoals de darmen en de vagina heeft aangetoond dat melkzuurbacteriën plaatselijke ontsteking kunnen beïnvloeden en kunnen concurreren voor nutriënten en receptoren met de pathogenen. Daarom onderzochten we ook deze meer indirecte effecten tegen (A)OM pathogenen. Veelbelovende resultaten werden verkregen: *L. rhamnosus* GG kon de adhesie van *M. catarrhalis* en *S. pneumoniae* aan nasofaryngeale Calu-

3 epitheelcellen verminderen en bleek in staat te zijn om de inductie van pro-inflammatoire cytokines (IL-8, IL-1β, TNF-α) door *M. catarrhalis* te verlagen.

Deze *in vitro* resultaten zorgden voor de uitwerking van een interventiestudie waar de helft van een groep OM patiënten gevraagd werd om een oliesuspensie met *L. rhamnosus* GG en *Bifidobacterium animalis* subsp. *lactis* BB-12 (Probactiol® Mini) dagelijks gedurende 4 weken te consumeren. De andere helft van de groep nam geen probiotisch product. Er werd onderzocht of de probiotische bacteriën zich konden verplaatsen naar verschillende niches van de bovenste luchtwegen, of ze daar konden overleven en of ze de plaatselijke microbiota konden beïnvloeden. Zes patiënten werden reeds geïncludeerd, op het moment van dit schrijven. *B. animalis* subsp. *lactis* BB-12 werd in geen enkel staal gedetecteerd in tegenstelling tot *L. rhamnosus* GG die in de stalen van twee patiënten werd gedetecteerd met behulp van een geoptimaliseerde qPCR methode. Bovendien werd de aanwezigheid van *L. rhamnosus* GG ook bevestigd met behulp van *16S rRNA* amplicon analyse (Illumina MiSeq). Deze waarneming is erg interessant voor verdere toepassingen aangezien het aantoont dat oraal ingenomen lactobacillen op een natuurlijke wijze kunnen migreren naar andere plaatsen in de bovenste luchtwegen. Of ze ook *in vivo* een impact kunnen hebben op de concentratie van OM gerelateerde pathogenen moet nog verder onderzocht worden.

ABSTRACT

The human body is occupied by a vast number of microorganisms, which live in close contact with their host and are collectively called the microbiota. The human body can be divided in at least four fundamental microbial niches with a different composition, which play an important role in the entire life of an individual. These niches include the skin, upper respiratory tract (URT), genital tract and gastrointestinal tract. The microbiota present in each of the niches provides to the host a vast number of health effects. The URT microbiota, which forms the focus of this PhD thesis, protects the host from bacterial pathogenic colonisation by better adaptation of the symbiotic microorganisms to the nutrients in the human environment than the pathogens, by competition for adhesion sites and by stimulation of immune responses that activate production of antimicrobial and anti-inflammatory components. Interestingly, also lactic acid bacteria are members of the URT microbiota and are even linked to a 'healthy' URT ecosystem. These observations have formed the basis of our investigations towards the search for probiotics in the upper respiratory tract. As the nasopharyngeal niche is quite unexplored in probiotic research, little is known about the potential of well-documented lactobacilli in this niche.

One of the URT diseases most often encountered in children is acute otitis media (AOM). Recent data indicate that up to 50% of children less than 1 year of age have already suffered at least one episode of AOM. The common cause of all forms of OM is dysfunction of the Eustachian tube due to physical obstruction or to inflammation of the mucous membranes in the nasopharynx. This inflammation is mostly caused by infections with bacterial pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis*. While antibiotics are still considered as standard therapy, they have many side-effects including the induction of antibiotic resistance. In this thesis the following questions regarding the potential of probiotic lactobacilli were addressed:

1. Can lactobacilli inhibit the three main (A)OM pathogens?
2. What are important antimicrobial compounds?
3. Can lactobacilli mediate more indirect mechanisms such as pathogen exclusion and the inhibition of production of inflammation markers by the (A)OM pathogens?

4. Can orally administered *Lactobacillus rhamnosus* GG transfer to URT niches and influence the local microbiota?

To address these questions, we first developed a screening platform, consisting of both agar, planktonic and biofilm assays, to test the antimicrobial activity of lactobacilli against the three main (A)OM pathogens. Almost all lactobacilli tested showed a clear antimicrobial activity against *M. catarrhalis* while activity against *H. influenzae* and *S. pneumoniae* was less pronounced. Both supernatant and living lactobacilli were able to inhibit the growth of *M. catarrhalis.* Planktonic growth as well as biofilm formation of the pathogen was affected by the (supernatant of) the lactobacilli tested. Subsequently, we aimed to investigate the major antimicrobial compound in the supernatant which was responsible for this antimicrobial activity. For this purpose, supernatant of the lactobacilli was treated with proteinase K or heat or was made pH neutral to investigate whether respectively proteins/peptides and/or acids could be involved. As the activity of the supernatant disappeared after pH neutralisation, lactic acid was considered as an important antimicrobial metabolite. Indeed, lactobacilli which produced more lactic acid (D and/or L) showed more activity against the (A)OM pathogens and addition of L-lactic acid or D-lactic acid inhibited growth in suspension (MIC values between 0.5 and 25 g/L depending on the pH) and biofilm (addition of 0.5 g/L D-lactic acid). Nevertheless, lactic acid is probably not the only active molecules as several results also point to the presence of other effector molecules, probably small antimicrobial peptides such as bacteriocins However, investigations towards such peptides are more complex and we did not succeeded in inducing their production. Yet, not only the production of antimicrobial compounds is important against (A)OM. Lactic acid bacteria have already been shown to influence local inflammation in the presence of pathogens and to compete with them for nutrients and receptors in other human body sites such as the gut and vagina. For that reason, we also investigated these more indirect mechanisms of action against the (A)OM pathogens. Promising results were obtained: *L. rhamnosus* GG was able to inhibit the adhesion of *M. catarrhalis* and *S. pneumoniae* to the nasopharyngeal epithelial Calu-3 cell line and showed to be able to reduce the induction of pro-inflammatory cytokines (IL-8, IL-1β, TNF-α) by *M. catarrhalis*.

These promising *in vitro* results stimulated the set-up of an intervention study with OM patients where half of the group was asked to administer an oil suspension containing *L. rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 (Probactiol® Mini) daily for 4 weeks and the other half of the group not. The capacity of the probiotic bacteria to transfer to the URT niches, to persist there and to influence the local pathogen concentration was investigated. Six patients were yet included in the study at the time of the redaction of this PhD manuscript. *B. animalis* subsp. *lactis* BB-12 could not be detected in any of the URT samples, while *L. rhamnosus* GG could be detected in two patient's URT samples using a dedicated and optimised qPCR approach. Furthermore, the presence of *L. rhamnosus* GG was substantiated by the microbial *16S rRNA* amplicon analysis (Illumina MiSeq). This observation is very interesting for further applications as it indicates that orally applied lactobacilli can naturally migrate to the upper respiratory tract. Whether they can impact on the OM-related pathogen concentration *in vivo* remains to be substantiated.

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LIST OF ABBREVIATIONS

AI-2	Autoinducer 2
AOM	Acute otitis media
ASV	Amplicon sequence variants
BHI	Brain heart infusion
CFU	Colony forming units
COM	Chronic otitis media
D-LA	D-lactic acid
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
GIT	Gastrointestinal tract
HI	*Haemophilus influenzae*
IL	Interleukin
LAB	Lactic acid bacteria
LGG	*Lactobacillus rhamnosus GG*
L-LA	L-lactic acid
Llp	Lectin-like protein
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LTA	lipoteichoic acid
MC	*Moraxella catarrhalis*
MEF	Middle ear fluid
MEM	Minimum essential medium
MH	Mueller Hinton
MIC	Minimal inhibitory concentration
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MOA	Mechanism of action
MRS	de Man, Rogosa and Sharpe

MUC	Mucin
NGS	Next-generation sequencing
NTHi	Nontypeable *H. influenzae*
OD	Optical Density
OM	Otitis media
OME	Otitis media with effusion
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
q(RT-)PCR	Quantitative (real time) polymerase chain reaction
RNA	Ribonucleic acid
mRNA	Messenger Ribonucleic acid
RGs	Reference genes
rRNA	Ribosomal Ribonucleic acid
SCS	Spent culture supernatant
SEM	Scanning electron microscopy
SPN	*Streptococcus pneumoniae*
TH	Todd Hewitt
TNF	Tumor necrosis factor
URT	Upper respiratory tract

1 INTRODUCTION AND SCOPE

1.1 INTRODUCTION

Because various physical, chemical and infectious agents can enter the human body via the human airways, humans are prone to upper respiratory tract (URT) infections. In children, the most common URT infection is otitis media (OM). OM encompasses a spectrum of disease conditions characterized by accumulation of fluid in the middle ear cavity and inflammation of the middle ear cleft (Figure 1.1). A common pathway to all forms of OM is impaired function of the Eustachian tube and inflammation of the middle ear (Dhooge *et al.*, 2005). Eustachian tube dysfunction may be caused by congestion and inflammation of the mucosal lining (*e.g.* following an URT infection), by mechanical obstruction from enlarged adenoids (Figure 1.2) (Buzatto *et al.*, 2017) or by a primary dysfunction of the muscles involved in the Eustachian tube opening such as in children with repaired cleft palate. Dysfunction of the Eustachian tube may cause accumulation of middle ear fluid and thus create an ideal environment for bacterial growth evolving in inflammation. Based on culture-dependent data, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* have long been described as the three main pathogens related to AOM (Rovers *et al.*, 2004), but next- generation-sequencing (NGS) approaches and comparison with healthy subjects, have recently highlighted that other bacteria can also be involved as discussed in the next paragraphs.

OM can occur in different forms, as defined by Bluestone *et al*. (2002). Acute otitis media (AOM) is the rapid onset of acute infection within the middle ear, characterised by signs and symptoms such as otalgia and fever. Otitis media with effusion (OME) is characterised by inflammation of the middle ear without signs or symptoms of acute infection and accompanied by accumulation of fluid. Otitis media with effusion is the most common cause of hearing impairment in childhood and resolution of hearing loss is the main treatment goal for OME (Boudewyns *et al.*, 2012). Middle ear effusion (MEF) is a liquid in the middle ear which may be serous, mucoid or purulent. The duration of the effusion may vary from less than three weeks (acute), three weeks up to two-three months (subacute) or > three months (chronic). Chronic otitis media (COM) is defined as chronic inflammation of the mucosa and submucosa of the middle ear and may result in changes not only to the mucosa and submucosa but also to the tympanic membrane and ossicles. COM is the most severe form of OM but is very uncommon in developed countries (Verhoeff *et al.*, 2006).

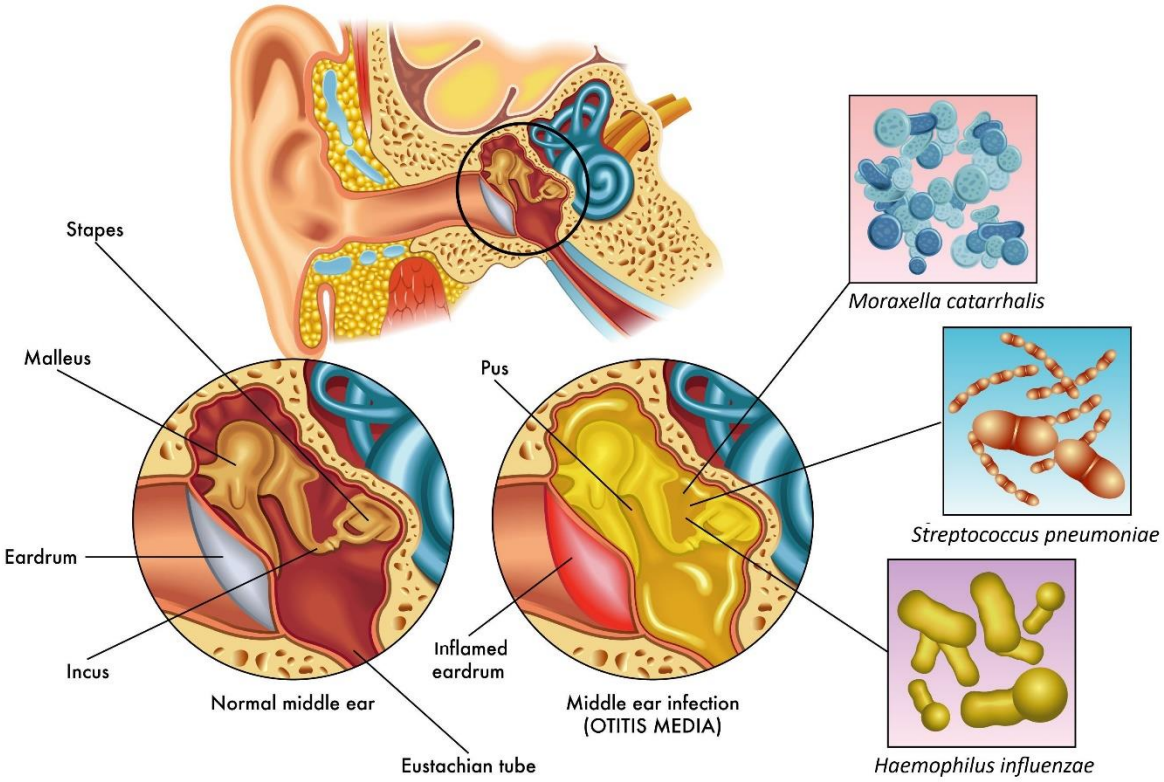


Figure 1.1: Anatomy of the ear and characteristics of otitis media (OM). *In healthy conditions, the middle ear is filled with air while OM is characterised by the presence of fluid (pus) in the middle ear and the inflammation of the middle ear cleft. Dysfunction of the Eustachian tube prevents the middle ear fluid from normal drainage and thus creates an ideal environment for bacterial growth evolving in inflammation The three main pathogens in (A)OM are* Moraxella catarrhalis, Streptococcus pneumoniae and Haemophilus influenzae*, but other pathogens are emerging such as* Turicella *and* Alloiococcus*, especially for more chronic forms of OM (see text). Figure was purchased from 123RF.com and slightly adapted.*

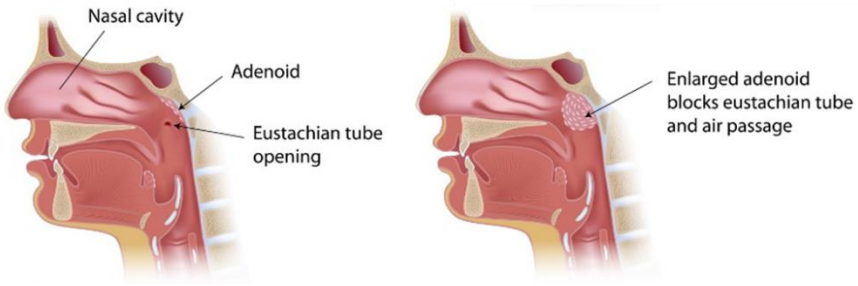


Figure 1.2: Blocking of Eustachian tube by swollen adenoids. *Adenoids can play an important role in OM. In normal conditions (left) they are small and the Eustachian tube is free for ventilation. When adenoids are swollen (right), they can block the Eustachian tube opening and hamper its function. Source: https://aimatinfections.com/aom/?more=true.*

Most children experience at least one episode of AOM (Rovers *et al.*, 2004) with a peak incidence between 6 and 12 months. Monasta *et al.* (2012) estimated the yearly global AOM incidence rate (*i.e.* the number of new episodes per hundred people per year) to be 10.9, with an average of 3.6 for Europe and 43.4 for Sub-Saharan West Africa and Central Africa. More than half of these episodes occur in children younger than 5 years. Recent monitoring data indicate that 46% of US children less than 1 year of age have already suffered at least one episode of AOM (Chonmaitree *et al.*, 2016). OME typically has a bimodal distribution in prevalence with a first peak around two years and a second peak around five years of age (Zielhuis *et al.*, 1989). It represents the most common form of OM with a point prevalence in screening studies of around 20% in young children (Rovers *et al.*, 2004).

OM is a multifactorial condition including anatomical, host-related and environmental factors (Rosenfeld and Bluestone, 2003) (Figure 1.3). The risk of infection is, for instance, influenced by age, season, genetic predisposition, being breastfed or not, exposure to cigarette smoke, among other factors (as reviewed by Schilder *et al.*, 2016). Although many factors are suggested to be involved, the pathogenesis is yet not fully understood. Interestingly, AOM is considered to be a polymicrobial infection. Indeed, based on decades of culture-based analysis, bacterial pathogens such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* have long been the major focus of pathogenic research in AOM (Ngo *et al.*, 2016). Importantly, viruses, commonly detected via immunological and molecular techniques also play a role in OM. Studies indicate that the influenza A virus (Chonmaitree *et al.*, 2008), respiratory syncytial virus (Patel *et al.*, 2007), human rhinovirus (Nokso-Koivisto *et al.*, 2004) and adenovirus (Chonmaitree *et al.*, 2008) could predispose to bacterial infection in AOM. These viruses can create changes in Eustachian tube functioning by initiating inflammation (Patel *et al.*, 2009), altering the biochemical and rheological properties of airway mucus (Bakaletz, 2010) and compromising the mucociliary clearance action (Pittet *et al.*, 2010). Furthermore, by upregulating expression of eukaryotic receptors, viruses can increase bacterial adherence and colonisation (Avadhanula *et al.*, 2006; Bakaletz, 2010). The use of NGS approaches (especially shotgun sequencing) to map the community of viruses and bacteriophages, also called the virome, has thus far mostly focused on the gut. Recently, the human respiratory virome is gaining more interest with studies on cystic fibrosis (Willner *et al.*, 2009, 2012), lower respiratory tract infections (Lysholm *et al.*, 2012; Yang *et al.*, 2011) and severe acute respiratory infections (Wang *et al.*, 2016b; Zoll

et al., 2015) being reported. In the latter disease, a more diverse ecosystem of viruses was observed in children with URT infection compared to healthy children. More specifically, the diseased children were more colonised by viruses from the *Paramyxoviridae, Coronaviridae, Parvoviridae, Anelloviridae, Orthomyxoviridae, Picornaviridae,* and *Adenoviridae* families, while healthy controls were dominated by *Anelloviridae* and bacteriophages (Wang *et al.*, 2016b). However, to the best of our knowledge, no metagenomic URT virome data are yet available for children suffering from OM. Similarly, little is documented about the community of URT fungi present during OM. There is thus a clear need for more dedicated metagenomic studies that will give a better global overview of the total URT microbial community (bacteria, viruses, bacteriophages and fungi). Since the information about the URT virome and fungome is limited, we will focus in this literature study on the bacterial microbiome and the potential bacterial interactions between OM pathogens and probiotics.

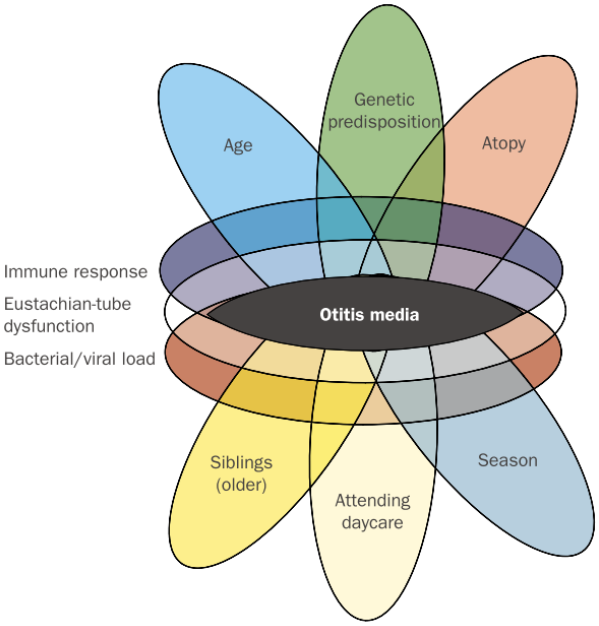


Figure 1.3: Factors involved in OM pathogenesis *(Rovers et al., 2004).*

1.2 THE POLYMICROBIAL BACTERIAL MICROBIOME OF OTITIS MEDIA PATIENTS

The relationship between bacterial community composition in the URT, risk of pathogen colonisation, and OM symptoms is increasingly being studied via culture-independent approaches such as NGS. Although these NGS approaches certainly have their limitations in rather low-biomass niches such as the respiratory tract, including the presence of inhibitors

and contaminants, the difficulty in discriminating between live and dead bacteria and the lack of information about viruses and fungi, these culture-independent approaches have yet revealed novel insights on potential pathogenic and beneficial bacteria as will be discussed below. It should be noted, however, that most approaches only identify the bacteria on genus level while pathogenicity is expressed at strain-level. This makes the distinction between commensal and potential pathogenic species challenging. Furthermore, inconsistencies in microbiome studies can be due to differences in disease parameters, geographical location (Ngo *et al.*, 2016), sampling, storage, DNA extraction (Vandeputte *et al.*, 2017), sequencing approach, bio-informatic analysis (Table 1.1), among others, that can all favour certain species. The next paragraphs aim to map the current knowledge about the bacterial microbiome differences between AOM, OME and COM, but more work in larger study cohorts is certainly warranted.

INFOBOX – Reverse Koch's postulates for probiotics

In 1890, Robert Koch published his four criteria to establish a causative relationship between a microbe (pathogen) and a disease. These postulates, although they have their limitations, had an enormous influence in microbiology. Underneath this box, a comparison is given for Koch's postulates and possible translated reverse postulates for the search for 'next-generation probiotics' based on comparative microbiome research combined with experiments about their causative relationship with improved health (O'Toole *et al.*, 2017). A good framework to investigate the potential of interesting bacteria that are present only or in higher abundance in healthy persons is thus warranted.

Koch's postulates for pathogens

1. The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
2. The microorganism must be isolated from a diseased organism and grown in pure culture.
3. The cultured microorganism should cause disease when introduced into a healthy organism.
4. The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

Possible reverse Koch's postulates for probiotics

1. The microorganism must be found in high abundance in healthy organisms and decreased in the ones suffering from a disease.
2. The microorganism must be isolated from a healthy organism and grown in pure culture.
3. The cultured organism should promote health when introduced into a diseased organism.
4. The microorganism must be reisolated from the healthy experimental host and identified as being identical to the original specific causative agent.

Microbiome insights indicate that Koch's postulate one is not entirely true. Many opportunistic pathogens, for example, can be found in the URT of healthy persons (De Boeck *et al.*, 2017). As this is also true for beneficial bacteria, the translated probiotic postulate would be less strict: potential probiotics can be present in diseased individuals but should be decreased in number.

1.2.1 Development of the healthy URT microbiome in children

The microbiome of the URT (nose and nasopharynx) is variable in time and depends on several, often environmental factors (Figure 1.4) (De Steenhuijsen Piters *et al.*, 2015; Man *et al.*, 2017). First of all, the mode of delivery seems to have a significant effect on the URT microbiota directly after birth. Indeed, a longitudinal study organised in the United States by Bosch *et al.* (2016) has followed 102 children in the first six months of life and analysed the bacterial DNA from nasopharyngeal swabs via Illumina MiSeq sequencing of the V4 variable region of the *16S rRNA* gene. These authors observed that children who were delivered vaginally versus via Caesarean-section, carried an URT microbiota resembling respectively the maternal vaginal environment versus the skin microbiota directly after birth. This study confirmed earlier observations by Dominguez-Bello *et al*. (2010) where babies were only sampled right after birth and their microbiota was compared with the microbiota of different niches of the mother's body via 454 pyrosequencing of the V2 variable region. Already after one day of life, Bosch *et al.* (2016) observed that the URT microbiota shifts towards a *Streptococcus viridans*-predominated profile, regardless of mode of delivery. After six months, a differentiation towards a *Corynebacterium pseudodiphteriticum/ propinquum-, Dolosigranulum pigrum-, M. catarrhalis/nonliquefaciens-, S. pneumoniae-, H. influenzae*-dominated community or a mixed community with these bacteria was observed. In total, 11 nasopharynx microbiota profiles (termed clusters) were identified using Illumina MiSeq sequencing (V4 region). Yet, children born by Caesarian-section showed a reduced colonisation with commensals like *Corynebacterium* and *Dolosigranulum* (Bosch *et al.*, 2016). *Dolosigranulum* is a rather unexplored lactic acid bacterium belonging to the family of *Carnobacteriaceae* while *Corynebacteria* are generally believed to be pathogenic as some of their species are involved in diseases such as diphtheria (Ton-That and Schneewind, 2003) and pneumonia (Tarr *et al.*, 2003). Both genera are gaining more interest recently as they seem to be prevalent members in the nose and nasopharynx of healthy adults (De Boeck *et al.*, 2017). In this view, postulate one of the probiotic Koch's postulates applies for *Dolosigranulum* and *Corynebacterium* (INFOBOX).

Earlier, researchers of the same group could also identify eight distinct clusters within a Dutch study group of 60 children at the ages of 1.5, 6, 12, and 24 months nasal swab sampling. The different sequencing approaches applied (454 sequencing and V5-V7 region) might be a reason

for the different amounts of clusters (8 versus 11) observed. Interestingly, Biesbroek *et al.* (2014) also observed associations between certain taxa and microbiota stability during the first two years of life. Less-stable profiles contained a high abundance of *Haemophilus* and *Streptococcus.* In contrast, an early presence and high abundance of *Moraxella* and *Corynebacterium*/*Dolosigranulum* in the first period of life were associated with a more stable pattern (Biesbroek *et al.*, 2014b), which was later confirmed by the same researchers (Biesbroek *et al.*, 2014a; Bosch *et al.*, 2016) using Illumina MiSeq sequencing (V4 region) as well. Santee and colleagues (2016), using a 16S rRNA PhyloChip sequencing approach focusing on the V5 region, observed an association between an enrichment of *Moraxella nonliquefaciens* in the nasopharynx of American children and acute sinusitis. The fact that, on the one hand, early colonisation of *Moraxella* is associated with a stable microbial pattern and on the other hand, *M. nonliquefaciens* is enriched in children suffering from acute sinusitis, highlights that association with health and disease should be studied at strain or species level. This is not always possible with the currently available NGS approaches, especially not with amplicon sequencing, although pipelines such as the DADA2 algorithm that take into account genuine amplicon sequence variants (Callahan *et al.*, 2016) and shotgun sequencing approaches are an important step forward.

In addition to delivery mode, feeding type also appears to be an important driver in the maturation of the URT microbiota, with breastfed infants showing to have a more health-related microbiota with *Dolosigranulum* and *Corynebacterium*-dominated profiles (Biesbroek *et al.*, 2014a; Bosch *et al.*, 2016). As can be expected, antibiotic use appears to decrease the number of these protecting commensals (Pettigrew *et al.*, 2012; Teo *et al.*, 2015). However, contrary to what you would expect, a study about the nasal microbiota in 89 twins, of which 46 monozygotic and 43 dizygotic, using broad-coverage quantitative polymerase chain reaction (qPCR) and *16S rRNA* gene sequencing revealed that host genetics did not affect the nasal microbiota composition (Liu *et al.*, 2015). On the other hand, it did appear to have a significant effect on the bacterial density in the nose, because monozygotic twins showed to have a significantly more correlated bacterial density compared to dizygotic twin pairs. Season, in contrast, does appear to influence a child's URT microbiota with more *Pasteurellaceae*, *Staphylococcaceae* and *Moraxella* observed in winter and more *Corynebacteriaceae*, *Haemophilus*, *Lactobacillus* and *(Brevi)bacillus* in summer (Bogaert *et al.*, 2011; Mika *et al.*,

2015). In addition, the presence of *Streptococcus* seems to be positively influenced by co-habiting with siblings, the use of antibiotics prior to the sampling, the attendance of day-care and the exposure to cigarette smoke (Greenberg *et al.*, 2006; Teo *et al.*, 2015), but this should be further explored at species and strain-level.

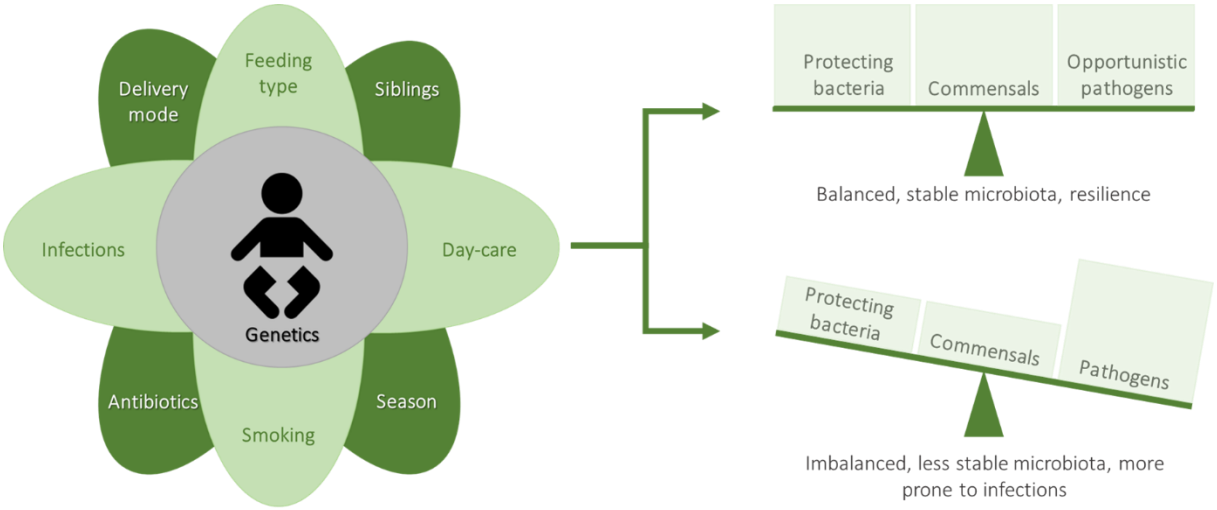


Figure 1.4: Factors influencing the respiratory microbiota and/or bacterial density. *First colonisation in early life takes place during birth. The mode of delivery (natural vs. Caesarian section) largely influences the microbial community in the newborn's respiratory tract. Afterwards, the dynamics and evolution of the microbiota is driven by many other environmental factors such as feeding type, having older siblings or not, attending day-care, the season, growing up in a smoking environment, taking antibiotics and having infections. Together with the host's genetics which influences the bacterial density in the nasopharynx, the microbiota can develop towards a balanced, stable microbiota where resilience, i.e. the ability of the host to remain healthy even when exposed to a stress, occurs. Conversely, the microbiota can also develop towards a community that is imbalanced, less stable and is more prone to infections and inflammation. Figure is based on* (Biesbroek *et al.*, 2014a; Man *et al.*, 2017; Pettigrew *et al.*, 2012; Schilder *et al.*, 2016; Teo *et al.*, 2015).

1.2.2 Acute otitis media (AOM)

Taking the influence of all these (environmental) factors into account, it is not surprising that the URT microbiota balance can be easily disturbed, resulting in health issues such as OM. In one of the first NGS-approaches on AOM, Laufer *et al.* (2011) investigated nasal swabs of 108 children with and without AOM via 454 sequencing (V1-V2 region). The authors observed a relationship between the presence of *S. pneumonia*, one of the main OM pathogens, and a less diverse and less even (*i.e.* how close in numbers each species in an environment is) microbial community. Furthermore, the presence of *Haemophilus, Rothia* and *Actinomyces* was associated with an increased risk of AOM. In contrast, a potentially protective microbiota

consisting of bacterial species such as *Corynebacterium, Dolosigranulum*, *Propionibacterium*, *Lactococcus* and *Staphylococcus* was associated with a decreased risk of pneumococcal colonisation and AOM. The same research group subsequently analysed nasal swabs of 240 children, now also taking the intake of antibiotics in the six months before sampling into account (Pettigrew *et al.*, 2012). The mean levels of the AOM-associated taxa *Rothia* and *Actinomyces* were higher in children with antibiotic use in the past six months. Of interest for potential probiotic applications (INFOBOX), *Lactococcus*, *Anoxybacillus*, and members of the family *Enterobacteriaceae* appeared negatively associated with colonisation by each of the three 'classical' bacterial AOM pathogens *M. catarrhalis, S. pneumoniae* and *H. influenzae,* and with AOM in children who used antibiotics in the past six months (Pettigrew *et al.*, 2012). However, such association does not necessarily imply a causal relation between these potential probiotic taxa and health (INFOBOX). Therefore, addition experimental substantiation is necessary, as will be further discussed below in more detail. Hilty *et al.* (2012) observed that the nasopharyngeal microbiota of children suffering from AOM contained most frequently bacteria from the families of *Moraxellaceae*, *Streptococcaceae* and *Pasteurellaceae,* in agreement with the three major AOM pathogens. In contrast to these potential pathogenic taxa, potential beneficial commensals, such as *Staphylococcaceae*, *Flavobacteriaceae*, *Carnobacteriaceae* and *Comamonadaceae* were less prevalent in AOM patients compared to the control children (Table 1.1).

In addition to the nasal and nasopharyngeal microbiota obtained via swab sampling, middle ear fluid (MEF) is also a specimen of interest for detailed microbiome analyses. It is however more difficult to obtain comparable samples from healthy control subjects since getting access to the middle ear is only ethical when medical problems occur. Sillanpää *et al*. (2017) investigated 90 MEF samples of 79 children between 5 and 42 months using a combination of nested PCR and Illumina MiSeq 16S amplicon sequencing (V4 region) and operational taxonomic unit (OTU) clustering via QIIME. They observed a dominance of *S. pneumoniae* in 14 samples (16%), *H. influenzae* in 15 (17%) and *M. catarrhalis* in 5 (5.6%), while the less well-known AOM pathogens *Turicella otitidis*, and *Staphylococcus auricularis* dominated each in two subjects. For comparison, based on culture-dependent data*, H. influenzae*, S*. pneumoniae* and *M. catarrhalis* were the detected pathogens in 22%, 19% and 10% of the cases, respectively. This study thus showed that both culture-dependent and -independent techniques confirm that the three

major AOM pathogens dominate MEF of children suffering from AOM, but that NGS can also point towards other emerging pathogens. *T. otitidis* and *Alloiococcus otitidis* are examples of these emerging pathogens. In the study of Sillanpää *et al*., they were found in 5 (5.6%) and 3 (3.3%) MEF samples, respectively. Before, they were only occasionally reported to occur in AOM based on culture-dependent data (Ashhurst-Smith *et al.*, 2007; Gomez-Garces *et al.*, 2004) and their role as main pathogenic driver of disease is currently still under debate.

1.2.3 Otitis media with effusion (OME) and chronic OM (COM)

Although less prevalent than AOM, OME and chronic OM are also yet characterised by NGS (Table 1.1). As one of the first studies in the field, Liu *et al.* (2011) investigated the microbiota of the tonsils, adenoids and middle ear specimens of one patient with COM via 454 sequencing (V3-V4 region). Interestingly, they saw overlapping communities in these three respiratory niches. The adenoids showed a more complex microbial profile containing *Pseudomonaceae, Streptococcaceae, Fusobacteriaceae* and *Pasteurellaceae*, while the middle ear and tonsils were dominated by just one family: *Pseudomonaceae* and *Streptococcaceae*, respectively. This observation adds support to the assumption of the adenoids being a source site for both the middle ear and the tonsil microbiota (Liu, 2011). Subsequently, Jervis-Bardy *et al.* (2015) provided a hallmark study for OME, because they observed by Illumina MiSeq sequencing of the 16S V1-V3 region that OTUs from the classic AOM pathogens *Streptococcus, Haemophilus* and *Moraxella* are also common in MEF, nasopharyngeal and adenoid samples of 11 children with OME. Two follow-up studies also observed similarities between MEF and adenoids of OME patients (Chan *et al.*, 2016, 2017). However, an important difference with AOM appeared, namely *A. otitidis* dominated the middle-ear-effusion microbiota (23% mean relative abundance), followed by *Haemophilus* (22%), *Staphylococcus* (11%), *Corynebacteria* (6%), *Moraxella* (5%), and *Streptococcus* (5%). These abundances were observed to be stable over time as they did not change drastically after one year (Chan *et al.*, 2017). Swabs of the adenoids, on the other hand, showed colonisation by *Haemophilus* (25% mean relative abundance), *Moraxella* (14%), *Streptococcus* (13%), *Fusobacteria* (11%) and *Neisseria* (7%). Interestingly, *Alloiococcus* was inversely correlated with *Haemophilus*, found in greater relative abundance in unilateral effusion and had a very low relative abundance in adenoid swabs (<1%) (Chan *et al.*, 2016). In the external auditory canal, the same *Alloiococcus* was found to have the highest relative abundance (28%), followed by *Staphylococcus* (20.8%) and *Pseudomonas* (3.2%) (Chan

et al., 2017). Thus, taken together, the current data suggest that dominance of *A. otitidis* is associated with OME, while dominance of *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* may favour AOM. Furthermore, the studies of Chan and colleagues suggest that the external auditory canal and adenoids can act both as bacterial reservoirs for middle ear infections. As perforations in the tympanic membrane often occur, this can give a free pass to bacteria that normally reside in the external auditory canal to move to the middle ear cavity. However, similar American research in which 55 children with chronic OM (COM) were sampled and *16S rRNA* amplicon sequencing via the Illumina MiSeq Platform (V4 region) was performed, resulted in different bacterial disease profiles. The six most abundant bacteria in the MEF samples of this study were *Haemophilus* (relative abundance 22.54%), *Moraxella* (11.11%), *Turicella* (7.84%), unclassified *Alcaligenaceae* (5.84%), *Pseudomonas* (5.40%) and *Alloiococcus* (5.08%) while *Streptococcus* accounted for 4.21% of the MEF bacterial reads (ranked as 8th most abundant genus) (Krueger *et al.*, 2016). Neeff *et al.* (2016) associated *Haemophilus, Staphylococcus* and *Alloiococcus* with an increased risk of COM using Illumina MiSeq sequencing (V3-V4 region) in 24 patients with COM and 22 healthy controls. Interestingly, a higher relative abundance of *Novosphingobium, Staphylococcus, Escherichia-Shigella, Burkholderia* and *Propionibacterium* were observed in the middle ear of healthy controls.

Table 1.1: Overview of URT and OM microbiome studies.

Focus	Sample type	Disease-associated bacteria*	Sequencing method	n (country)	Age (months)	Reference
Respiratory microbiota in healthy subjects	Nasal swab	*Haemophilus* *Streptococcus*	454 Sequencing V5-V7 region	60 (The Netherlands)	1.5-24	(Biesbroek *et al.*, 2014b)
	Nasal aspirates	*Moraxella* *Streptococcus* *Haemophilus*	Illumina MiSeq V4 region	234 (Australia)	2-12	(Teo *et al.*, 2015)
OM type	**Sample type**	**Disease-associated bacteria**	**Sequencing method**	**n**	**Age (months)**	**Reference**
AOM	Nasal swab	*S. pneumoniae* *Haemophilus* *Rothia* *Actinomyces*	454 Sequencing V1-V2 region	108 (USA)	6-78	(Laufer *et al.*, 2011)
	Nasal swab	*Moraxellaceae,* *Streptococcaceae* *Pasteurellaceae*	454 Sequencing V3-V5 region	153 (Switzerland)	0-24	(Hilty *et al.*, 2012)
	Nasal swab	*S. pneumoniae* *H. influenzae* *M. catarrhalis*	454 Sequencing V1-V2 region	240 (USA)	3-36	(Pettigrew *et al.*, 2012)
	MEF aspirate	*S. pneumoniae* *H. influenzae* *M. catarrhalis* *T. otitidis* *S. auricularis*	Illumina MiSeq V4 region	79 (Finland)	5-42	(Sillanpää *et al.*, 2017)
	Nasal swab	*Moraxella* *Haemophilus* *Streptococcus*	Illumina MiSeq V4 region	139 (USA)	1-12	(Chonmaitree *et al.*, 2017)
OME	MEF aspirate Adenoid and tonsil tissue	*Pseudomonaceae* *Streptococcaceae* *Fusobacteriaceae* *Pasteurellaceae*	454 Sequencing V3-V4 region	1 (USA)	96	(Liu, 2011)
	MEF aspirate Adenoid swab Nasal swab	*Alloiococcus* *Haemophilus* *Streptococcus* *Moraxella*	Illumina MiSeq V1-V3 region	11 (Australia)	3-10 years	(Jervis-Bardy *et al.*, 2015)
	MEF aspirate Adenoid swabs	*Alloiococcus* *Haemophilus* *Moraxella* *Staphylococcus* *Streptococcus*	Illumina MiSeq V3-V4 region	18 (Australia)	1-16 years	(Chan *et al.*, 2016, 2017)

		Pseudomonas *Corynebacteria*				
COM	Middle ear swab Mastoid swab	*Haemophilus* *Staphylococcus* *Alloiococcus*	Illumina MiSeq V3-V4 region	46 (New-Zealand)	6 months – 87 years	(Neeff *et al.*, 2016)
	MEF aspirate	*Haemophilus* *Moraxella* *Turicella*	Illumina MiSeq V4 region	55 (USA)	0-24: 25 subj >24: 30 subj	(Krueger *et al.*, 2016)

* Bacteria were significantly more abundant in this group as indicated by the authors

1.3 INFECTION MECHANISMS BY THE MAIN BACTERIAL OTITIS MEDIA PATHOGENS

Since both culture-dependent and culture-independent studies as reviewed above highlight *S. pneumoniae, H. influenzae* and *M. catarrhalis* as key otitis media pathogens (de Vries *et al.*, 2009; Duell *et al.*, 2016; Kadioglu *et al.*, 2008; van der Poll and Opal, 2009), we review subsequently their main pathogenesis mechanisms for host respiratory colonisation and disease. These virulence mechanisms can be divided in three – partially overlapping– disease mechanisms: interactions with the nasopharyngeal epithelium, interactions with the host immune system, and formation of polymicrobial biofilms. Such insights in the known pathogenic mechanisms is important for future studies on emerging pathogens such as *A. otiditis* and *T. otitis*, as well as on probiotic mechanisms of actions that could prevent or inhibit these key pathogenic steps as new alternative treatment strategies for OM. Of note, since pathogenicity is strain-specific, virulence factors can vary between distinct strains which results in different grades of pathogenicity. There is thus a clear need to substantiate these important mechanisms with specific strains. In the next paragraphs, the most commonly occurring virulence factors in the three main OM pathogens are discussed.

1.3.1 Interactions with nasopharyngeal epithelium

1.3.1.1 Impacting on mucin and toxin production

Before getting access to the receptors of the epithelial cells, pathogenic invading bacteria must traverse the mucus layer of the nasopharynx. This layer consist of a mixture of water, ions, glycoproteins, proteins, and lipids and serves as an important defence mechanism of the host against invading pathogens (Rose, 2006). Moreover, the epithelial barrier is also important to keep a beneficial symbiosis in the host–microbiota relationship (Man *et al.*, 2017). The

glycoproteins (with 70–80% O-linked glycosylation) in the mucus, also called mucins, are secreted by goblet cells. Although in healthy condition, the mucins help to protect the host mucosae, in diseased conditions such as OM, the mucociliary clearance becomes ineffective and an excessive production of mucins will occur (Komatsu *et al.*, 2008). In *S. pneumoniae*, the outermost capsule is well documented to prevent the entrapment in nasal mucus which increases the access possibilities to the epithelial surface. Effective opsonophagocytosis is furthermore prevented by the capsule (van der Poll and Opal, 2009). However, at least 98 different capsule serotypes are known to date, while only a limited number of these serotypes are associated with colonisation and disease (Ziane *et al.*, 2016). Strain-specific characteristics thus play a huge role in the pathogenicity of *S. pneumoniae*. To overcome the mucus defence layer, *S. pneumoniae* produces neuraminidases such as NanA and NanB which cleave host glycoproteins and mucins and thus increase adherence to epithelial receptors (Figure 1.5) (Jedrzejas, 2001). Protein D, on the other hand, is an outer membrane protein which is present on the surface of all *H. influenzae* strains. It is a lipoprotein displaying glycerophosphodiester phosphodiesterase activity and affinity for some human IgD proteins. The protein causes a decrease in frequency of ciliary movement and a loss of cilia in a human nasopharyngeal tissue culture model (Ahrén *et al.*, 2001a).

In humans, more than 20 mucin genes have been identified (Val, 2015). Especially upregulation of the *MUC5B*, *MUC5AC* and/or *MUC4* is linked with OM (Val, 2015). Interestingly, the main pathogens that are involved in OM can upregulate *MUC5AC*. A clinical isolate of nontypeable *H. influenzae* (NTHi), the variant of *H. influenzae* which does not express capsular polysaccharides and which is most commonly linked to OM (Murphy *et al.*, 2009; Van Eldere *et al.*, 2014), and *S. pneumoniae* strain R6 (a derivative of serotype 2 strain D39 (Lanie *et al.*, 2007)), when present together, can synergistically induce *MUC5AC* mucin transcription (Shen *et al.*, 2008). Furthermore, in a culture model of human middle ear epithelium, whole cell lysates of the three pathogens induced upregulation of *MUC2*, *MUC5AC* and *MUC5B*. A combination of whole cell lysate of NTHi strains 86028NP and 2019 and *S. pneumoniae* strains TIGR4 (serotype 4) and D39 (serotype 2) synergistically induced *MUC2* and *MUC5AC* gene expression while the lysate of *M. catarrhalis* did not participate in the synergistic upregulation (Kerschner *et al.*, 2014). Krueger and collegues (2016) monitored the presence of mucins MUC5AC and MUC5B in MEF of 55 children with COM via Western blotting. MUC5B was

present in 94.5% and MUC5AC in 65.5% of the MEFs. Interestingly, the dominance of the overall most abundant genus, *Haemophilus*, disappeared in samples containing only mucin MUC5B compared to samples containing both MUC5AC and MUC5B mucins suggesting MUC5AC to contribute to OM (Krueger *et al.*, 2016). No evidence of a protective role for mucin MUC5B is, however, documented yet. In mice, *MUC5B*, and not *MUC5AC*, appeared to be required for mucociliary clearance, for controlling infections in the airways and middle ear, and for maintaining immune homeostasis in mouse lungs, whereas *MUC5AC* was dispensable (Roy *et al.*, 2013).

The OM pathogens can also attack nasopharyngeal epithelial cells by production of toxins. As far as we know, of the three main OM pathogens, only *S. pneumoniae* produces such an exotoxin. Pneumolysin, a cholesterol-dependent cytolysin binds to cholesterol in cell membranes, forming oligomers and creating transmembrane pores (Johnson *et al.*, 1980). Pneumolysin is produced by almost all pneumococcal isolates and can decrease mucosal clearance in the upper airways (Hirst *et al.*, 2004). This latter decrease is due the synergistic action of pneumolysin with H_2O_2, produced by the pathogen, which impairs the ciliary function of respiratory cells (Feldman *et al.*, 2002; Marriott *et al.*, 2008). Furthermore, it also interacts with the host's immune system as discussed in the following paragraphs. *Haemophilus* and *Moraxella* are Gram-negative bacteria that have lipopolysaccharides (LPS) or endotoxins in their cell wall. LPS molecules are known to be strong inflammatory and barrier-disruptive bacterial molecules, but their role in pathogenesis of these two OM pathogens is currently elusive. Both pathogens appear to have an adapted LPS, containing shorter polysaccharides compared to enteric pathogens, lacking the O-antigen, and thus their LPS is termed LOS (lipooligosaccharides) (de Vries *et al.*, 2009). Both *M. catarrhalis* and *H. influenzae* use it for adhesion, biofilm formation and resistance to complement killing (de Vries *et al.*, 2009; Figueira *et al.*, 2007; Johnston *et al.*, 2008; Jurcisek *et al.*, 2005; Post *et al.*, 2016; Swords *et al.*, 2004). Not surprisingly, the presence of LOS is an important trigger for OM development in chinchilla models (Figueira *et al.*, 2007; Jurcisek *et al.*, 2005).

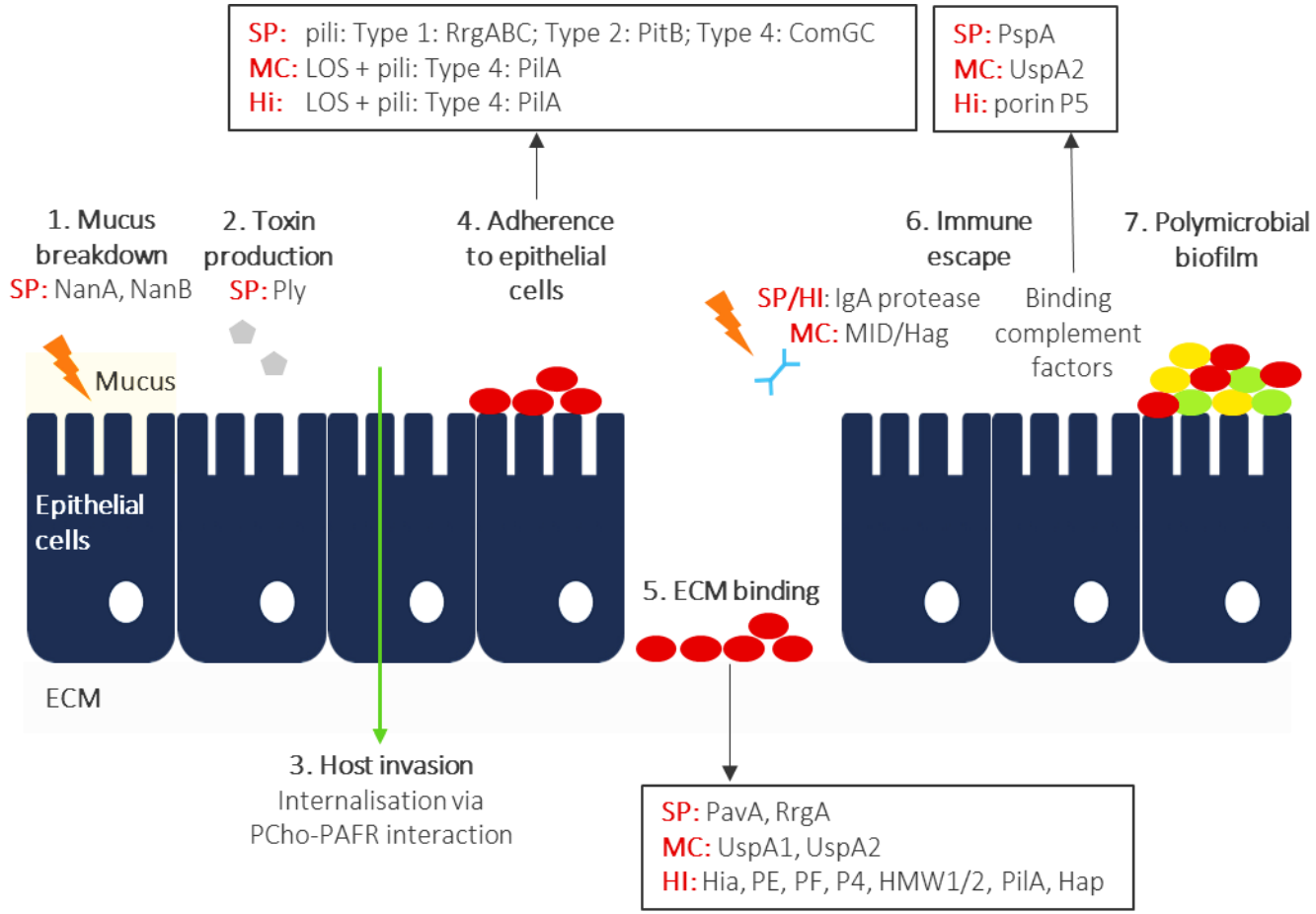


Figure 1.5: Pathogen interactions with nasopharyngeal epithelium and host immune system. *Different frequent occurring pathogenic effector molecules are specified per (A)OM pathogen. SP:* S. pneumoniae*; MC:* M. catarrhalis*; HI:* H. influenzae*; ECM: extracellular matrix; NanA/B: neuraminidases; Ply: pneumolysin; PCho: phosphorylcholine; PAFR: platelet-activating factor receptor; PavA: pneumococcal adhesion and virulence A; UspA: ubiquitous surface protein; Hia: H. influenzae adhesion; HMW1/2: high molecular weight molecules 1/2; Hap: Haemophilus adhesion protein; PspA: pneumococcal surface protein A. It should be noted that not all pathogenic strains or serotypes carry these effector molecules.*

1.3.1.2 Adhesion to epithelial cells and extracellular matrix (ECM)

As for most mucosal pathogens, adhesion to the nasopharyngeal epithelium is thought to be another key step in the pathogenesis. Many bacteria can contain long filamentous adhesive structures, termed pili, which play a key role in several physiological functions and *M. catarrhalis, H. influenzae* and *S. pneumoniae* are no exceptions to this fact. Pili are long and thin proteinaceous protrusions of the cell surface present on specific Gram-positive and Gram-negative bacteria, although their molecular structure can be very diverse. While pili in Gram-negative bacteria are typically formed by non-covalent homopolymerisation of major pilus subunit proteins into a pilus shaft, Gram-positive bacteria are formed by covalent polymerisation of pilin subunits in a process that requires a dedicated sortase enzyme (Proft and Baker, 2009). Two types of sortase-dependent pili have been reported in *S. pneumoniae* (Figure 1.6). Type 1 pili are composed of the major pilus shaft protein (RrgB) and two minor pilus proteins (RrgA and RrgC) (Barocchi *et al.*, 2006; Danne and Dramsi, 2012). Interestingly, these pili are thermosensitive as they are not induced in environments where the temperature is lower than 31°C (Basset *et al.*, 2017), which suggests that the pathogen uses different virulence mechanisms in colder anatomic sites such as the nares compared to warmer sites such as the nasopharynx/lungs. Furthermore, its expression promotes the bacterial penetration of the blood-brain barrier in a mouse model of meningitis (Iovino *et al.*, 2016). More recently, type 2 sortase-dependent pili were discovered which consist solely of a pilus islet two B (PitB) protein backbone (Bagnoli *et al.*, 2008). Both pili types have been shown to play an important role in adherence of *S. pneumoniae* to epithelial cells, although the corresponding host receptors are – to the best of our knowledge- not yet identified (Bagnoli *et al.*, 2008; Barocchi *et al.*, 2006). Furthermore, *S. pneumoniae* uses the sortase-independent type IV pili for binding and internalisation of exogenous DNA which can lead to incorporation of new genetic material and resistance to antibiotics and vaccines (Laurenceau *et al.*, 2013). This type IV pilus is only assembled during bacterial competence and is composed of the major pilin protein ComGC (Muschiol *et al.*, 2017). The role of these pili in adhesion is not known.

Type IV pili are also quite common in Gram-negative pathogens, with *M. catarrhalis* and *H. influenzae* expressing them as well (Figure 1.6) (Carruthers *et al.*, 2012; Luke-Marshall *et al.*, 2011). In both pathogens, adhesion to epithelial cells can be initiated by the main type IV pilus protein PilA (Luke *et al.*, 2004; Novotny and Bakaletz, 2016). Although the exact mechanism of

adhesion is not unraveled yet, PilA of *H. influenzae* has been shown to bind the ICAM-1 receptor (Novotny and Bakaletz, 2016).

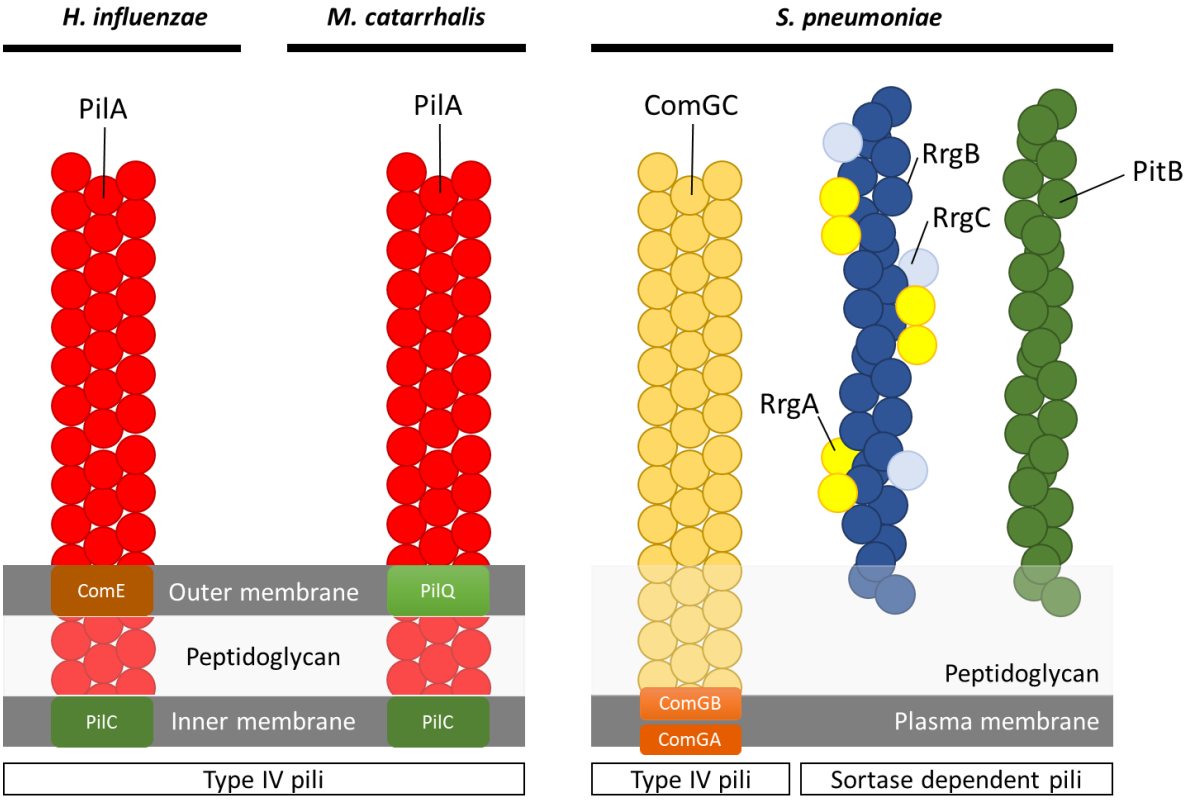


***Figure 1.6: Comparison of pili structure and composition of* H. influenzae, M. catarrhalis *and* S. pneumoniae.** *All three OM pathogens contain Type IV pili which are important for binding and internalisation of DNA and can be involved in adhesion to epithelial cells. The major pilin in the Gram-negative pathogens* (H. influenzae *and* M. catarrhalis*) is PilA. These pili are anchored in the inner membrane via PilC and in the outer membrane via ComE and PilQ for* H. influenzae *and* M. catarrhalis*, respectively. The Type IV pili in* S. pneumoniae*, on the other hand, are composed of ComGC pilin proteins and anchored in the plasma membrane via ComGB and ComGA proteins. In addition,* S. pneumoniae *also contains two types of sortase-dependent pili which are anchored in the peptidoglycan layer. The first one is composed out of a major pilin RrgB and two minor pilins RrgA and RrgC while the second one is only composed our of one backbone pilin, called PitB. The structures are simplified and based on references (Ayers et al., 2010; Bagnoli et al., 2008; Hilleringmann et al., 2008; Johnston et al., 2014).*

Adhesion to epithelial cells is also facilitated by the *Haemophilus* adhesion protein (Hap) (Duell *et al.*, 2016; Fink *et al.*, 2003). Furthermore, β-glucan receptors on the surface of monocytic cells and macrophages are involved in the adherence and non-opsonic entry of NTHi which do not express capsular polysaccharides (Ahrén *et al.*, 2001b). Such an entry in macrophage-like cells subepithelially of adenoid tissue was also observed by Forsgren *et al.* (1994). By residing intracellularly in these cells, NTHi can escape the local immune system responses. Moreover, phosphorylcholine (PCho) is a small, zwitterionic molecule that can be covalently attached

through its phosphate group to the LOS of *H. influenzae* (Duell *et al.*, 2016) similar as PCho can be bound to lipoteichoic acid (LTA) of *S. pneumoniae* (Cundell *et al.*, 1995). PCho enhances the survival in the respiratory tract as it increases the adherence (Johnson *et al.*, 2011; Young *et al.*, 2013) and is part of the recognition domain of the host protein platelet-activating factor. This protein binds to host platelet-activating factor receptor on epithelial cells and increases host invasion (Clark and Weiser, 2013). Strains containing PCho-decorated LOS/LTA are thus linked with a fitness advantage (Pang *et al.*, 2008).

Underneath the epithelial cells, the extracellular matrix (ECM) of the host appears to be a major target of colonisation of the key OM pathogens, because they all contain microbial surface components recognising adhesive matrix molecules (MSCRAMM). These molecules bind fibronectin, fibrinogen, laminin and/or collagen I. Several molecules of *S. pneumoniae* , such as pneumococcal adhesion and virulence A (PavA) protein and the previously mentioned pilus-associated adhesion (RrgA) have been shown to bind ECM molecules and facilitate increased adherence in this way (Figure 1.5) (Nelson *et al.*, 2007; Rosch *et al.*, 2008). Most strains have these proteins, but not all (Duell *et al.*, 2016). *H. influenzae* adhesin (Hia) and protein E, protein F, P4, high molecular weight molecules 1 and 2 (HMW1/2) also all bind ECM proteins of the host such as laminin, vitronectin, fibronectin and/or proteoglycan (Duell *et al.*, 2016; Hallström *et al.*, 2011; Singh *et al.*, 2013; Su *et al.*, 2016) as well as the previously mentioned PilA (Novotny and Bakaletz, 2016) and Hap (Duell *et al.*, 2016; Fink *et al.*, 2003). For *M. catarrhalis,* more intimate contact of the pathogen and the host appears to be mediated by the ubiquitous surface proteins (Usp) A1 and A2 which bind laminin and fibronectin of the ECM and the CEACAM-1 receptor on the epithelial cells (de Vries *et al.*, 2009).

1.3.2 Interactions with host immune system

1.3.2.1 Pro-inflammatory interactions in the host

Once the OM pathogens have invaded and crossed the epithelial barrier, they interaction with antigen-presenting cells (APC) and stimulate them to secrete different cytokines which play a pivotal role in the inflammatory responses. IL-1β and tumour necrosis factor-α (TNF-α), for example, have been thought to initiate the acute inflammatory response in OM (Skotnicka and Hassmann, 2008). Moreover, in a chinchilla model, both IL-1β and TNF-α appear to regulate mucin production in a dose- and time- dependent way, especially the *MUC5AC* gene (Kerschner

et al., 2004). IL-8 on the other hand is an important attractant for neutrophils (Leibovitz *et al.*, 2000). Si *et al*. (2014) observed an increase in the mRNA levels of interferon-γ (IFN-γ), TNF-α, IL-1β and IL-6, while protein analysis via ELISA only recorded higher TNF-α and IL-1β concentrations in MEF of OME children compared to non-OME children. Similarly, ELISA on MEF sample of OME children showed a positive correlation between the concentration of the pro-inflammatory cytokines IL-1 β, IL-6 and IL-8, and TNF- α and the amount of OM pathogens in the MEF (Zielnik-Jurkiewicz and Stankiewicz-Szymczak, 2016).

Immune responses can be activated by specific pattern recognition receptors (PRRs), often Toll-like receptors (TLRs), which are found on epithelial cells, mast cells and dendritic cells, and other APCs. These receptors are trained to trigger host immune response in response to bacterial ligands. In the middle ear mucosa of both OM and non-OM patients, TLR2, TLR4, TLR5 and TLR9 were found at mRNA and protein level, but the correlation between expression levels and OM phenotype differs in different studies. Si *et al.* (2014) observed that the mRNA and protein expression of TLR2, TLR4 and TLR5 was not changed in COM patients compared with non-COM patients, while the expression was significantly lower in chronic suppurative OM patients compared to the other two groups. Similarly, Lee *et al.* (2013) noted a decrease in TLR-2, -4, -6 and -9 gene expression levels in OME children, without information about the protein level. Nevertheless, both studies suggest that TLR expression might be decreased in OM patients. This would be in agreement with, for example, the outer membrane ubiquitous surface protein A1 (UspA1) of *M. catarrhalis* being able to inhibit the TLR2- NF-κB pro-inflammatory response in the host (Slevogt *et al.*, 2008). On the other hand, in *H. influenzae*-associated infections, the TLR2-interacting lipoproteins seem to be major triggers of the immune system (Duell *et al.*, 2016). Lipoprotein P6, has been shown to induce IL-8 and TNF-α production by macrophages while (more anti-inflammatory) IL-10 production was induced by both P4, P5 and P6 (Berenson *et al.*, 2005). Moreover, both LTA of *S. pneumoniae* and PCho of *S. pneumoniae* and *H. influenzae* can also bind the platelet-activating factor receptor, inducing inflammation in the host via a TLR2-independent mechanism (Gisch *et al.*, 2013; van der Poll and Opal, 2009; Weidenmaier and Peschel, 2008).

As already mentioned, pneumolysin, is normally a key virulence factor of most serotypes of *S. pneumoniae*. It can activate $CD4^+$ T-cells, by impairing the respiratory burst of phagocytic cells, by inducing production of chemokines and cytokines, by stimulating complement fixation and

by activating inflammation (Hirst *et al.*, 2004; van der Poll and Opal, 2009; Zhang *et al.*, 2007). However, some strains and serotypes have evolved mechanisms to evade the immune responses of the inflammasome. For instance, allele 5 pneumolysin expressed by serotype 1 MLST306 and some serotype 8, which does not activate the inflammasome, in contrast to allele 1 pneumolysin, induces a lower IL-1β response in the host and thus serves as a potential immune evasion factor (Rabes *et al.*, 2016).

1.3.2.2 Immune-escape factors

To protect itself against the host's adaptive immune defense, many pathogens directly target antibodies from the host. For instance, the IgA1 proteases which are produced by *S. pneumoniae* and *H. influenzae* cleave human secretory antibodies such as sIgA (Figure 1.5) (Jalalvand *et al.*, 2013; Kadioglu *et al.*, 2008). In addition, the *M. catarrhalis* immunoglobulin D (IgD) binding protein/hemagglutinin (MID/Hag) binds soluble IgD from the host (Forsgren *et al.*, 2001) and PCho protects *H. influenzae* against bactericidal antibody (IgG) binding (Clark *et al.*, 2012). The branched LOS of *H. influenzae,* which is pro-inflammatory as discussed above, is also highly variable and, as a consequence, serves as camouflage that covers the bacterial surface and protects from antibody recognition (Clark *et al.*, 2013). The previously mentioned lipoprotein P6 has a different strategy to protect itself against antibodies: by having two different orientations in the outer membrane it can evade an antibody response directed at it (Michel *et al.*, 2013). Furthermore, *H. influenzae* can neutralise host's antimicrobials and immune responses by extracellular DNA (eDNA) which neutralises human β-defensin 3 and PCho decorated-LOS, which causes a reduced IL-1β and TNF-α production (Jones *et al.*, 2013; Jurcisek and Bakaletz, 2007; Webster *et al.*, 2006; West-Barnette *et al.*, 2006). In addition, the production of PCho seems to decrease the susceptibility of *H. influenzae* to the human antimicrobial peptide LL-37 (Lysenko *et al.*, 2000). The pneumococcal surface protein A (PspA) of pneumococci, on the other hand, can bind lactoferrin, an antibacterial component of the human immune system (Rosenow *et al.*, 1997).

Pathogens can also evade the host's immune system by for instance binding complement factors. The pneumococcal surface protein C (PspC or CbpA) can prevent formation of C3b, while PspA interferes with the fixation of complement component C3, both preventing complement-mediated opsonisation (Iannelli *et al.*, 2004; Quin *et al.*, 2005; Rosenow *et al.*, 1997). On the surface of *M. catarrhalis*, especially UspA2 is important as it blocks the

complement-mediated opsonisation pathways by binding C4bp (Nordström *et al.*, 2004), C3 (Nordstrom *et al.*, 2005) and vitronectin (Attia *et al.*, 2006). Evasion of the immune system by NTHi can be mediated in most strains by porin P5, which is able to bind human factor H, a complement control protein, and as a consequence avoids C3 complement deposition (Rosadini *et al.*, 2014). Prevention of attacks by the complement system is additionally executed by the ability to bind vitronectin, which protects from host serum factors (Duell *et al.*, 2016).

1.3.3 Polymicrobial biofilm formation

A pathogenesis mechanism that only rather recently receives a lot of attention in COM and OME is mono- and polymicrobial biofilm formation by the OM bacterial pathogens (Figure 1.5) (Gu *et al.*, 2014; Hall-Stoodley *et al.*, 2006). These studies hypothesise that de presence of biofilms causes OM episodes to recur more often. By investigating middle ear mucosa biopsy specimens of OME children with confocal scanning laser microscopy and fluorescence *in situ* hybridisation (FISH) with specific probes, Hall-Stoodley *et al*. (2006) observed the presence of all three main OM pathogens in the biofilms. Interestingly, Broides *et al*. (2009) noticed that *M. catarrhalis* was more frequently present in polymicrobial OM infections than in single species OM infections suggesting that other bacterial pathogens can impact the persistence of *M. catarrhalis* or the severity of the disease caused by it. Indeed, although bacteria often compete with each other for e.g. nutrients and receptors, in many cases they collaborate for 'the greater common good'. The formation of an extensive exopolysaccharide (EPS) matrix, for example, causes a general protection of the inhabitants of the biofilm. Additionally, in a polymicrobial biofilm, β-lactam resistant *H. influenzae* and *M. catarrhalis* can protect *S. pneumoniae* against β-lactam antibiotics while *S. pneumoniae*, on its turn, protects the other two pathogens against macrolide killing (Perez *et al.*, 2014a; Weimer *et al.*, 2011). Furthermore, when growing together in a biofilm, Cope *et al*. (2011) observed an upregulation of type IV pili of *H. influenzae* and an increased H_2O_2 production of *S. pneumoniae*. The exact functions of these molecules in a polymicrobial biofilm are, however, not clear yet.

1.4 POTENTIAL OF PROBIOTICS AGAINST OM AND THEIR MOLECULAR MECHANISMS

As yet mentioned (§1.2.1), among the bacteria that are more prevalent in healthy subjects than in OM patients are potential probiotics that actually can contribute to better ear and upper respiratory tract health (INFOBOX). Similar as it is important for the OM pathogens to know

their pathogenesis mechanisms and virulence factors reviewed in earlier paragraphs, in order to apply probiotics against OM, it is important to identify potential probiotics and their probiotic mechanisms. Probiotics are officially defined as "live micro-organisms that, when administered in adequate amounts, confer a health benefit to the host" (Hill *et al.*, 2014). Thus, for a microbial strain to be probiotic, its health benefits should be documented in clinical trials that can substantiate causal health relations for the specific probiotic applied, in addition to more descriptive microbiome studies. Interestingly, as mentioned above, lactic acid bacteria, which are widely applied as gastrointestinal probiotics, are also among the interesting probiotic candidates for the URT based on various NGS studies mentioned above (§1.2.1), so that various examples will be given for these bacteria.

1.4.1 Documented health benefits of probiotics with focus on lactobacilli

Probiotic bacteria have already proven to confer a lot of benefits to the host in different niches of the human body. A large meta-analysis (including 74 studies with a total of 10351 participants) of probiotic efficacy for gastrointestinal diseases confirmed that probiotics are effective in the treatment or prevention of pouchitis, infectious diarrhoea, irritable bowel syndrome, *Helicobacter pylori*, *Clostridium difficile*-disease and antibiotic-associated diarrhoea. Of the 11 species of mixed cultures that were used in these studies, four included strains of *Lactobacillus*. Especially *L. rhamnosus* GG and *L. casei* showed positive significant effects while *L. plantarum* and *L. acidophilus* were less conclusive (Ritchie and Romanuk, 2012). Also for non-GIT diseases, oral administration of probiotics has already shown efficacy: two meta-analyses showed that probiotics are better than a placebo in reducing the number and duration of acute URT infection episodes, the antibiotic use and the cold-related school absence (Hao *et al.*, 2015; King *et al.*, 2014). Single *Lactobacillus* strains compared in these meta-analyses included *L. rhamnosus* HN001, *L. rhamnosus* GG, *L. acidophilus* NCFM and *L. casei* DN 114001. Only *L. rhamnosus* HN001 did not seem to have a significant positive effect on acute URT infections, while the other three lactobacilli did show significantly better results than the placebo group. In addition, probiotics can play a preventive role in the development of atopic diseases. A meta-analysis compared 17 studies (comprising 4755 children) and observed that infants treated with probiotics had a significantly lower risk ratio for eczema compared to controls, while no significant difference was observed for asthma, wheezing or rhinoconjunctivitis (Zuccotti *et al.*, 2015). Three single *Lactobacillus* strains included in this meta-analysis were *L. reuteri* ATCC

57730, *L. rhamnosus* GG and *L. acidophilus* LAVRI-A1. Only for *L. rhamnosus* GG, two out of the five trials reported showed a significant lower risk ratio. No significant positive effects were observed for the other two lactobacilli. Furthermore, when comparing 20 studies to determine the efficacy of probiotics for prevention and/or treatment of urogenital infections in adult women, Hanson *et al.* (2016) observed significant positive effects in the treatment and prevention of bacterial vaginosis, prevention of recurrences of candidiasis and urinary tract infections, and clearing human papillomavirus (HPV) lesions. Most studies used combinations of different lactobacilli. Six single-species probiotic interventions were tested from which two studies showed significant positive results: *L. rhamnosus* GG usage to lower the vaginal pH in patients suffering from bacterial vaginosis (Rossi *et al.*, 2010) and *L. casei* Shirota to enhance the HPV clearance (Verhoeven *et al.*, 2013). Both lactobacilli were effective but the strength of the evidence was stated as low in the meta-analysis. A topic which is recently gaining more interest is the effect of probiotics on neurological symptoms such as depression. A meta-analysis comparing 5 clinical trials suggested a significant positive effect on the population under 60 years of age while they had no effect on people aged over 65 (Huang *et al.*, 2016). More studies are however necessary to confirm these findings. These positive results show the potential of probiotics in several niches. However, the topical application of probiotics in the URT and the mechanisms of action of their beneficial effects are less investigated and will be discussed in next paragraphs.

1.4.2 Clinical studies with topical application of probiotics

Currently, only a limited number of clinical trials have been performed with 'potential probiotics' in relation to health benefits to the URT of the host (Table 1.2). Furthermore, the current data on the clinical efficiency of probiotics for OM are not univocal. Both oral and topical intake of probiotics has been explored in recent years (as reviewed in Marom *et al.*, 2016; Niittynen *et al.*, 2012). It seems that the oral administration route especially aims at enhancing immune responses systemically (mainly via the gastrointestinal immune cells). On the other hand, topical application of the probiotic strains directly in the URT, *e.g.* via a nasal spray, might be a better administration route to directly target the OM pathogens, but this is only explored so far for a limited number of probiotic species. Although lactobacilli are increasingly explored, one of the best documented lactic acid bacteria (LAB) probiotics for topical application are α-haemolytic *Streptococcus* (AHS) bacteria (Niittynen *et al.*, 2012). A

combination of two species of *Streptococcus mitis* and *S. sanguis* and one species of *S. oralis* were used in two Swedish studies. In the first one, 108 otitis-prone children were investigated after daily nasal administration of the AHS mix (7.5 x 10^7 CFU per intake) or placebo during 10 consecutive days. The AHS-treatment group experienced less recurrences of AOM compared to the placebo group as monitored in a three month period (Roos *et al.*, 2001). The second study which tested the same mix of AHS (5 x 10^5 CFU per intake) for 43 children with recurrent OM for four months, however, did not see a difference of AOM recurrences and did not detect significant changes in the nasopharyngeal colonisation (Tano *et al.*, 2002). This difference could be due to the lower amount of administered streptococci in the latter study (see Table 1.2). Skovbjerg *et al*. (2008) used lactobacilli in a similar study. They compared the administration of *S. sanguinis* NCIMB 40104, *L. rhamnosus* NCIMB 40564 or a placebo in 60 children with serous OM. In both treated groups, ca. 50% of the children showed improvements or were cured (9/19 in *Streptococcus* group and 9/18 in *Lactobacillus* group) while this number decreased to only 18% (3/17) in the placebo group. The spray treatment did not alter the composition of the nasopharyngeal microbiota (although only monitored with cultivation techniques) or the cytokine patterns (IL-1β, IL-6, IL-8 and IL-10) in the middle ear fluid (Skovbjerg *et al*. 2009). More recently, a different AHS strain, *S. salivarius* 24SMB, also showed promising results (Marchisio *et al.*, 2015). Children who administered the strain in each nostril twice per day for five consecutive days each month for three consecutive months showed less episodes of AOM and received less antibiotics as monitored for a six month period. In addition, Mårtensson *et al*. (2016) reported the successful nasal administration of promising *Lactobacillus* and *Bifidobacterium* strains, isolated from honey bees to healthy adults. The spray did not increase URT inflammation as was tested with a cytokine microarray representing 30 cytokines/chemokines and mediators involved in type 1 and 2 inflammatory responses. However, no adverse effects were observed after administration.

Table 1.2: Overview of clinical trials with topical probiotic application.

URT Disease	Probiotic strain(s)	Type	n	Dose and duration	Results	Reference
OM	*S. mitis, S. sanguis, S. oralis*	Saline spray	108	Dose: Intranasal 150 µL per nostril, 1x/day of saline suspension $5x10^8$ CFU/ml Duration: parallel 10 days + 10 days	Cured ↑: *Streptococcal* group 42% vs. placebo group 22% (p = 0.02) Recurrence of OM ↓: 40% vs. 51% (p = 0.04) Nasopharyngeal flora: ns.	(Roos *et al.*, 2001)
OM	*S. mitis*, *S. sanguis*, *S. oralis*	Saline spray	43	Dose: Intranasal 50 µL per nostril, 1x/day of saline suspension 10^7 CFU/ml Duration: 4 months	Episodes of OM: Streptococcal group 44% vs. placebo 40% (ns.) Nasopharyngeal flora: ns.	(Tano *et al.*, 2002)
OM	*S. sanguinis* NCIMB 40104, *L. rhamnosus* GG	Saline spray	60	Dose: Intranasal 100 µL per nostril, 2x/day of saline suspension $5x10^9$ CFU/ml) Duration: 10 days	Recovery: 7/19 patients in *S. sanguinis* group vs. 1/17 in placebo group ($p < 0.05$); 3/18 patients in *L. rhamnosus* group (p = 0.60 compared with placebo) Nasopharyngeal flora: ns.	(Skovbjerg *et al.*, 2008)
OM	*S. salivarius* 24SMB	Saline spray	100	Dose: Intranasal 50 µL per nostril, 2x/day of saline suspension 10^{11} CFU/ml) Duration: spray 5 days per month for 3 consecutive months.	Recurrence of OM ↓: Streptococcal group 14.9% vs. placebo group 30% (p = 0.076) Antibiotic use ↓: 70% vs 83.0 % (p=0.13) Recurrence of OM after colonisation of *S. salivarius 24* SMB 13.6% vs. 42.8% without colonisation	(Marchisio *et al.*, 2015)
Pharyngitis and OM	*S. salivarius* K12	Buccal tablet	82	Dose: 10^9 CFU per tablet, 1x/day Duration: 90 days	Recurrence of pharyngitis ↓ by ca. 90% in probiotic group* Recurrence of OM ↓ by ca. 40% in probiotic group* * calculated by comparing with infection rates in the previous year	(Di Pierro *et al.*, 2012)

General inflammation	**URT**	A mixture of 9 *Lactobacillus spp.* and 4 *Bifidobacterium spp.*	Honey and 22 pollen in water spray	Dose: Intranasal 200 µL per nostril, of suspension 10^{11} CFU/ml) Duration: one dose administrated	No untoward effects. No significant difference in SNOT-22 scores obtained postchallenge with LAB and sham Nasopharyngeal flora: ns.	(Mårtensson *et al.*, 2016)

As summarised above and in Table 1.2, although various clinical benefits have been reported, the randomised-controlled studies with probiotics do not all show efficacy. This could be explained by the fact that the applied probiotic strain was not optimally selected – or administered (e.g. too low dose, too short) for the URT condition targeted or because most of the 'host' study participants were not responsive to the probiotics. Detection methods, host genetics, too severe inflammation or too severe microbiome dysbiosis could indeed influence (measured) responses to probiotic treatment, as also shown for gastrointestinal applications of probiotics, highlighting the need for patient stratification (*e.g.* Claes *et al.*, 2015). Therefore, it can be anticipated that knowledge about the molecular mechanisms of action of the probiotics in the URT– and better molecular knowledge of OM pathogenesis– will facilitate the selection of the most optimal probiotic strain for each condition. In the next paragraphs, potential probiotic mechanisms of action against infection of OM pathogens are discussed. Although little is yet documented about the potential protecting characteristics of nasopharyngeal probiotics, we have rationalised these mechanisms similar as for the gastrointestinal tract (GIT) (Lebeer *et al.* 2008). Desired properties are divided in adaptation factors that allow optimal adaptation to the URT niche and processing conditions for the specific application, and probiotic factors that directly relate to the desired health- promoting effects of the probiotics. Since many clinical studies with URT probiotics performed so far have been done with lactobacilli, and since current microbiome studies also suggest a potential role for LABs potential mechanisms of action will be explored here mainly for lactobacilli. Moreover, lactobacilli have an advantage over other less well studied health-related taxa such as Corynebacteria, because they have a long history of safe use ("Generally Recognized As Safe" (GRAS) and "Qualified Presumption of Safety" (QPS)-status) in fermented foods which is important for future applications.

1.4.3 Adaptation mechanisms rationalised for URT probiotics

Considering the fact that most URT pathogens strongly adhere to the nasopharyngeal epithelium – at least temporarily in their infection process (as discussed in earlier paragraphs), it is reasonable to envisage that probiotics to be applied in the URT should – at least temporarily- be able to colonise the mucosa to compete with these pathogens, especially considering the nasal clearance being less than 20 minutes (Sherly and Prathibha, 2014).

Although there is no strong consensus in the literature that gastrointestinal probiotics should be able to strongly adhere to the mucosa, selecting highly adherent probiotic strains is often part of the screening platforms. Successful gastrointestinal probiotics such as *Lactobacillus rhamnosus* GG show a high adhesion capacity to human intestinal epithelial cells and mucus due to the presence of adhesive heteromeric SpaCBA pili (Kankainen *et al.*, 2009; Segers and Lebeer, 2014). More specifically, the tip pilin SpaC acts as a mucus binding protein (MUB). Interestingly, *L. rhamnosus* GG has also been shown to colonise the tonsils when administered in a dairy formulation containing 10^{10} CFU daily for three weeks (Kumpu *et al.*, 2013). In addition, *L. rhamnosus* GG could also be recovered from adenoids (100% recovery by qPCR) and middle ear fluid (MEF) (21% recovery by qPCR) after oral consumption in a dairy formulation for three weeks in ca. 1.6 x 10^{10} CFU/dose (Swanljung *et al.*, 2015; Tapiovaara *et al.*, 2014). Whether the SpaCBA pili are also important for the adherence to respiratory and nasopharyngeal epithelium cells is at present not known. In addition to pili, other sortase-dependent proteins (SDPs) could promote adherence of lactobacilli – and related potential probiotics- to the respiratory tract epithelium (Call and Klaenhammer, 2013). For instance, we recently found indications for a novel type of SDP-dependent pili in the nasopharyngeal *Lactobacillus casei* AMBR2 strain (Wuyts *et al.*, 2017). Other surface proteins that are linked to adherence to the host epithelium are lectins, *i.e.* proteins that bind carbohydrates with high specificity. For instance, the lectin-like protein 1 (Llp-1) of *L. rhamnosus* GR-1 has been shown to play a tissue-specific role in adhesion to vaginal (Petrova *et al.*, 2016b) epithelium, but not gastrointestinal and endocervical cells, suggesting that lectins could also mediate tissue-specific adhesion to the URT niche.

Being able to strongly adhere to the nasopharyngeal epithelium will probably not be sufficient to efficiently compete with the OM pathogens and to sufficiently interact with the human host cells to confer beneficial effects. It can be hypothesised that the applied probiotics should also be able to adapt to the specific host nutritional environment and specific stress conditions of the URT. Indeed, the conditions in the gut and the URT are not comparable as the oxygen level, the pH, the relative humidity, the 'travel' distance and time, temperature etc. differ substantially (Man *et al.*, 2017). The thickness of the EPS layer of *L. rhamnosus* GG, for example, has been shown *in vitro* to increase in a neutral pH (cfr. URT) compared to an acidic environment (cfr. gut), which causes pili of *L. rhamnosus* GG to unfold and be more accessible

for interaction with proteins (Burgain *et al.*, 2015), but whether this is also true *in vivo* remains to be substantiated. Further mechanistic studies are certainly needed to define the most important characteristics of candidate probiotic bacteria in the URT. At present, a standard model is lacking for *in vitro* URT adhesion assays, but several cell lines are used such as lung epithelial A549 (Lafontaine *et al.*, 2004) and Calu-3 cells (Allonsius *et al.*, 2017), hypopharyngeal FaDu cells (Guglielmetti *et al.*, 2010), pharyngeal Detroit 562 cells (Su *et al.*, 2016) and laryngeal CCl-23 cells (Wong *et al.*, 2013). In contrast to the interaction with the gut epithelium, mucosal adhesion of lactobacilli to the nasopharyngeal epithelium has not been extensively studied. However, by *in vitro* assays Guglielmetti *et al.* (2010) observed that *L. helveticus* MIMLh5 was able to adhere to FaDu hypopharyngeal carcinoma cells and antagonise the typical sore-throat pathogen *S. pyogenes.* The model gastrointestinal probiotic *L. rhamnosus* GG has also been shown to inhibit the adherence of *S. pneumoniae* to the laryngeal cell line CCL-23 in a time- and dose-dependent way (Wong *et al.*, 2013).

Less pH-stress (pH 6.3 and 7 in nasal cavity and nasopharynx, respectively), lower temperature and more oxygen (Man *et al.*, 2017) compared to the GIT can be hypothesised to favour different probiotics than the 'classical GIT ones'. At present, the available nutrients and other stress factors in the URT are not well characterised, but it can be rationalised that the probiotics will have to adapt to low concentrations of free carbohydrates and iron (Siegel and Weiser, 2015), as well as to the presence of antimicrobial molecules in the mucus such as lysozyme, lactoferrin and PLUNC (palate, lung, and nasal epithelial clone)-proteins (Underwood and Bakaletz, 2011). For instance, our recently isolated *L. casei* AMBR2 strain from the nasopharynx is catalase-positive (while all other *Lactobacillus* species are catalase-negative), suggesting a role for catalase in adaptation to the oxidative environment of the URT (Wuyts *et al.*, 2017). Indeed, URT lactobacilli will have to withstand other stresses than in the GIT: they will not have to resist to gastric digestive enzymes and bile acid stress, unless also immunomodulatory effects are aimed for via the gastrointestinal immune system.

1.4.4 Probiotic mechanisms rationalised for URT probiotics

1.4.4.1 Direct antimicrobial actions against OME pathogens

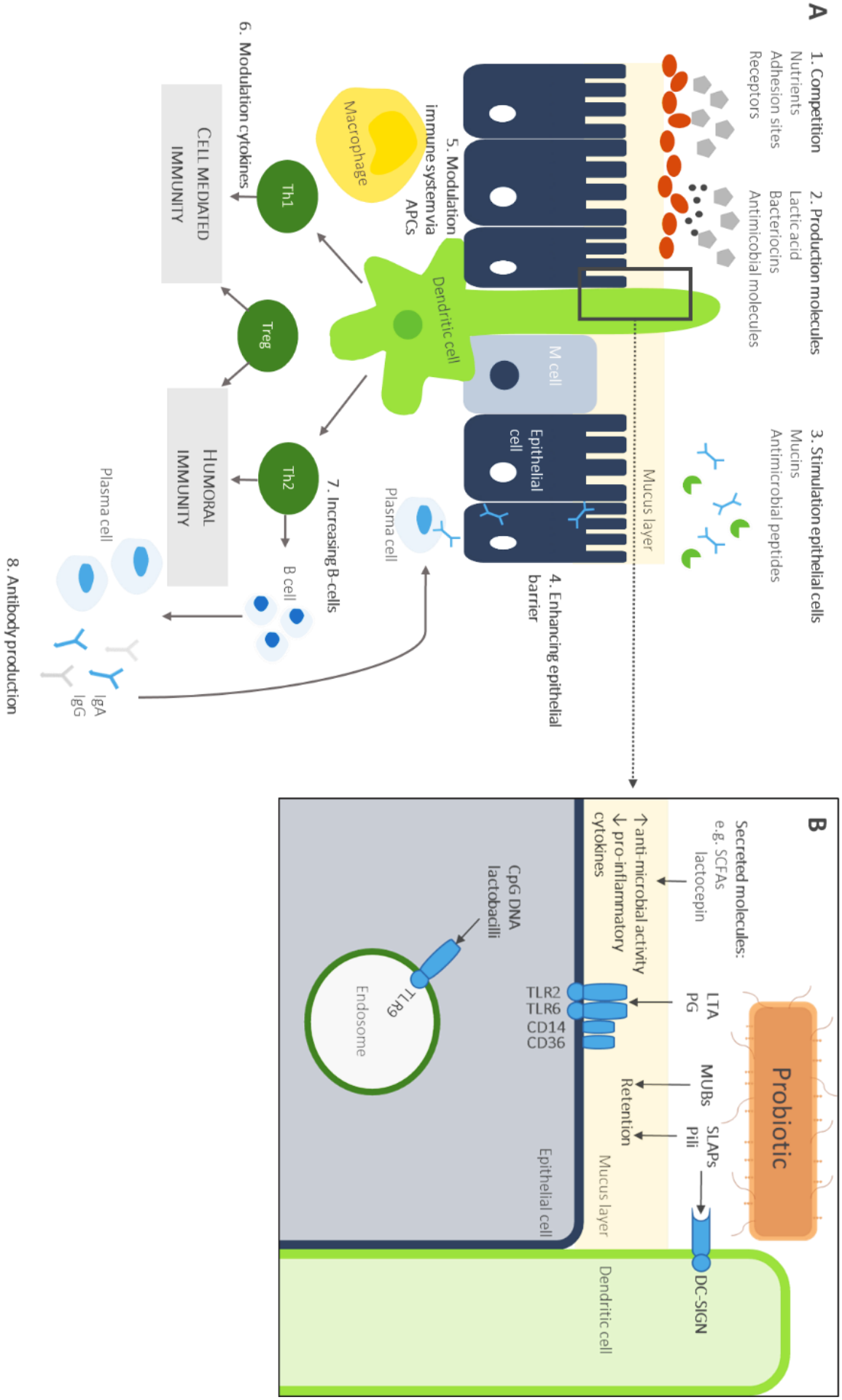
In addition to competition for adhesion sites, probiotics can directly inhibit pathogens through the production of antimicrobial substances, such as lactic and acetic acid, bacteriocins and

hydrogen peroxide (Lebeer *et al.*, 2008b). These substances exert inhibitory effects on both Gram-positive and Gram-negative bacteria, but of course the most active mechanism will depend on the exact pathogen(s) that are targeted by probiotic application. Organic acids such as lactic and acetic acid can work mainly inhibitory against Gram-negative bacteria as their undissociated form can enter the bacterial cell and dissociate in the cytoplasm (Alakomi *et al.*, 2005; De Keersmaecker *et al.*, 2006; Makras *et al.*, 2006). In 2006, lactic acid was documented to be the active antimicrobial molecule of lactobacilli against *Salmonella enterica* serovar Typhimurium (De Keersmaecker *et al.*, 2006; Hütt *et al.*, 2006; Makras and De Vuyst, 2006). However, lactic acid has also been shown to permeabilise the Gram-negative outer membrane of pathogens such as *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, and *S.* Typhimurium by utilising a fluorescent-probe uptake assay and sensitisation to bacteriolysis (Alakomi *et al.*, 2005). Furthermore, in spent culture supernatant of lactobacilli, lactic acid was shown to play a crucial role in the antibacterial activity against *M. catarrhalis* (van den Broek *et al*., accepted for publication in Beneficial Microbes). This makes it a promising molecule to circumvent the attack of Gram-negative URT pathogens such as *M. catarrhalis* and *H. influenzae.* But lactic acid is not the only active molecule which is produced by *L. rhamnosus* GG as the antimicrobial effect seems not to be merely dependent on the lactic acid concentration (Marianelli *et al.*, 2010). Indeed, seven heat-stable peptides with antibacterial activity against enteroaggregative *E. coli* strain EAEC042, *S.* Typhimurium, and *Staphylococcus aureus* were isolated from *L. rhamnosus* GG supernatant (Lu *et al.*, 2009). The genome sequence of *L. rhamnosus* GG revealed bacteriocin-related genes what suggests possible production of these antimicrobial peptides (Kankainen *et al.*, 2009). Bacteriocins are produced by many lactobacilli: *L. acidophilus*, for example, produces lactacin B (Barefoot and Klaenhammer, 1983) and *L. plantarum* plantaricin (Gonzalez *et al.*, 1994). By the formation of pores or inhibition of cell wall synthesis, bacteriocins exert their antimicrobial action against (often closely related) bacteria. As far as we know, no bacteriocin of lactobacilli has been shown to have antimicrobial activity against OM pathogens although *S. pneumoniae* is sensitive to nisin, a bacteriocin produced by *Lactococcus lactis* (Goldstein *et al.*, 1998). Furthermore, lactobacilli such as *L. rhamnosus* GG and *L. rhamnosus* GR-1 contain lectin-like proteins which are shown to inhibit and/or structurally disrupt pathogenic biofilms (Petrova *et al.*, 2016a, 2016b). In addition, Pericone *et al.* (2000) observed the bactericidal effect of H_2O_2, produced by *S. pneumoniae*, against its co-inhabitants of the URT such as *H. influenzae* and *M. catarrhalis*, suggesting this mechanism of probiotics might

also be mediated in the URT, however little evidence is available. The production of H_2O_2 by lactobacilli has been suggested to be a third important antimicrobial mechanism, especially in the vagina of healthy women (Servin, 2004), although this is controversial and the molecule is instable (Petrova *et al.*, 2015).

Another way of looking at production of antimicrobial molecules is the production of molecules that interact with cell-cell communication of pathogens. Quorum sensing, a system of stimuli and responses correlated to population density, might interfere with pathogen infection (de Kievit and Iglewski, 2000). The *luxS* gene is responsible for the production of autoinducer-2 (AI-2), an important interspecies quorum sensing molecule, in both Gram-negative and Gram-positive bacteria (de Kievit and Iglewski, 2000). Some lactobacilli, contain this gene (*e.g.* (Lebeer *et al.*, 2007) for *L. rhamnosus* GG), as do the OM pathogens *S. pneumoniae* (Vidal *et al.*, 2011) and *H. influenzae* (Armbruster *et al.*, 2009). AI-2 is an important factor in biofilm formation by *S. pneumoniae* and *H. influenzae* (Armbruster *et al.*, 2009; Vidal *et al.*, 2011). Furthermore, a mutation in the *luxS* gene causes a reduction in the virulence and persistence in a murine model of nasopharyngeal carriage of *S. pneumoniae.* A *luxS* mutation also increased the virulence of *H. influenzae* in a chinchilla model (Daines *et al.*, 2005; Joyce *et al.*, 2004; Stroeher *et al.*, 2003). In contrast to these pathogens, *M. catarrhalis* cannot produce AI-2 itself as it does not contain the *luxS* gene. However, interestingly, its biofilm formation is promoted by the production of AI-2 by *H. influenzae* (Armbruster *et al.*, 2010). Disrupting the AI-2 transport, antagonising of the signalling, inhibition of the AI-2 production or quenching of AI-2 would thus possible strategies to interfere with the interspecies communication in OM infections. However, since the AI-2 synthase LuxS also interferes with the cell metabolism (Vendeville *et al.*, 2005), the role of quorum sensing in pathogen exclusion is difficult to investigate.

Figure 1.7: Postulated beneficial modes of action of URT probiotics. *(A) In agreement with beneficial activities in the gut, probiotics could also perform such activities in the URT for instance by (1) competing with pathogens for nutrients, adhesion sites and receptors; (2) the production of antimicrobial molecules such as bacteriocins and lactic acid; (3) stimulation of epithelial cells to modulate the mucin and antimicrobial peptide production; (4) enhancing the epithelial barrier; (5) modulating the immune system via APCs; (6) modulation of cytokine production; (7) increasing B-cell production; and (8) stimulation of antibody production. (B) Interaction of several probiotic effector molecules with their receptors localised on the epithelial/dendritic cells or endosomes. APC: antigen presenting cell; LTA: lipoteichoic acid; MUB: mucus binding protein; PG: peptidoglycan; SCFA: short-chain fatty acids; SLAP: surface-layer-associated protein; Th1/2: T helper 1/2 cell; Treg: regulatory T cell. Figure based on* Lebeer *et al.*, 2008b; Niittynen *et al.*, 2012; Sanders *et al.*, 2018.


A
1. Competition
Nutrients
Adhesion sites
Receptors
2. Production molecules
Lactic acid
Bacteriocins
Antimicrobial molecules
3. Stimulation epithelial cells
Mucins
Antimicrobial peptides
Mucus layer
M cell
Epithelial cell
4. Enhancing epithelial barrier
5. Modulation immune system via APCs
Macrophage
Dendritic cell
6. Modulation cytokines
Th1
Treg
Th2
CELL MEDIATED IMMUNITY
HUMORAL IMMUNITY
7. Increasing B-cells
B cell
Plasma cell
Plasma cell
IgA
IgG
8. Antibody production
B
Probiotic
Secreted molecules:
e.g. SCFAs
lactocepin
↑anti-microbial activity
↓ pro-inflammatory cytokines
LTA
PG
MUBs
Pili
SLAPs
Retention
DC-SIGN
TLR2
TLR6
CD14
CD36
CpG DNA
lactobacilli
TLR9
Endosome
Epithelial cell
Mucus layer
Dendritic cell

1.4.4.2 Enhancement of the nasopharyngeal epithelial barrier

Another documented probiotic mechanism for the GIT is the enhancement of the epithelial barrier function as reviewed recently by (Bron *et al.*, 2017). The mechanisms include, for instance, enhancement of tight junction functioning. In an *in vivo* study, *L. plantarum* WCFS1 was shown to induce changes in the intestinal epithelial tight junctions, which was demonstrated by an increased presence of zonula occludens-1 and occludin, two tight junction proteins (Karczewski *et al.*, 2010). In addition, two soluble proteins, produced by *L. rhamnosus* GG, Msp1/p75 and Msp2/p40 were demonstrated to protect the tight junctions in Caco-2 cell monolayers from hydrogen peroxide-induced disruption (Seth *et al.*, 2008). Furthermore, these proteins also prevented TNF-induced apoptosis of epithelial cells in cultured cells and *ex vivo* colon organ culture models (Yan *et al.*, 2007). Whether probiotics can exhibit this beneficial mechanism as well in the upper respiratory tract is underexplored, but would be probably health promoting as the epithelial barrier is disturbed in nasopharyngeal infections (Steelant *et al.*, 2016; Yeo and Jang, 2009).

Another epithelial barrier function promoting mechanism is the stimulation of antimicrobial peptides such as defensins, whose function is to protect the mucosal surfaces against invasion of microorganisms (Madsen, 2012; Underwood and Bakaletz, 2011). The mechanism of the antimicrobial activity of defensins is multiple: the construction of pores in the membrane of pathogens is the most important one, but they can also inhibit bacterial toxins such as pneumolysin of *S. pneumoniae* (Lehrer *et al.*, 2009). On the other hand, defensins can influence the immune system to produce pro-inflammatory cytokines and chemokines. Of the human β-defensins (HBD), HBD-2 is the most potent antimicrobial peptide. Its production is regulated by the cytoplasmic receptor NOD2 which recognises muramyl dipeptide motifs found on all lactobacilli (Delcour *et al.*, 1999; Wehkamp, 2004). Such a muramyl dipeptide in the peptidoglycan of *L. salivarius* Ls33, M-tri-Lys, has been shown to activate the NOD2 receptor while being MyD88 independent (Fernandez *et al.*, 2011). HBD-2-mediated killing of some strains of *S. pneumoniae, H. influenzae* and *M. catarrhalis* has been reported at low concentrations (Maxson and Yamauchi, 1996) and can be induced by probiotics (Schlee *et al.*, 2008). Two pathways can be responsible for the production of HBD-2: IL-1β can trigger the production via the Raf-MEK1/2 MAP kinase pathway (Moon *et al.*, 2006) as well as the TLR2 induction of the MyD88 MAP kinase pathway (Lee *et al.*, 2008). In addition to HBD-2, human α-

defensins 1-4, which are expressed by neutrophil granules, are important in the phagycytosis mediated killing of bacteria. Especially *H. influenzae* is sensitive to this kind of defensins (Bishop-Hurley *et al.*, 2005).

Furthermore, human epithelial cells can produce other antimicrobial proteins such as lysozymes, cathelicidins, C-type lectins and ribonucleases which often attack cell wall structures and/or the bacterial membrane. Lysozyme degrades the peptidoglycan of the bacterial cell wall and can kill *S. pneumoniae* synergistically with HBD-2 (Lee *et al.*, 2004). Cathelicidins are cationic antimicrobial peptides which also trigger the host's immune system. In a chinchilla model, a cathelicidin was observed to be able to kill of NTHi 86028-NP and *M. catarrhalis* 1857, however *S. pneumoniae* serotype 14 seemed to be less sensitive (McGillivary *et al.*, 2007). *L. rhamnosus* GG can upregulate cathelicidin-related antimicrobial peptides (CRAMP) in mice (Bu *et al.*, 2006), but little is known about similar effects in humans. Other examples are the induction of mucus and the induction of cytoprotective molecules (reviewed in Madsen, 2012).

1.4.4.3 Enhancement of the (systemic) immune system

Besides the stimulation of the production of antimicrobial molecules of the host, probiotic application can also modulate host immune responses, both innate and adaptive immunity (Lebeer *et al.*, 2010). Probiotic bacteria can for instance modulate the maturation of dendritic cells (DC) towards an anti-inflammatory IL-10 profile. The protein SlpA of *L. acidophilus* NCFM, a surface-layer-associated protein (SLAP), has been shown to fulfil this immunostimulating role by the interaction with DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Konstantinov *et al.*, 2008) (Figure 1.7). In addition, the stimulatory role of probiotics on regulatory T-cell activity is well explored and seems an important probiotic mechanism in controlling overt inflammatory conditions, although there exist large strain differences in this capacity (Borchers *et al.*, 2009; Feleszko *et al.*, 2007). In asthmatic mice, oral administration of *L. rhamnosus* GG (10^9 CFU every second day for 8 consecutive weeks) suppressed allergen-induced proliferative responses and was associated with an induction of T-regulatory cells in both mesenteric and peribronchial lymph nodes (Feleszko *et al.*, 2007). Other properties include the stimulation of mucosal immunoglobulin A (IgA) levels and allergen-specific B and T cell responses, which might especially modulate allergic diseases, e.g. in the URT (reviewed in (Toh *et al.*, 2012)). Guglielmetti *et al*. (2010) explored the immunomodulatory effect of the probiotic strain *L. helveticus* MIMLh5 in FaDu hypopharyngeal carcinoma cells *L. helveticus* MIMLh5, could reduce

the induction of IL-6, IL-8 and TNF-α while it enhanced the expression of the heat shock protein coding gene *hsp70* (Guglielmetti *et al.*, 2010). Interestingly, in mice, intranasal administration of *L. rhamnosus* GG during three days increased the cytotoxic activity of pulmonary natural killer (NK) cells after infection with influenza virus H1N1. This probiotic strain was also shown to increase the secretion of IL-1β and TNFα, which resulted in a better survival of the mice after 15 days.

The exact molecules by which probiotics can exert these immunomodulatory effects are only fragmentarily documented and the research towards these molecules has primarily focused on the GIT environment. Immune priming molecules include microbe-associated molecular patterns (MAMPs) such as LTA and EPS that can interact with pattern recognition receptors such as TLRs (Lebeer *et al.*, 2010) (Figure 1.7). In intestinal cell and monocytic models, pili of *L. rhamnosus* GG were observed to have an anti-inflammatory effect on the cells as an increased exposure of the pili decreased the IL-8 mRNA induction twice compared to the wild-type (Lebeer *et al.*, 2012; Vargas García *et al.*, 2015). Other molecules such as TAs and EPS show related characteristics in intestinal assays. LTAs of several *Lactobacillus* strains can bind TLR2 and activate pro-inflammatory cytokine release (Balzola *et al.*, 2011; Claes *et al.*, 2011; Grangette *et al.*, 2005; Matsuguchi *et al.*, 2003) while EPS molecules of both *L. casei* Shirota and *L. plantarum* seemed to be more immunosuppressive in the gut (Remus *et al.*, 2012; Yasuda *et al.*, 2008). TLR9, present on endosomes, can, on the other hand, be triggered by unmethylated cytosine-guanine (CpG)-containing DNA. Such CpG-rich DNA is carried by many *Lactobacillus* species and can thus stimulate Th1 responses leading to cell mediated immunity. Next to MAMPs, the secretion of probiotic effector molecules can influence the host's immune system as well. In a mouse model, the production of the protease lactocepin in the gut by *L. paracasei* was shown to selectively degrade pro-inflammatory cytokines (von Schillde *et al.*, 2012). Interestingly, a large genome comparison highlighted the presence of these kind of proteases in many *Lactobacillus* strains, suggesting that it is an overarching feature among several strains (Sun *et al.*, 2015). Other *Lactobacillus*-genus wide produced metabolites, short-chain fatty acids, exhibit also immunostimulatory effects. In macrophages and neutrophils, for instance, they inhibit IL-6 and TNF-α but stimulates IL-10 production (Maslowski *et al.*, 2009; Park *et al.*, 2007; Vinolo *et al.*, 2011a, 2011b). In addition, lactate is also metabolised to butyrate

in the gut by lactate-utilising, butyrate-producing bacteria such as *Eubacterium hallii* and *Anaerostipes caccae* where it can have additional beneficial functions (Louis and Flint, 2009)

Until now we only discussed the direct immunomodulating effects of living probiotic bacteria. However, probiotics or certain of their surface molecules are also investigated for their potential to ameliorate humoral responses to vaccines when applied as an adjuvant. Several studies, for example, show that administration of *L. rhamnosus* GG before and/or after vaccination can increase the specific antibody production in the human host leading to enhanced protection rates (Boyle *et al.*, 2011; Davidson *et al.*, 2011; de Vrese *et al.*, 2005; Isolauri *et al.*, 1995). Induction of a higher concentration of such pathogen-specific antibodies can for instance help to inhibit the pathogen adhesion to the host's epithelial cells as observed for *S. pneumoniae* (Amerighi *et al.*, 2016). However, although the URT mucosal immune system is an interesting route for *e.g.* vaccination, modulation of immune responses through microbial/probiotic modulations at the URT (for example via nasal application) is currently underexplored.

1.5 CONCLUSION

OM is a leading cause of health care visits and antibiotic prescription in childhood (Pettigrew *et al.*, 2012). Thanks to major advances in bacterial community analyses, our knowledge about the bacterial composition of this infection is steadily increasing. The Human Microbiome Project has already done major efforts for standardisation of microbiome-focused studies in order that variation in the microbiome can be systematically studied. However, due to the use of different sequencing approaches and biological sample material, it is still difficult to compare all the results and define a 'healthy core microbiome'. A uniform sampling protocol and analysis technique, both algorithms and hardware, would thus be preferred to compare everything adequately as small details in sample handling can cause large differences in the outcome and interpretation as reviewed by Vandeputte *et al*. (2017) for gut samples.

Nevertheless, NGS approaches have now substantiated a key role for the 'classical' OM pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in samples of diseased children. In addition, other potential pathogens such as *Turicella* and *Alloiococcus* arise, while there is currently little knowledge about their virulence factors and pathogenic impact on the human immune system and barrier function (see INFOBOX for the 'classical' Koch's postulates). More

knowledge, individually and in a biofilm, is thus necessary to target specific causal activities of pathogens in the different disease states.

In addition, different of these NGS studies have correlated the presence of certain bacteria in the nasopharynx of infants, with a more 'healthy' status in later childhood. *Corynebacterium* and the lactic acid bacterium, *Dolosigranulum*, for example, are often referred to as protective bacterial genera (Biesbroek *et al.*, 2014b; Laufer *et al.*, 2011; Pettigrew *et al.*, 2008). The underlying mechanisms of their potential protective roles are yet not understood. Furthermore, to define adequate probiotic strains, the importance of validation experiments cannot be underestimated, as they causal relations between the bacteria and an increased health need to be demonstrated (see INFOBOX for the 'reverse' Koch's postulates). However, the targeting of the microbiome by health-promoting bacteria, such as probiotics, will gain more interest in the future as the awareness of the negative consequences of antibiotics is rising. Promising clinical results highlight much potential for these probiotic bacteria in the fight against OM, although variation in the study outcome is still observed as well. Selecting the optimal health-promoting bacteria against OM pathogens will benefit from more molecular insights in both pathogenic as well as probiotic mechanisms of action, as exemplified above.

1.6 SCOPE OF THIS THESIS

Otitis media (OM) is an URT infection with a high incidence and antibiotic prescription rate. It is generally caused by a dysfunction of the Eustachian tube due to inflammation of the mucous membranes in the nasopharynx. This inflammation often originates from infections with bacterial pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis* (Rovers *et al.*, 2004). Next-generation sequencing studies towards the URT microbiome in health and disease suggested that the absence or imbalance of lactic acid bacteria is frequently linked to more URT infections (Biesbroek *et al.*, 2014b; Bogaert *et al.*, 2011). Therefore, in this PhD project probiotics defined as "live micro-organisms that, when administered in adequate amounts, confer a health benefit to the host" (Hill *et al.*, 2014), are explored as innovative therapeutic strategies for OM.

In chapter 2, an *in vitro* screening platform was developed and potential probiotic lactobacilli were screened for their direct antimicrobial activity against the three main (A)OM pathogens. This screening platform consisted of agar, planktonic and biofilm-based assays.

In chapter 3, we investigated which effector molecules of lactobacilli are important in the activity against the (A)OM pathogens. For this purpose, supernatant of lactobacilli was treated in different ways and minimal inhibitory concentration (MIC) assays were determined.

As lactobacilli are well-documented to modulate host immune responses to gastro-intestinal pathogens, in chapter 4, indirect mechanisms of action of lactobacilli against the (A)OM pathogens through modulation of URT host cell interactions were investigated. Lactobacilli were screened for their adhesion capacity to respiratory epithelial cells, resulting in the selection of *L. rhamnosus* GG for further investigations towards pathogen exclusion and immunomodulation activity. Several gene deletion mutants of *L. rhamnosus* GG were included as well to screen for important surface molecules in these activities.

To translate some of these *in vitro* results towards *in vivo* applications, chapter 5 describes the development of an intervention study in which patients with OM were asked to administer orally an oil suspension of *L. rhamnosus* GG and *Bifidobacterium animalis* subsp. lactis BB-12 (Probactiol® Mini) daily for 4 weeks. Swabs, tissue samples and middle-ear-fluid aspirates were collected and investigated to detect a possible transfer of the probiotics from the oral cavity to these URT niches. Furthermore, via *16S rRNA* amplicon sequencing, possible influences on the URT microbiome were investigated. The outline of this doctoral thesis is depicted in Figure 1.8.

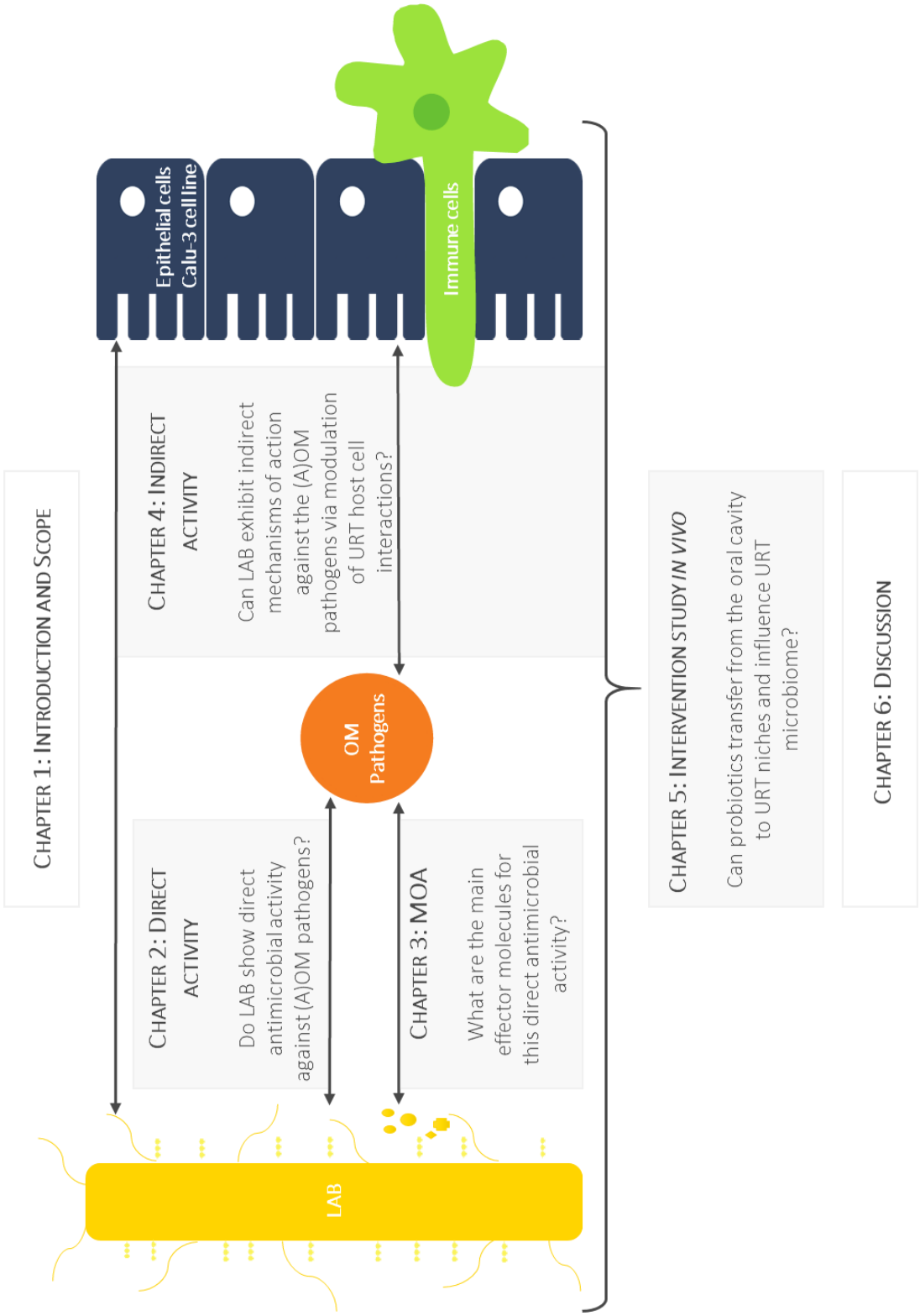


Figure 1.8: Outline of this thesis. *(A)OM: (acute) otitis media; LAB: lactic acid bacteria; MOA: mechanism of action; URT: upper respiratory tract.*

2 ANTIMICROBIAL ACTIVITY OF LACTIC ACID BACTERIA AGAINST (A)OM PATHOGENS IN DIRECT INTERACTION ASSAYS

Part of this chapter is accepted for publication: **van den Broek, M. F. L.,** De Boeck, I., Claes, I. J. J., Nizet, V. and Lebeer, S. 2017. Multifactorial inhibition of lactobacilli against the respiratory tract pathogen Moraxella catarrhalis. Beneficial microbes. Accepted for publication (Reference: BM-2017-07-0101.R2).

2.1 INTRODUCTION

As reviewed in Chapter 1, the common cause of all forms of OM is dysfunction of the Eustachian tube due to inflammation of the mucous membranes in the nasopharynx. This inflammation is mostly caused by infections with bacterial pathogens such as *Streptococcus pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis* (Rovers *et al.*, 2004). While antibiotics are still considered as standard therapy, they have many side-effects including the induction of antibiotic resistance (Goossens *et al.*, 2005). Indeed, a high proportion of *H. influenzae* and *M. catarrhalis* strains are now resistant to beta-lactam antibiotics such as penicillin and amoxicillin (Harrison *et al.*, 2009). Moreover, antibiotics disturb the endogenous 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 diarrhoea 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, there exists an urgent need for alternatives to classical antibiotic therapy for OM.

Lactobacilli are typically linked to beneficial properties in food fermentation and in health applications as probiotics, which are defined as "live micro-organisms that, when administered in adequate amounts, confer a health benefit to the host",(Hill *et al.*, 2014). Some profiling studies now indicate that presence of lactic acid bacteria, such as lactobacilli, in the URT may be associated with reduced occurrence of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (Biesbroek *et al.*, 2014b; Pettigrew *et al.*, 2012). However, these DNA-based microbiome studies have not yet been backed up with mechanistic studies on the direct interactions between lactobacilli and the corresponding URT pathogens.

In this chapter, the growth and biofilm inhibiting activities of various *Lactobacillus* strains, including several well-documented probiotic strains for gastrointestinal or vaginal application, and their spent culture supernatant were explored vs. the most important OM pathogens. The analyses were conducted using assays optimised to allow detailed insights in 1:1 competitions between potential probiotic and pathogen.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and growth conditions

Lactobacillus strains (Table 2.1) were grown at 37°C in de Man, Rogosa and Sharpe (MRS) medium (Difco). *M. catarrhalis* ATCC25238 was inoculated in Mueller Hinton (MH) (LabM Limited) broth and cultured aerobically at 37°C. *H. influenzae* ATCC49247 was grown in MH broth enriched with 0,5% yeast extract, 15 mg/L hemin and 15 mg/L NAD (Sigma Aldrich) in 5% CO_2 atmosphere and *S. pneumoniae* in Todd Hewitt broth (Sigma Aldrich) in 5% CO_2 atmosphere. Solid media contained 1.5% (w/v) agar. For antimicrobial assays, the respective media for these pathogens were, when indicated, enriched with glucose (Sigma Aldrich) to a final concentration of 5 g/L.

Table 2.1: Inventory of bacterial strains used in this chapter.

Species	Strain	Relevant genotype or description	Reference and/or source
		LACTOBACILLI	
Lactobacillus bulgaricus	AMB-1	Single colony isolate from Colombian yoghurt	Yoghurt
Lactobacillus casei	ATCC334	Single colony isolate obtained from a stock culture of ATCC334	ATCC
Lactobacillus casei*	Shirota	Single colony isolate obtained from a commercially available fermented drink containing *L. casei* Shirota (Yakult®), confirmed by sequencing	Commercial probiotic product
Lactobacillus 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	Commercial probiotic product
Lactobacillus casei*	MCJ	Single colony isolate from spontaneously fermented carrot juice	Fermented carrot juice
Lactobacillus helveticus	AMB-2	single colony isolate	Commercial probiotic product
Lactobacillus parabuchneri	AB17	Single colony isolate from spontaneously fermented beet juice	Fermented beet juice
Lactobacillus parabuchneri	NM63-3	Single colony isolate from spontaneously fermented carrot juice	Fermented carrot juice
Lactobacillus paracasei	LMG12586	Single colony isolate obtained from a stock culture of LMG12586	BCCM/LMG
Lactobacillus pentosus	KCA1	Single colony isolate from KCA1 (vaginal origin)	(Anukam *et al.*, 2013)
Lactobacillus plantarum	LMG1284	Single colony isolate from *L. plantarum* ATCC8014 or LMG1284	BCCM/LMG

Lactobacillus plantarum	CMPG5300	Single colony isolate (vaginal origin)	(Malik *et al.*, 2014)
Lactobacillus plantarum	5057	Single colony isolate of *L. plantarum* 5057	(Danielsen, 2002)
Lactobacillus plantarum	WCFS1	Single colony isolate of *L. plantarum* WCFS1	(Kleerebezem *et al.*, 2003)
Lactobacillus reuteri	RC14	Single colony isolate from a commercially available probiotic supplement containing *L. reuteri* RC14, 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 (urethra origin)	(Chan *et al.*, 1984, 1985; Reid and Bruce, 2001; Reid and Reid, 1999), ATCC
Lactobacillus rhamnosus	GG	Single colony isolate of wild-type strain, isolated from human faeces	(Kankainen *et al.*, 2009)

*** According to a recent large-scale comparative genomics study in our lab by Wuyts *et al.* (2017), these strains classify actually in the *L. paracasei* clade of the *L. casei* group.**

OTHER			
Escherichia coli	LMG2093	Single colony isolate from LMG2093	BCCM/LMG
PATHOGENS			
Haemophilus influenzae	ATCC49247	Single colony isolated from expectorated sputum from a 76 year-old male with pneumonia	ATCC
Moraxella catarrhalis	ATCC25238	Type strain, nasal cavity	ATCC
Streptococcus pneumoniae	ATCC49619	Single colony isolated from sputum of 75-year-old male	ATCC

2.2.2 Preparation of spent culture supernatant (SCS) of lactobacilli

Lactobacilli were incubated for 19 h to a final concentration of ±2 x 10^9 CFU/mL. Spent culture supernatant (SCS) was obtained by centrifugation for 15 min at 4000 rpm at 4°C. Afterwards, the SCS was filter sterilised (0.20 µm cellulose acetate, VWR) and the pH (Mettler-Toledo AG) and the concentration of D- and L-lactic acid (Roche Yellow Line) were measured.

2.2.3 Spot and streak-line antimicrobial assays with live lactobacilli against OM pathogens

The antimicrobial activity of live lactobacilli against the OM pathogens was explored by standard antimicrobial tests with specific modifications towards the pathogen and potential probiotics under study (Figure 2.1). For the streak-inoculation assay, lactobacilli were streak-inoculated from a colony on a starter plate (MRS medium) onto a round test plate (69.4 cm^2, respective medium of the pathogen under study supplemented to reach 5 g/L glucose) and incubated at 37°C for 48 h. Then, the pathogens were streak-inoculated from a colony on a

starter plate onto the test plates in triplicate. The plates were incubated overnight at 37°C and the inhibition zone was measured(Schillinger and Lücke, 1989). In addition, the antimicrobial activity of *Lactobacillus* cultures was explored by modified spot assays (Ghequire *et al.*, 2012; Lie *et al.*, 1997). Briefly, 2 µL of each *Lactobacillus* culture was spotted on a square agar plate (144 cm², 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 48h at 37°C. Ca. 10^6 CFU/mL of an 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.

2.2.4 Radial diffusion antimicrobial assay for SCS of lactic acid bacteria against OM pathogens

The antimicrobial activity of SCS was investigated with a modified protocol based on previously described competition assays between lactobacilli and gastro-intestinal pathogens (Figure 2.1) (De Keersmaecker *et al.*, 2006). Approximately 10^6 CFU/mL of the pathogen was added to 60 mL of 1.5% (w/v) agar and poured in a square agar plate (144 cm²). After the plates dried, wholes of 0.4 cm diameter were made in the agar with a Pasteur pipette 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, France) and sterile MRS medium brought to pH 4.3 were used as a positive and negative control, respectively.

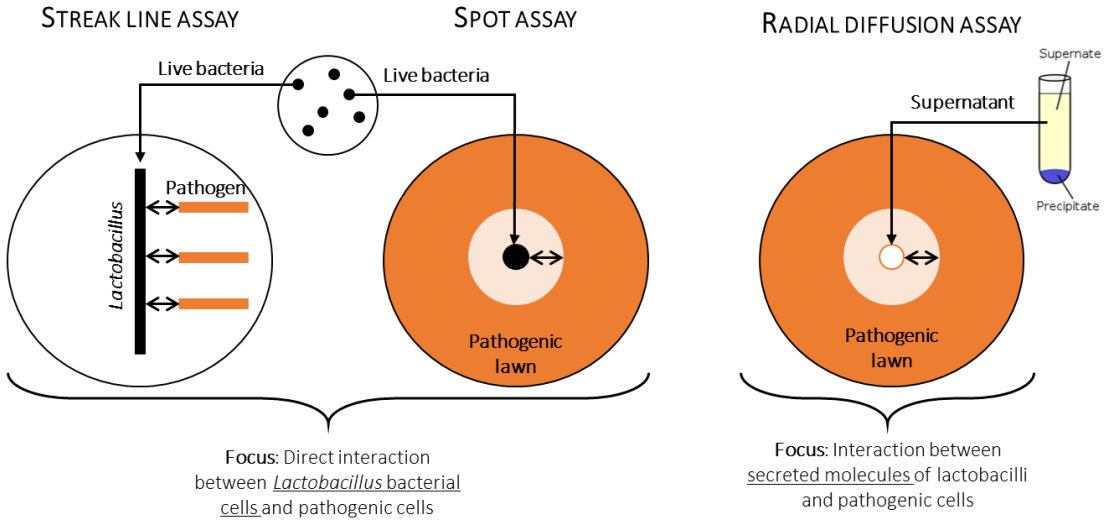


Figure 2.1: Rationale for using three different agar assays to test antimicrobial activity of lactobacilli against (A)OM pathogens. *In both streak line and spot assay, live bacterial cells are used and the direct bacterial interaction between* Lactobacillus *and the (A)OM pathogens are evaluated. In the radial diffusion assay, the spent culture supernatant (SCS) of an overnight* Lactobacillus *culture is used and the activity of the secreted molecules against (A)OM pathogens is evaluated. Arrows indicate inhibition zones.*

2.2.5 Time-course analysis of the antimicrobial activity of SCS of lactobacilli against OM pathogens

A time-course analysis of the growth inhibition in suspension was performed as described previously (Paparella, 2007) 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 optical density (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.

2.2.6 Biofilm assays of OM pathogens

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 *et al.* (2006) with minor modifications. An 10^8 CFU/mL suspension of an overnight culture of *M. catarrhalis* was made in a 1:20 diluted BHI broth. A volume of 170 µL 1:20 BHI, 10 µL active molecules and 20 µL of the bacterial suspension (final concentration 10^7 CFU/mL) was loaded into a 96-well microplate and incubated at 37°C for 19 h. 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 the wells were washed once with 220 µL dH_2O (slowly added to avoid disruption of the biofilm). Then, the plate was incubated at 60°C for 90 min. to fix the biofilm and 220 µL of 0.1% crystal violet solution (in 18:20 phosphate buffered saline (PBS), 1:20 methanol and 1:20 isopropanol) was gently added and incubated at room temperature for 30 min. The wells were subsequently washed several times with 300 µL dH_2O until blank well was not coloured anymore. A 220-µL volume of 95% ethanol was then added to each well, mixed and a 100 µL portion of this ethanol solution was transferred to a new 96-well plate. The absorbance at 570 nm was measured using a Synergy HTX multi-mode reader.

2.2.7 Spray drying of lactobacilli to enhance storage at room temperature and 4°C

Lactobacilli were spray dried as described earlier (Broeckx *et al.*, 2017). Briefly, a *Lactobacillus* suspension was centrifuged at 3983 x g for 10 min at 20°C. Afterwards, the obtained pellet was

resuspended to its original volume in PBS. The suspension was either used as such or enriched with a saccharide excipient (lactose or trehalose) with a dry probiotic:saccharide ratio (w/w) of 1:2 and 1:7. Spray drying was carried out with a laboratory-scale spray dryer (B-290, Büchi, Flawil, Switzerland). A co-current spray dryer configuration was used and the feed was sprayed into the drying chamber using a two-fluid nozzle. The spray drying parameters were held constant during the experiments: inlet temperature of 135 °C, and feed rate of 25% (∼7.5 ml/min), resulting in an outlet temperature of approximately 55–60 °C. The spraying air flow was set at 831 l/h and the aspirator at 80%. The spray-dried powder was collected from a single cyclone separator. The samples were stored at room temperature or at 4°C upon use.

2.2.8 Statistics

Data are represented as mean values ± standard deviation. One- and two-way ANOVA and t-tests were used to determine statistical significance. Differences were considered statistically significant at $P<0.05$.

2.3 RESULTS

2.3.1 Set-up of a screening platform to explore direct antimicrobial activities of lactic acid bacteria against the main AOM pathogens

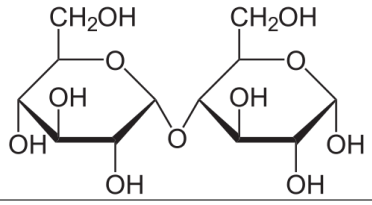
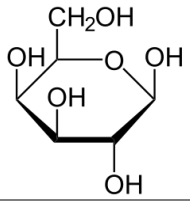
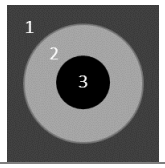
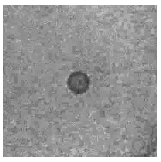
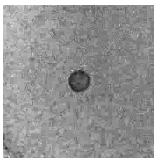
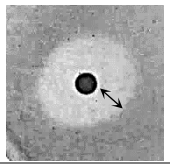
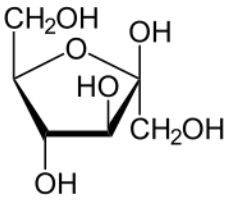
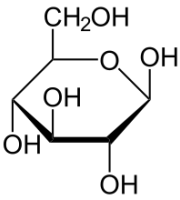
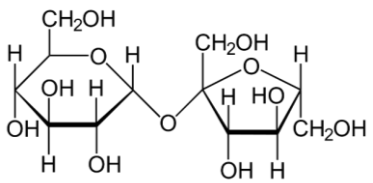
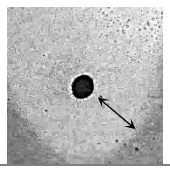
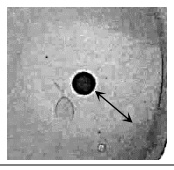
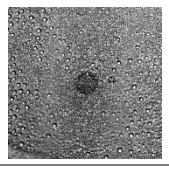
2.3.1.1 Determination of optimal media for agar-based assays

Agar-based assays for the interaction between lactobacilli and *M. catarrhalis* were first done with two media: BHI and MH. Better growth of *M. catarrhalis* was observed for MH so this medium was further used. Agar assays for *S. pneumoniae* and *H. influenzae*, however, showed to be more complicated. These pathogens normally need blood for optimal growth, but the blood-based agar showed to transform to opaque agar after contact with the SCS of the lactobacilli tested. This made it difficult to disentangle and measure the inhibition zones. For this reason, first different concentrations of blood and other media were explored. Finally, MH agar plates enriched with 0.5% yeast extract, 15 mg/L hemin and 15 mg/L NAD (Sigma Aldrich) were used for *H. influenzae.* Todd Hewitt agar plates (Sigma Aldrich) seemed to work best for *S. pneumoniae* and were chosen for further assays.

In the streak line and spot assays, lactobacilli were first grown on MRS plates before adding the AOM pathogen. However, MRS inhibited the growth of the pathogens and thus was not the

optimal medium for these assays. Therefore, the capacity of lactobacilli to grow on the pathogen's specific growth media was tested. Interestingly, lactobacilli could grow on all the media but they could not produce inhibition zones, without addition of a sugar source. In contrast, inhibition zones could be produced after addition of several sugars (to total final concentration of 5 g/L), as shown in Table 2.2 for *L. rhamnosus* GG. Addition of galactose, fructose or glucose, resulted in clear inhibition zones against *M. catarrhalis*. As glucose is the sugar component in MRS, this supplement was chosen for the follow up experiment to enhance optimal growth and metabolite production.

Table 2.2: Impact of different sugar supplements on inhibition zone against M. catarrhalis*. Legend: 1: pathogen grown on agar, 2: inhibition zone (indicated by arrows), 3:* L. rhamnosus GG *spot.*

Legend	No sugar	Maltose	Galactose
			
			
Fructose	**Glucose**	**Sucrose**	
			
			

In the beginning of the inhibition assays, standard, round 69.4 cm² petri dishes (see §2.2.3 and §2.2.4 for more details) were used for the spot and radial diffusion antimicrobial interaction assays. To make the screening more high throughput, we changed to 144 cm² square petri dishes (see $2.2.3 and §2.2.4 for more details) with the same concentration of pathogen used.

2.3.1.2 Time-course analysis of the activity of spent culture supernatant of LAB against (A)OM pathogens grown under planktonic conditions

In addition to agar-based antimicrobial interaction studies, we also wanted to explore the antimicrobial activity of spent culture supernatant of the lactobacilli on the growth of AOM pathogens under planktonic growth conditions. To optimise such assays under liquid conditions, different inocula (10^3-10^6 CFU/mL) of *M. catarrhalis* were first grown in their standard growth medium (*i.e.* Mueller Hinton broth) and their growth was monitored for 168 h (7 days). As shown in Figure 2.2A for OD measurements, all concentrations give rise to well-established growth curve and 10^5 CFU/mL was for further experiments. Because the OD measurements do not allow a discrimination between the lactic acid bacterium and pathogen added to the liquid broth (or any other species added), we could only explore the activity of the antimicrobial compounds produced in the broth during a specified growth period – termed here as spent culture supernatant (SCS). To minimise the influence of the plain MRS medium on the growth of *M. catarrhalis*, different amounts (20%, 10% and 5% of the total volume) of MRS medium (*i.e.* the standard growth medium of the lactobacilli) were added to the inoculum of the pathogen (Figure 2.2B). Addition of 20% MRS broth resulted in a total inhibition of *M. catarrhalis* in Mueller Hinton medium, while adding 10% MRS broth delayed the growth with ca. 24 h. Only addition of 5% did not inhibit or delay the growth of *M. catarrhalis*. Hexetidine, a commercial oral antiseptic, was chosen as positive control, because its well documented activity as antimicrobial compound. All concentrations tested were able to inhibit *M. catarrhalis* for 7 days (Figure 2.2C). A concentration of 0.005% hexetidine was chosen for further experiments, since this correlated with the added volume for the MRS control (*i.e.* 5% of 0.1% hexetidine).

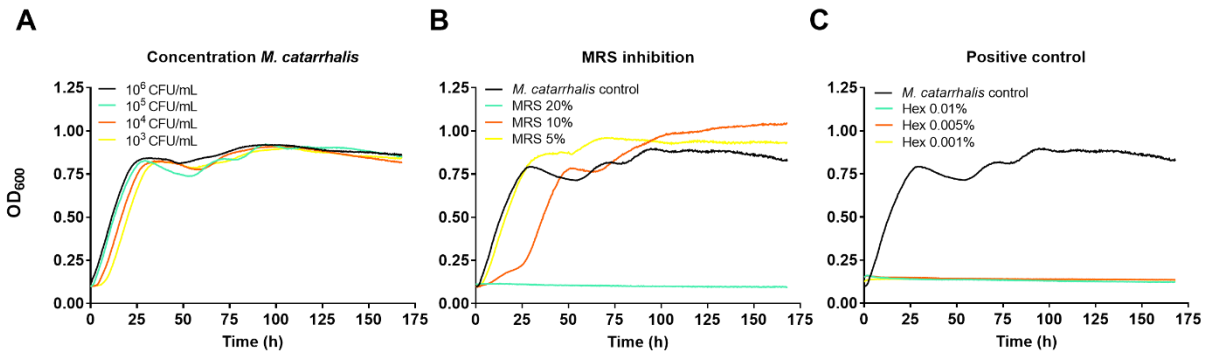


Figure 2.2: Optimisation of set-ups to explore the antimicrobial activity against M. catarrhalis ***under planktonic conditions.*** *(A) Four different starter concentrations (10^3-10^6 CFU/mL) of* M. catarrhalis *were monitored during growth of 168 h. (B) Influence of different amounts of plain MRS (20, 10 and 5%) on the growth curve of* M. catarrhalis*. (C) Effect of different concentrations of the positive control, hexetidine (hex) on the growth of* M. catarrhalis*.*

2.3.1.3 Activity of spent culture supernatant of LAB against (A)OM pathogen grown in biofilms

Since OM pathogens can grow as biofilms in the middle ear (Gu *et al.*, 2014; Hall-Stoodley *et al.*, 2006), we also aimed to develop and optimise assays to explore the antimicrobial activity of the LAB under biofilm conditions. As with the time-course analysis assay, crystal violet staining does not allow discrimination between the lactic acid bacterium and pathogen added to the liquid broth (or any other species added). Therefore, we could only explore the activity of the SCS.

In the first experiments, we used normal, flat bottom 24 or 96 well plates and 96-well PEG plates (Calgary Biofilm device) to grow biofilms of the (A)OM pathogens, starting with *M. catarrhalis*. However, *M. catarrhalis* cells could not be grown to a confluent biofilm under these conditions In contrast, *M. catarrhalis* could form a confluent biofilm on the walls of round bottom 96-well plates, as monitored by crystal violet staining (OD_{570} measurements). Since many bacteria form more biofilms under nutrient-poor conditions (Donlan and Costerton, 2002), both BHI medium as such, as well as a 1:10 diluted version were tested for optimal biofilm growth, with the richer growth medium promoting more biofilm growth (Figure 2.3A). In addition, two different inocula of starting material of *Moraxella* were explored with 10^6 CFU/ml resulting in more biofilm formation than 10^5 CFU/ml (Figure 2.3A). Also for *H. influenzae* and *S. pneumoniae,* similar assays were performed to optimise the initial concentration for overnight biofilm growth in a 5% CO_2 and 37°C environment (Figure 2.3B and C). Starting concentrations of 10^6 CFU/mL showed slightly better biofilm formation while non-diluted

medium was the most effective. The blank in the experiments with *S. pneumoniae*, however, showed the same absorbance (OD_{570}) as the inoculated wells, suggesting that no biofilms were formed.

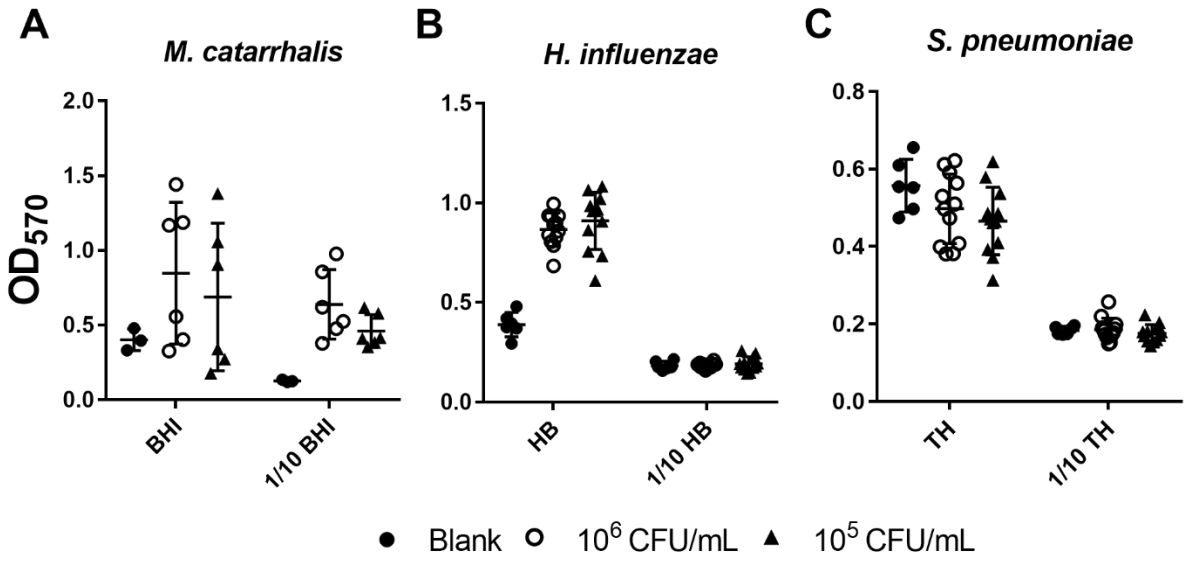


Figure 2.3: Optimisation optimal initial concentration of (A) M. catarrhalis*, (B)* H. influenzae*, and (C)* S. pneumoniae *biofilms. Two concentrations were tested: 10^6 and 10^5 CFU/mL. A blank was included to test for unspecific crystal violet staining due to the medium.*

Because still quite some variability in the biofilm formation capacity was observed, the staining procedure was further adapted. During staining, wells with biofilms are washed to avoid staining of non-bacterial particles and unattached cells. Unfortunately, this first washing step also caused a lot of the biofilm to detach. For this reason, we checked two fixating methods to protect the biofilms: 90 min at 60°C and the addition of 200 µL methanol. As shown in Figure 2.4, the exposure to heat increased biofilm stability significantly both for *M. catarrhalis* and *H. influenzae* biofilms (adjusted P-values 0.0019 and <0.0001, respectively). The blank controls in the biofilm assays of *S. pneumoniae* gave similar OD_{570} values as the inoculated wells, indicating *S. pneumoniae* did not form a proper biofilm (adjusted P–values 0.41, 0.97 and 0.23 for the control, heat and methanol treatments, respectively). Biofilm assays were repeated with the addition of several concentrations of horse blood to the TH medium, however no clear biofilm formation of *S. pneumoniae* could be observed.

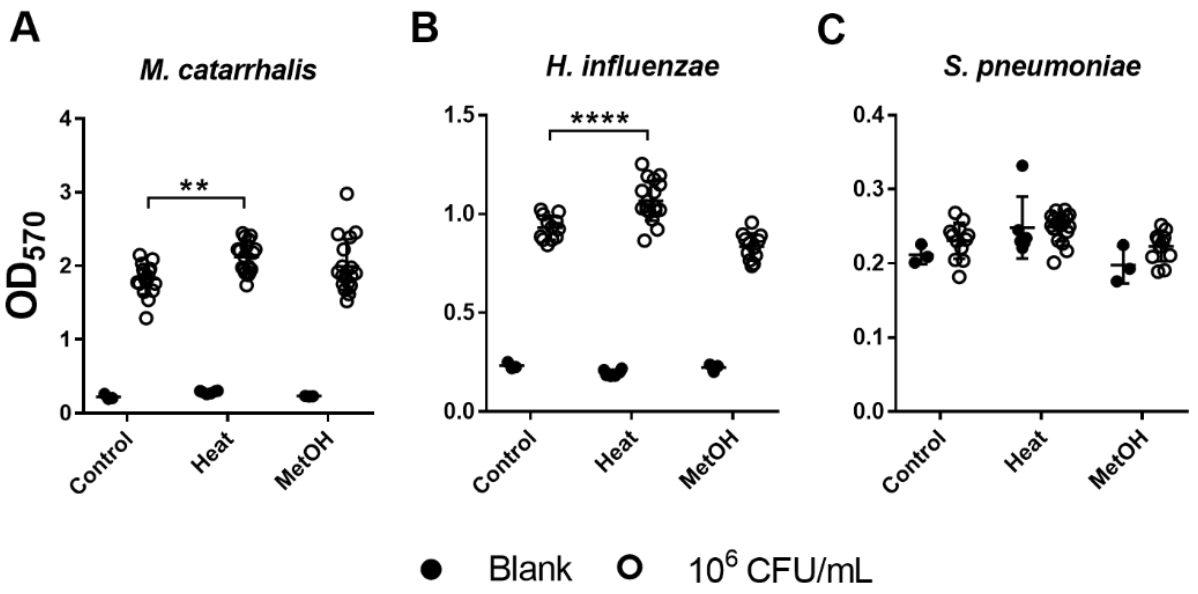


Figure 2.4: Comparing fixation methods of OM pathogen biofilms. 90 min on 60°C (Heat) or the addition of methanol (MetOH) for (A) M. catarrhalis*; (B)* H. influenzae *and (C)* S. pneumoniae *biofilms*

We further focused on *M. catarrhalis*. Different media (BHI, 1:20 BHI and BHI + 15 mg/L hemin), different concentrations of the pathogen inoculum, different growth conditions and different incubation times were tested to increase biofilm formation. As shown in Figure 2.5, an increased starting concentration of 10^7 CFU/mL overall showed better biofilms while increasing the incubation time also increased the variation between the technical repeats. Biofilms grown in 1:20 BHI medium, on the other hand, always showed stable results with the highest pathogen concentration. When comparing the means and standard deviations of the different growth conditions for overnight incubation with diluted BHI, incubation at 37°C showed the highest absorbance while incubation at 26°C showed the least variation (SD of 0.07 compared with 0.12 and 0.11 for CO_2 and 37°C, respectively). For this reasons, we chose to incubate 96-well plates in further experiments in diluted BHI for 19h at 37°C.

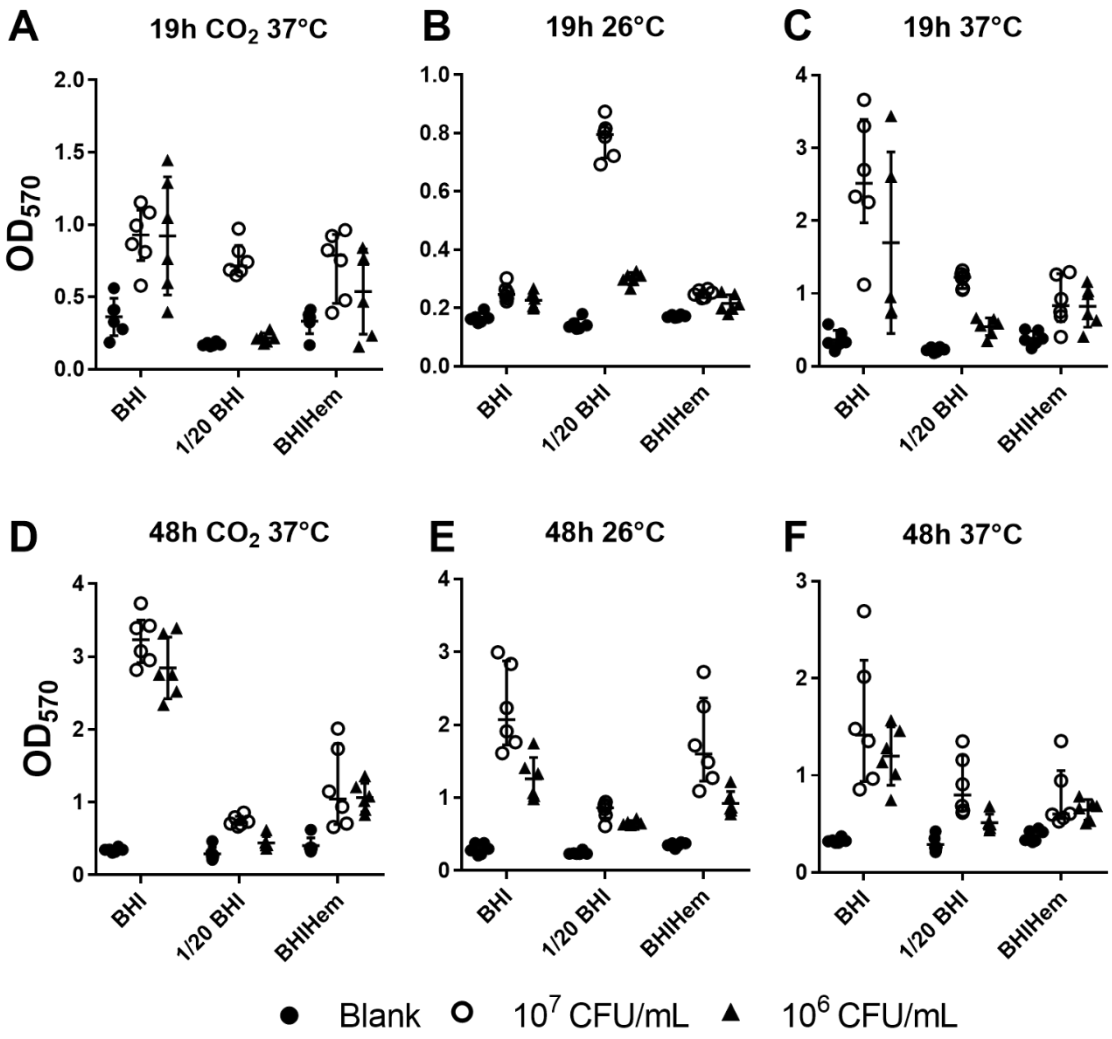


Figure 2.5: Optimisation biofilm set-up for M. catarrhalis*. Two initial concentrations of* M. catarrhalis *(10^7 and 10^6 CFU/mL) were grown overnight (A-B-C) or during 48h (D-E-F) to form biofilms. Different environments were tested: 5% CO_2 in 37°C (A and D), 26°C (B and E) and 37°C (C and F). A blank was included to test for unspecific crystal violet staining due to the medium.*

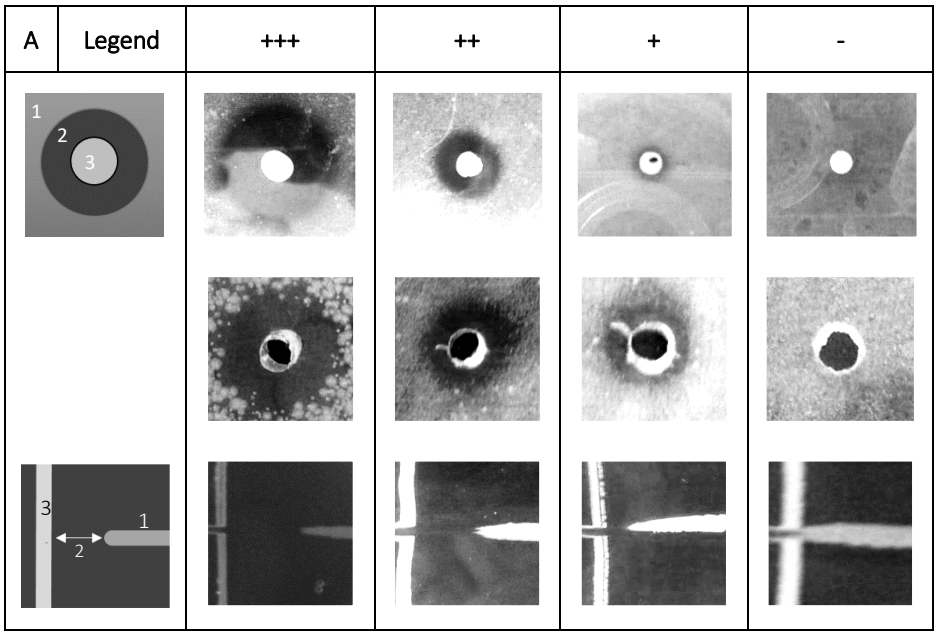
2.3.2 Several *Lactobacillus* strains show a direct antimicrobial activity against *M. catarrhalis* in agar-based, planktonic and biofilm growth conditions

Having optimised various assays for the direct interaction studies between lactobacilli and OM pathogens, we subsequently aimed to screen the activity of a selection of *Lactobacillus* strains (Table 2.1). The selection of the *Lactobacillus* strains was made on the availability in house, but included several model probiotics strains, as well as (in Belgium) commercially available strains, such as *L. rhamnosus* GG (various products available in Flanders such as Vifit from FrieslandCampina, *L. casei* Shirota (Yakult), *L. casei* DN 114001 (Actimel from Danone) and *L. plantarum* WCFS1 (recently commercially available through Winclove). The direct interaction

between these living lactobacilli and *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* was first explored via the agar-based antimicrobial assays optimised in paragraph 2.3.1.1, including the streak-line inoculation and spot assay. Streak-line inoculation assays indicated some inhibitory activity of several lactobacilli against all pathogens tested, but they were difficult to reproduce. In the spot assays, all lactobacilli tested demonstrated a strong activity against *M. catarrhalis,* with a clear, but less pronounced, activity against *H. influenzae* and *S. pneumoniae.* Interestingly, several commercially available strains, such as *L. rhamnosus* GG, *L. casei* Shirota and *L. casei* DN-114001, *L. plantarum* LMG1284 and *L. plantarum* WCFS1 were active with clear inhibition zones in the range of the positive control 0.1% hexetidine (Table 2.2). As a control, non-*Lactobacillus* microbial species such as *E. coli* and the pathogens themself were also included, but they expressed no activity against *M. catarrhalis*.

Subsequently, the activities of the secreted metabolites of the best performing *Lactobacillus* strains were explored by testing the antimicrobial activity of their respective SCS in agar-based well diffusion assays. The SCS of almost all *Lactobacillus* strains tested showed a strong activity, with inhibition zones larger than 0.5 cm against *M. catarrhalis* (Table 2.3). Several lactobacilli were also active with inhibition zones up to 0.2 cm against *H. influenzae* and *S. pneumoniae.* Of note, not all *Lactobacillus* strains tested exhibited the same antimicrobial activity. For instance, the model probiotic strain *L. rhamnosus* GG generated clearly larger inhibition zones against all pathogens tested than the fermented-food isolate *L. parabuchneri* AB17.

Table 2.3: Agar-based antimicrobial screening of the interaction between lactobacilli and (A)OM pathogens*. (A) examples of inhibition zones in radial diffusion test. Legend: 1: grown pathogen, 2: inhibition zone, 3: empty well or Lactobacillus spot/streak line (B) inhibition zones of various* Lactobacillus *strains against the three OM pathogens tested in three different assays. All experiments were executed at least in triplicate and the median is shown in the table. Legend: - : no inhibition, +: 0-0,2 cm inhibition; ++: 0,2-0,5 cm inhibition; +++: > 0,5 cm: inhibition. Abbreviations: HI:* H. influenzae *ATCC49247 ; MC:* M. catarrhalis *ATCC25238; SPN:* S. pneumoniae *ATCC49619. N.a.: not applicable.*



B		Spot assay			Radial diffusion assay			Streak line assay		
Species	Strain	HI	MC	SPN	HI	MC	SPN	HI	MC	SPN
Lactobacillus bulgaricus	AMB-01	n.a.	n.a.	n.a.	-	++	-	+	+++	+++
Lactobacillus casei	ATCC334	++	+++	++	++	++	++	+++	+++	+++
*Lactobacillus casei**	Shirota	++	+++	++	++	+++	++	+++	+++	+++
*Lactobacillus casei**	DN-114001	++	+++	++	++	+++	++	+++	+++	+++
*Lactobacillus casei**	MCJ	+	+++	+	-	++	-	+++	+++	++
Lactobacillus helveticus	AMB-02	-	-	-	-	-	-	-	-	-
Lactobacillus parabuchneri	AB17	+	++	-	-	-	-	++	++	-
Lactobacillus parabuchneri	NM63-3	++	+++	++	-	+	+	+++	+++	-
Lactobacillus paracasei	LMG12586	+++	+++	++	++	++	++	++	+++	+++
Lactobacillus pentosus	KCA1	-	+++	++	++	+++	++	++	++	+++
Lactobacillus plantarum	LMG1284	++	+++	++	++	+++	++	+++	+++	+++
Lactobacillus plantarum	CMPG5300	++	+++	++	++	+++	++	++	+++	+++
Lactobacillus plantarum	5057	+++	+++	++	++	+++	++	++	++	+++

Lactobacillus plantarum	WCFS1	+++	+++	++	++	+++	++	++	+++	+++
Lactobacillus reuteri	RC14	++	+++	++	++	+++	+	+++	+++	+++
Lactobacillus rhamnosus	GR-1	+++	+++	++	++	+++	++	++	++	+++
Lactobacillus rhamnosus	GG	+++	+++	++	++	+++	++	+++	+++	+++
Escherichia coli	LMG2093	-	+	+	-	-	-	n.a.	n.a.	n.a.
Streptococcus pneumoniae	ATCC49619	-	-	-	-	-	-	n.a.	n.a.	-
Moraxella catarrhalis	ATCC25238	-	-	-	-	-	-	-	n.a.	n.a.
Haemophilus influenzae	ATCC49247	-	-	-	-	-	-	n.a.	-	n.a.
0.1% hexetidine		n.a.	n.a.	n.a.	++	+++	++	n.a.	n.a.	n.a.

***** According to a recent large-scale comparative genomics study in our lab by Wuyts *et al.* (2017), these strains classify actually in the *L. paracasei* clade of the *L. casei* group.

2.3.3 Extension of the antimicrobial assays to explore the impact of probiotic formulation: example of *L. rhamnosus* GG and GR-1 after spray drying

In parallel with the screening of the *Lactobacillus* strains with the most promising antimicrobial activity against the (A)OM pathogens, formulation experiments were carried out in collaboration with the lab of prof. Filip Kiekens (Department of Pharmaceutical Sciences, UAntwerpen), to explore preservation and formulation of the lactobacilli in application forms that are more suitable for the upper respiratory tract (*e.g.* finer powder particles) than the current orogastro-intestinal dosage forms for probiotics. Therefore, spray drying is explored in the PhD project of Geraldine Broeckx and the IWT-SBO ProCure project in which both Prof. Lebeer and Prof. Kiekens are participating (www.procureproject.be). Since currently, mainly preservation of bacterial viability after spray drying is monitored (see Broeckx *et al.*, 2017), here, we explored whether the spray drying does impact on the antimicrobial activity of the lactobacilli. These experiments were done because the possibility to screen the functional activity of different probiotic formulations is an example of a validation of the above optimised direct interaction studies and because the antimicrobial activity of the probiotics is postulated to be crucial for later clinical effectiveness. *L. rhamnosus* GG and *L. rhamnosus* GR-1 were spray dried as such or in addition of trehalose or lactose. Dried *Lactobacillus* powder was stored at room temperature or at 4°C. The powder was resuscitated in 0.85 % NaCl and a spot assay was performed with the three (A)OM pathogens. Interestingly, the two lactobacilli tested remained active against especially *M. catarrhalis* (Table 2.4). Trehalose seemed to have a protective effect during the drying and storage process at room temperature.

Table 2.4: Impact of spray drying on the antimicrobial activity of lactobacilli against OM pathogens. *RT: room temperature; HI:* H. influenzae *ATCC49247; MC:* M. catarrhalis *ATCC25238; SPN:* S. pneumoniae *ATCC49619*

Species	Spray drying condition	Storage time	Storage conditions	HI	MC	SPN
L. rhamnosus GG	As such	1.5 years	RT	-	-	-
		1 weekend	4 °C	+	++	-
	1:7 trehalose	1.5 years	RT	-	+	-
		1 weekend	4 °C	+	++	-
	1:2 lactose	1 weekend	4 °C	-	++	-
L. rhamnosus GR-1	As such	1 weekend	4 °C	+	++	-
	1:2 trehalose	1 weekend	4 °C	-	++	-
	1:2 lactose	1 weekend	4 °C	+	++	-

2.3.4 Spent culture supernatant of several lactobacilli has a bacteriostatic antimicrobial activity of against *M. catarrhalis* in planktonic and biofilm growth conditions

Since the antimicrobial activity of the *Lactobacillus* strains discussed above was most remarkable for *M. catarrhalis,* and since – to the best of our knowledge – the interaction between lactobacilli and this pathogen was not yet explored in detail, we decided to focus on this interaction for further mechanistic studies. First, we performed a time-course analysis of the antimicrobial activity of SCS and secreted metabolites of different *Lactobacillus* strains. A prominent strain-specific effect of *Lactobacillus* SCS against *M. catarrhalis* was observed (Figure 2.6A). Remarkably, addition of the SCS of the model probiotic strain *L. rhamnosus* GG inhibited the growth of *Moraxella* for 72h 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 2.6A). The negative control, non-inoculated MRS medium brought to pH 4.3, only produced a small delay in growth, indicating that the effect is not merely pH related. Subsequent survival and plating assays indicated that the effect of SCS on the growth of *M. catarrhalis* was bacteriostatic (data not shown). In addition, we explored the capacity of *Lactobacillus* SCS to prevent biofilm formation using the optimised assays described previously. As shown in Figure 2.6B, SCS of *L. rhamnosus* GG could reduce the biofilm formation of *M. catarrhalis* by up to 70% under the tested conditions (adjusted P-value: 0.0001). Various other

Lactobacillus strains tested such as *L. rhamnosus* GR-1 and *L. plantarum* CMPG5300 also showed the potential to reduce biofilm formation (adjusted P-values: 0.0081 and 0.0158, respectively), but their activity appeared less reproducible. For the strain *L. casei* ATCC334, we could not observe a significant reduction under the tested conditions.

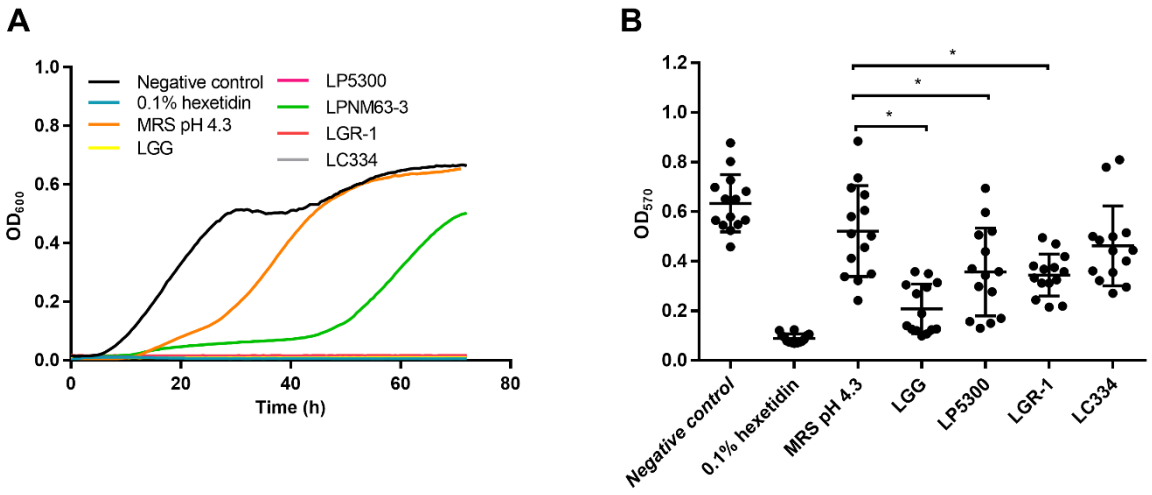


Figure 2.6: Antimicrobial effect for planktonic growth and antibiofilm effect of Lactobacillus ***SCS against*** M. catarrhalis. *(a) The effect of SCS of different* Lactobacillus *strains on the growth of* M. catarrhalis *in planktonic phase was measured by continuous monitoring the OD at 600 nm for 72h. Non-inoculated MRS medium and 0,1% hexetidine were used as negative and positive control, respectively. (b) The effect of SCS of different* Lactobacillus *strains on the growth of* M. catarrhalis *as biofilms for 19 h was measured by crystal violet staining (OD_{570}).*

2.4 DISCUSSION

In this chapter, we have shown for the first time – to the best of our knowledge- detailed data supporting a direct antimicrobial role for LAB against common (A)OM pathogens. Hereto, several in-house protocols and pipelines were optimised to allow the study of this interaction between LAB and (A)OM pathogens. Incubation times and environments, culture media, material to make holes in agar, size of petri dishes and related growing methods were all investigated and optimised. Furthermore, by doing a research stay in the lab of prof. Nizet at the University of California San Diego (http://nizetlab.ucsd.edu/), a lab with an enormous expertise with several pathogens such as streptococci and *Haemophilus* spp., additional important insights in pathogenic growth and antimicrobial assays were acquired. Currently, the screening platform is up-and-running, used by several other colleagues and available for fee-

for-service collaborations with industry. The assays have also been further extended towards pathogens from other human niches such as the skin and vagina, although it is not always straightforward to translate due to different growth condition conditions for each pathogen.

As mentioned before, spray drying results in powder characteristics suitable for nasal applications of probiotics (also reviewed in Broeckx *et al.*, 2016). As an example of the applicability of our antimicrobial screening platform, we explored the impact of probiotic formulation on probiotic functionality (*i.e.* antimicrobial activity), as a parameter to monitor. Here, we explored whether the antimicrobial activity of *L. rhamnosus* GG and *L. rhamnosus* GR-1, remained present after spray drying. Even after storage for 1.5 years at room temperature, inhibition zones against *M. catarrhalis* appeared in spot assays. The enhancement of the viability of *L. rhamnosus* GG after spray drying and during storage is recently described in Broeckx *et al.* (2017) (with co-authorship of Marianne van den Broek). This research paper describes more in detail that addition of lactose or trehalose in the spray drying medium enhances the viability of *L. rhamnosus* and its long time storage capacity. These are important findings as incorporation of probiotic species as live bacteria in nasopharyngeal sprays or related URT formulation strategies will largely depend on the success of drying techniques that preserve their long term viability and activity, as recently reviewed (Broeckx *et al.*, 2016).

One drawback of the current screening platform is the lack of methods to screen interaction between the two types of living bacterial cells (probiotic and pathogen) in the planktonic and biofilm assays. Since OD and crystal violet staining measurements do not discriminate between two different strains/species of bacterial cells, there is thus a need for differential or fluorescent labelling of several bacteria of interest to explore these 1:1 interactions. Recently, another PhD student associated to our group, Irina Spacova succeeded in the fluorescent labelling of several *L. rhamnosus* strains with different fluorescent colours such as mCHERRY (red) and mTagBFP2 (blue) (Spacova *et al.*, 2017). These strains were then used in biofilm interaction studies between the lactobacilli and green-fluorescently labelled *Salmonella* Typhimurium where a reduction of pathogenic biofilm was observed. These experiments pave the way for similar interaction studies of lactobacilli and *Moraxella*.

Nevertheless, the screening platform optimised in this PhD work could deliver reproducible and convincing data for the potential of *Lactobacillus* strains to inhibit the growth of (A)OM pathogens in suspension, on agar and as biofilm, especially that of the gram-negative pathogen

M. catarrhalis. Both actively growing lactobacilli and their secreted metabolites in SCS showed a clear antimicrobial activity in agar-based assays. The presence of fermentable sugars appeared to be essential for the lactobacilli to inhibit pathogens in the spot assay. Of the monosaccharides tested, fructose, glucose and galactose showed to function as adequate carbon sources for antimicrobial molecule production. Indeed, Kankainen *et al.* (2009) reported successful utilisation and metabolisation/fermentation via the EMP pathway of these sugars by *L. rhamnosus* GG. Under our tested conditions, the less- or non- fermentable disaccharides (maltose and sucrose) showed to be non-optimal for *L. rhamnosus* GG to produce antimicrobial compounds. As investigated by Kankainen *et al.* (2009), sucrose can be utilised by *L. rhamnosus* GG, but its fermentation to lactic acid is less efficient than for glucose. Maltose cannot be utilised by *L. rhamnosus* GG (Kankainen *et al.*, 2009). The question about which molecules in the SCS are responsible for the antimicrobial activity is further explored in next chapter.

3 LACTIC ACID AS MAJOR ANTIMICROBIAL COMPOUND OF LACTIC ACID BACTERIA AGAINST (A)OM PATHOGENS

Part of this chapter is accepted for publication: van den Broek, M. F. L., De Boeck, I., Claes, I. J. J., Nizet, V. and Lebeer, S. 2017. Multifactorial inhibition of lactobacilli against the respiratory tract pathogen Moraxella catarrhalis. Beneficial microbes. Accepted for publication (Reference: BM-2017-07-0101.R2).

3.1 INTRODUCTION

As shown in Chapter 2, several lactobacilli – and their spent culture supernatant- showed promising antimicrobial activities against the (A)OM pathogens *M. catarrhalis, H. influenzae* and *S. pneumoniae* in direct inhibition assays. In this chapter, we aimed to identify the main antimicrobial compound(s) expressed by the lactobacilli tested. As yet mentioned in Chapter 1, based on research against gastro-intestinal pathogens, one of the most important and best-documented antimicrobial metabolite of fermentative lactic acid bacteria is lactic acid, which has antimicrobial characteristics against Gram-negative pathogens such as *Salmonella* Typhimurium by permeabilising the membrane (Alakomi *et al.*, 2005; De Keersmaecker *et al.*, 2006; Sun and O'Riordan, 2013). In addition, many lactobacilli such as several *L. plantarum* strains produce bacteriocins (*e.g.* plantaricin) (Gonzalez *et al.*, 1994). These molecules comprise a huge family of ribosomally synthesised peptides that have antibacterial activity towards, mostly, closely related strains (Perez *et al.*, 2014b). The bacteriocins produced by lactobacilli are mostly small, heat-stable proteins with a high isoelectric point (also called class II bacteriocins) that cause membrane permeabilisation and subsequent leakage of molecules from target bacteria (Eijsink *et al.*, 2002). Other non-bacteriocin proteins such as reuterin, produced by *L. reuteri*, affect a more broad range of bacteria such as *E. coli* and *Listeria monocytogenes* and is a topic of study in food preservation (Langa *et al.*, 2018). Furthermore, lectin-like-proteins Llp1 and Llp2 of *L. rhamnosus* GG and *L. rhamnosus* GR-1 have been demonstrated by our group to inhibit pathogenic biofilm formation of the model gastro-intestinal and urogenital pathogen *E. coli* and *S.* Typhimurium (Petrova *et al.*, 2016a, 2016b) and they could thus also be of relevance in direct inhibition studies.

In this chapter, spent culture supernatant of several lactobacilli was treated by various approaches to investigate possible loss (or enhancement) of the antimicrobial activity. More in-depth-analysis towards specific *Lactobacillus* effector molecules and their effects on the cell wall of *M. catarrhalis* were also performed.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and growth conditions

Lactobacillus strains (Table 3.1) were grown at 37°C in de Man, Rogosa and Sharpe (MRS) medium (Difco). *M. catarrhalis* ATCC25238 was inoculated in Mueller Hinton (MH) (LabM Limited) broth and cultured aerobically at 37°C. *H. influenzae* ATCC49247 was grown in MH broth enriched with 0,5% yeast extract, 15 mg/L hemin and 15 mg/L NAD (Sigma Aldrich) in 5% CO_2 atmosphere and *S. pneumoniae* in Todd Hewitt broth (Sigma Aldrich) in 5% CO_2 atmosphere. Solid media contained 1.5% (w/v) agar. For antimicrobial assays, the respective media for these pathogens were, when indicated, enriched with glucose (Sigma Aldrich) to a final concentration of 5 g/L. *Lactococcus lactis* MG1363 was grown at 30°C in MH17 broth supplemented with 10 g/L glucose.

Table 3.1: Inventory of bacterial strains used in this chapter.

Species	Strain	Relevant genotype or description	Reference and/or source
		LACTOBACILLI	
Lactobacillus bulgaricus	AMB-1	Single colony isolate from Colombian yoghurt	Yoghurt
Lactobacillus casei	ATCC334	Single colony isolate obtained from a stock culture of ATCC334	ATCC
Lactobacillus casei*	Shirota	Single colony isolate obtained from a commercially available fermented drink containing *L. casei* Shirota (Yakult®), confirmed by sequencing	Commercial probiotic product
Lactobacillus 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	Commercial probiotic product
Lactobacillus casei *	MCJ	Single colony isolate from spontaneously fermented carrot juice	Fermented carrot juice
Lactobacillus helveticus	AMB-2	single colony isolate	Commercial probiotic product
Lactobacillus parabuchneri	AB17	Single colony isolate from spontaneously fermented beet juice	Fermented beet juice
Lactobacillus parabuchneri	NM63-3	Single colony isolate from spontaneously fermented carrot juice	Fermented carrot juice
Lactobacillus paracasei	LMG12586	Single colony isolate obtained from a stock culture of LMG12586	BCCM/LMG
Lactobacillus pentosus	KCA1	Single colony isolate from KCA1 (vaginal origin)	(Anukam *et al.*, 2013)

Lactobacillus plantarum	LMG1284	Single colony isolate from *L. plantarum* ATCC8014 or LMG1284	BCCM/LMG
Lactobacillus plantarum	CMPG5300	Single colony isolate (vaginal origin)	(Malik *et al.*, 2014)
Lactobacillus plantarum	5057	Single colony isolate of *L. plantarum* 5057	(Danielsen, 2002)
Lactobacillus plantarum	WCFS1	Single colony isolate of *L. plantarum* WCFS1	(Kleerebezem *et al.*, 2003)
Lactobacillus plantarum	CMPG11201	*msl* mutant of *L. plantarum* CMPG5300, Cm^R	(Malik *et al.*, 2016)
Lactobacillus plantarum	CMPG5376	*srtA* mutant of *L. plantarum* CMPG5300, Cm^R	(Malik *et al.*, 2013)
Lactobacillus reuteri	RC14	Single colony isolate from a commercially available probiotic supplement containing *L. reuteri* RC14, 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 (urethra origin)	(Chan *et al.*, 1984, 1985; Reid and Bruce, 2001; Reid and Reid, 1999), ATCC
Lactobacillus rhamnosus	GG	Single colony isolate of wild-type strain, isolated from human faeces	(Kankainen *et al.*, 2009)

***** According to a recent large-scale comparative genomics study in our lab by Wuyts *et al.* (2017), these strains classify actually in the *L. paracasei* clade of the *L. casei* group.

OTHER

Lactococcus lactis	MG1363	Single colony of the plasmid-free *Lactococcus* strain MG1363	(Wegmann *et al.*, 2007)

PATHOGENS

Haemophilus influenzae	ATCC49247	Single colony isolated from expectorated sputum from a 76 year-old male with pneumonia	ATCC
Moraxella catarrhalis	ATCC25238	Type strain, nasal cavity	ATCC
Streptococcus pneumoniae	ATCC49619	Single colony isolated from sputum of 75-year-old male	ATCC

3.2.2 Preparation of spent culture supernatant of lactobacilli

Lactobacilli were incubated for 19 h to a final concentration of ±2 x 10^9 CFU/mL. Growth was measured using Synergy HTX multimode plate reader (BioTek, Vermont, USA) by measuring OD at 595 nm. Path length correction was applied and Vmax was measured by the corresponding the Gen5 software as the maximum slope (of 4 consecutive points) of the curve. Spent culture supernatant (SCS) was obtained by centrifugation for 15 min at 4000 rpm at 4°C. Afterwards, the SCS was filter sterilised (0.20 µm cellulose acetate, VWR) and the pH (Mettler-Toledo AG)

and the concentration of D- and L-lactic acid (Roche Yellow Line) 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: (i) heating at 70°C for 30 min, (ii) treatment with proteinase K (50 µg/mL) (Sigma-Aldrich) and incubation for 60 min at 37°C, and (iii) neutralisation to pH 7.

3.2.3 Bacteriocin induction by coculture with related bacteria

Induction of bacteriocin production was tested based on Maldonado *et al.* (2004). Briefly, lactobacilli were grown overnight at 37°C in the presence of 0.5% of (i) living or, (ii) heat killed *Lactococcus lactis* MG1363 or (iii) its SCS. Standard antimicrobial assays were performed with the SCS of the (mixed) culture.

3.2.4 Spot and streak line antimicrobial assays with live lactobacilli

These assays were done as described in Chapter 2.

3.2.5 Radial diffusion antimicrobial assay for SCS of lactobacilli

These assays were done as described in Chapter 2.

3.2.6 Time-course analysis of the antimicrobial activity of SCS

These assays were done as described in Chapter 2.

3.2.7 Minimal inhibitory concentration (MIC) and checkerboard assay

The minimal inhibitory concentration (MIC) values were determined based on the procedure described earlier (Nizet *et al.*, 2001). The pathogens were grown in their appropriate media as described above and a concentration of 5×10^6 CFU was made. In triplicate, 10 µL of 10x concentrated active molecule in sterile H_2O, 10 µL of the bacterial suspension and 80 µL of bacterial medium were combined in the wells to obtain a final bacterial concentration of 5×10^5 CFU/mL. The microplate was incubated overnight at 37°C and the OD at 600 nm was measured. The checkerboard assay was done similarly. However, in this assay, the activity of the combination of different concentrations of two molecules was monitored in a 96 well plate. Different concentrations of molecule 1 were put horizontally, while different concentrations of molecule 2 were put vertically.

3.2.8 Biofilm assays

These assays were done as described in Chapter 2.

3.2.9 Scanning electron microscopy

5 µL of a 5 mL overnight culture of *M. catarrhalis*, grown under different conditions, was spotted on a cellulose acetate filter with pore size of 0,45 µm (Sartorius). The membrane was fixated by letting it float on fixative (2.5 % glutaraldehyde in 0.1 M Na^+-cacodylate) for 1 h at room temperature. Then, the membrane was submersed in the same fixative and incubated overnight at 4°C. The fixated membrane was rinsed with 0.1 M Na^+-cacodylate buffer and 7.5% saccharose thrice for 20 min at room temperature. Subsequently, the membrane was dehydrated in an ascending series of ethanol and submitted to critical point drying in a Leica EM CPD030 after which the membranes were mounted on a stub and gold coated in a sputter coater. Scanning electron microscopy imaging was performed with an SEM 515 (Philips, The Netherlands)

3.2.10 LIVE/DEAD stain and fluorescence microscopy

Tubes with bacteria were centrifuged for 1 min at 10000 rpm. Afterwards, the supernatant was discarded and the pellet was washed with 0,1 mL in 0,85% NaCl and centrifuged again for 1 min at 10000 rpm. After discarding the supernatant, the pellet was resuspended in 0,85% NaCl. 0,3 µL of a mixture of equal volumes of SYTO9 (3.34 mM) and propidium iodide (20 mM) (LIVE DEAD BacLight Viability Kit) was added to the suspension and mixed thoroughly. The samples were conserved in aluminum foil to eliminate contact with light and put at 4°C. The samples for the fluorescence microscope (EVOS FL Cell Imaging System, ThermoFisher Scientific) were made by putting 5 µL of the suspension on a glass slide and covering it with glass.

3.2.11 Statistics

Data are represented as mean values ± standard deviation. One-way ANOVA and t-tests were used to determine statistical significance in GraphPad Prism. Differences were considered statistically significant at $P<0.05$.

3.3 RESULTS

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

In a first attempt to identify the nature of the active antimicrobial molecules in the SCS of the lactobacilli, different classes of active molecules in the SCS (with a focus on acids and proteins) were inactivated or removed. We focused on SCS of *L. rhamnosus* GG as our model probiotic strain, because this SCS was very active in the experiments in Chapter 2.

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 neutralizing the pH (Figure 3.1A). Also in the time-course analysis of the effect on planktonic growth of the (A)OM pathogen, 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 major antimicrobial activity (Figure 3.1B). 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 3.1C).

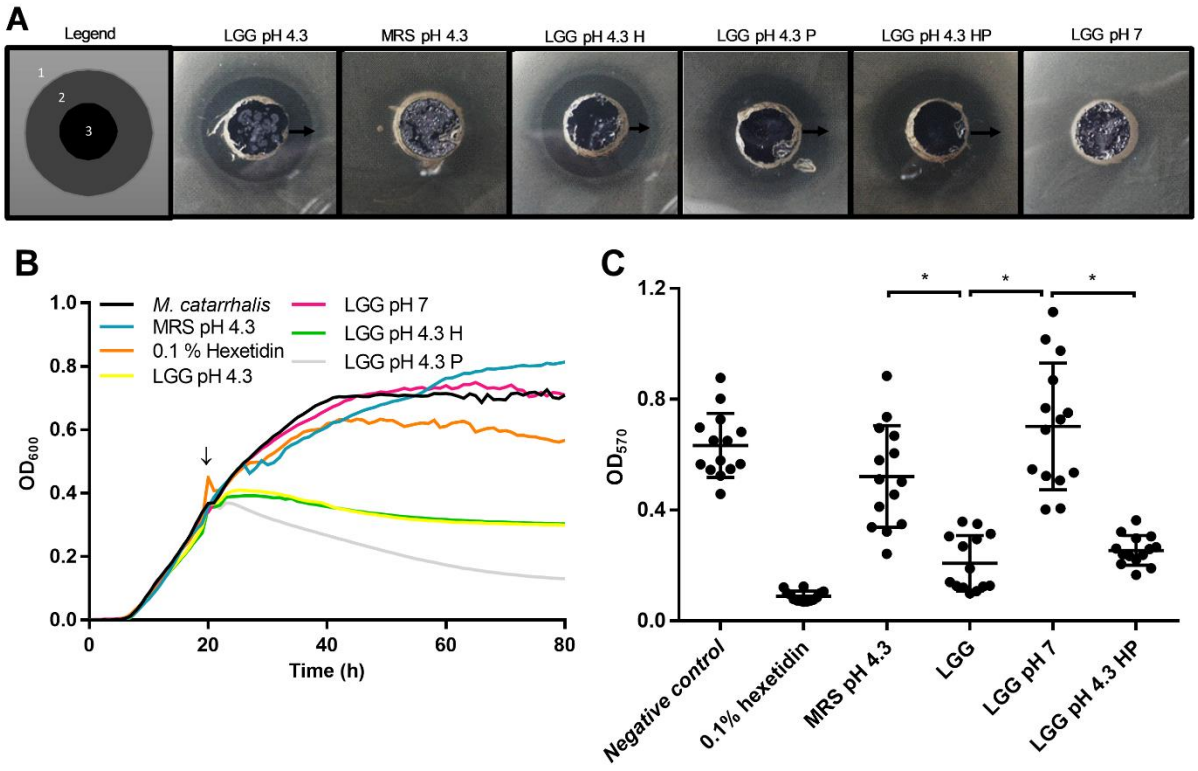


Figure 3.1: Impact of various treatments on the antimicrobial and antibiofilm effect of SCS of L. rhamnosus ***GG against*** M. catarrhalis*. (A) radial-diffusion assay with SCS treated as described in Material and Methods, Legend: 1: growth of pathogen; 2: inhibition halo; 3 well where SCS was added. (B) time-course analysis of* M. catarrhalis*. The arrow indicated 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, and (C) biofilm formation* 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. Significances had an adjusted P-value of <0.0001.*

Although the previous results do not point towards bacteriocins because they are generally not low-pH-sensitive and yet protease-sensitive. Nevertheless, it could also be that the bacteriocins were not expressed under the tested conditions. In fact, it is documented that environmental factors may play an important role in the regulation of bacteriocin production (Brurberg *et al.*, 1997; Risøen *et al.*, 2002). For this reason, we aimed to induce bacteriocin production of lactobacilli by co-incubating them with living, heat killed *L. lactis* MG1363 or its SCS, because Maldonado *et al.* (2004) have described that this related bacterium could induce bacteriocin production in *L. plantarum* NC8 Control and modified (heat or proteinase K) SCS of the (mixed) cultures were then tested in radial diffusion assays with *H. influenzae, M. catarrhalis* and *S. pneumoniae*. Unfortunately, no conclusions could be made based on these results because the inhibition zones were poorly reproducible (data not shown).

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

Since the antimicrobial activity of lactic acid against certain gastro-intestinal pathogens has been previously documented to be pH-dependent (De Keersmaecker *et al.*, 2006), we explored subsequently the role of lactic acid as major *Lactobacillus* metabolite in the inhibition of OM pathogens. 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 3.2). Interestingly, the graph clearly shows that high pH and low lactic acid concentration is linked to decreased activity against OM pathogens. A threshold line was drawn at a concentration of lactic acid of 13.7 g/L and at pH 4. As depicted in the figure, all lactobacilli which showed higher lactic acid production and a lower pH than this threshold, showed a strong antimicrobial activity. *L. bulgaricus* AMB-1, however, produced a high amount of lactic acid and an acid SCS, but exhibited a poor antimicrobial activity under our tested conditions.

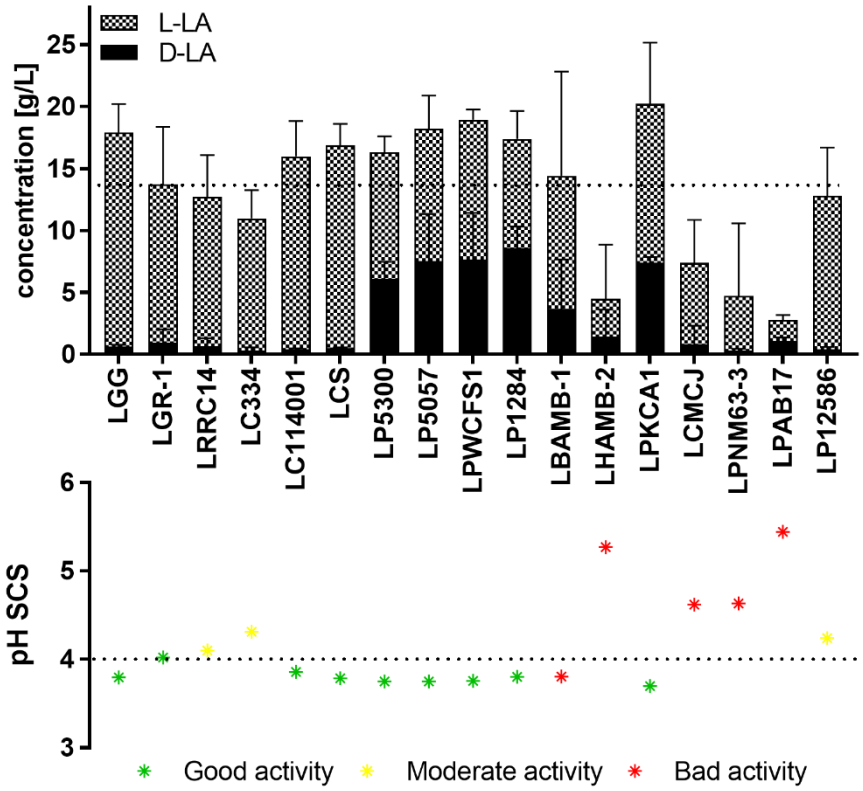


Figure 3.2 pH and concentration of D- and L-lactic of the Lactobacillus ***strains.*** *(A) Concentration of D- and L-LA produced by the* Lactobacillus *strains tested after growth for 19 h in MRS medium. (B) pH (stars) and overall antimicrobial activity (indicated by colour of stars: green: good; yellow: moderate; and red: bad activity) are depicted as well. Names of the lactobacilli can be found in Table 3.1.*

To confirm the antimicrobial activity of D- and L-lactic acid, MIC assays were performed against *M. catarrhalis*, *S. pneumoniae* and *H. influenzae*. Both D- and L-lactic acid showed a strong activity against *M. catarrhalis* with an MIC of ca. 0.5 g/L (Table 3.2). Interestingly, D- and L-lactic acid were almost 10-fold less active against the three pathogens tested when they were dissolved in a neutral solution, confirming the pH-dependent activity of lactic acid. A possible synergistic activity of D-and L-lactic acid was explored via checkerboard assays. However, no consequent synergistic activity was observed (data not shown).

Table 3.2: MIC of lactic acid under different pH conditions for M. catarrhalis, S. pneumoniae *and* H. influenzae. *Upper and lower limit are indicated between brackets LA: lactic acid; D-LA: D-lactic acid; L-LA: L-lactic acid.*

		M. catarrhalis	*H. influenzae*	*S. pneumoniae*
L-LA (g/L)	pH 2	0.5 (0.25- 1.0)	2 (1.0 -4.0)	2 (2.0 -4.0)
	pH 4	2	3 (2.0 - 4.0)	4
	pH 7	25 (20.0-40.0)	16	18 (16.0 -20.0)
D-LA (g/L)	pH 2	0.5	1 (1.0 - 2.0)	2
	pH 4	1 (1.0-2.0)	4 (2.0 - 4.0)	4
	pH 7	13 (2.5 – 20.0)	16 (4.0 - 30.0)	18 (16.0 - 20.0)

In addition, to confirm that an important activity in SCS could be attributed to the presence of lactic acid and not merely because of a low pH, a time course analysis of *M. catarrhalis* planktonic growth in the presence of several MRS combinations was performed. More specifically, MRS brought to pH 4 with L- and/or D-lactic acid (total final concentration of ca. 1.5% which is comparable with that in the SCS of lactobacilli) was compared with MRS brought to pH 4 with HCl and MRS at a neutral pH (Figure 3.3A). Afterwards (at t=70 h), the cultures were stained via a LIVE/DEAD staining and visualised under the fluorescence microscope (Figure 3.3B-G). The growth curves indicate that MRS at pH 4 induced a prolongation of the lag phase but not as extensively as MRS acidified with lactic acid, confirming an important role of lactic acid and not just any acidic mixture. Similarly, with LIVE/DEAD staining, more dead bacteria appeared in the samples treated with MRS supplemented with lactic acid.

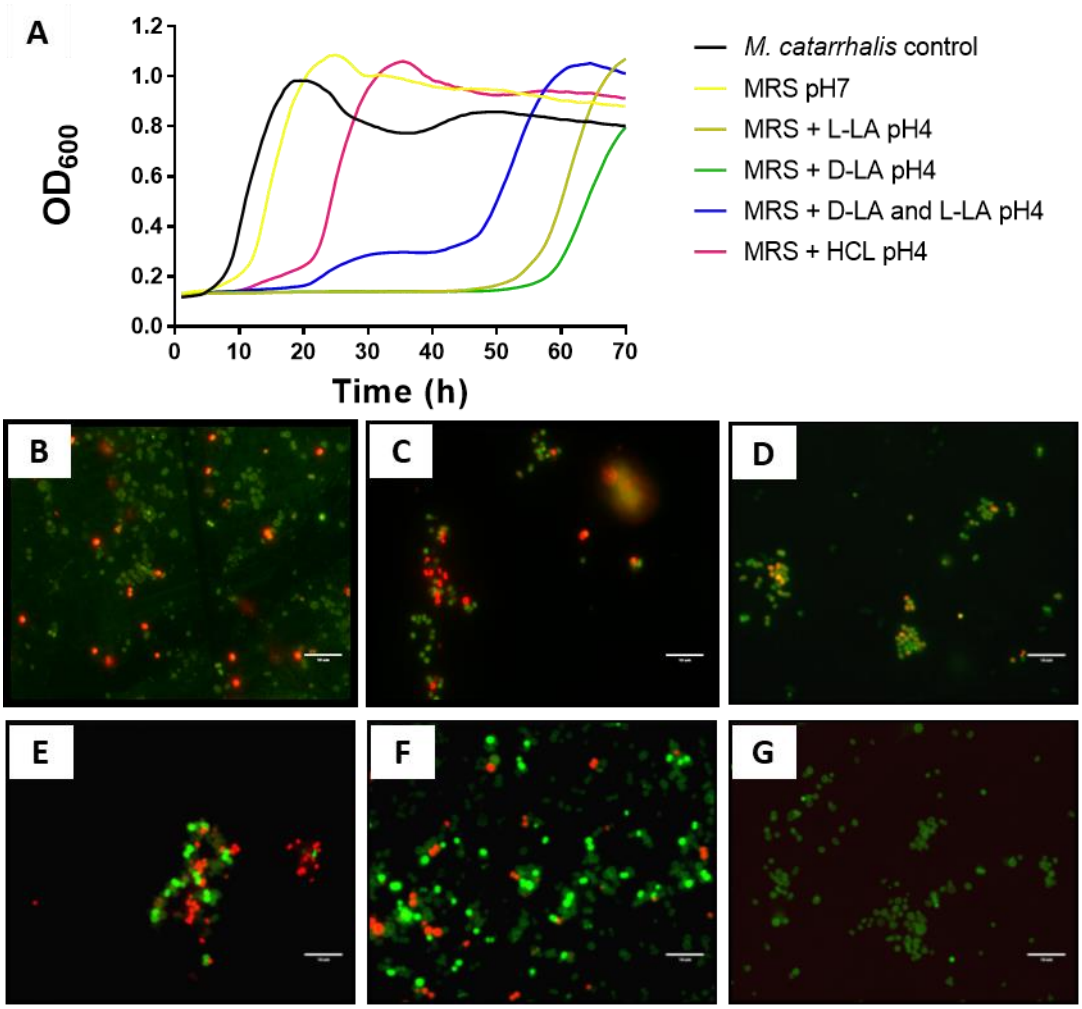


***Figure 3.3: Comparison between MRS brought to pH 4 by lactic acid and HCl and their impact in the growth of* M. catarrhalis.** *(A) Time-course analysis of* M. catarrhalis*. LIVE (green)/DEAD (red) staining after 70 h of incubation of (B)* M. catarrhalis *control, (C) MRS + L-LA pH4, (D) MRS + D-LA pH4, (E) MRS + L- and D-LA pH4, (F) MRS + HCl pH4, and (G) MRS pH7.*

In addition, the effect of D- and L-lactic acid (0.5 g/L and 0.25 g/L) on the growth of biofilms by *M. catarrhalis*, was monitored. Interestingly, only addition of 0.5 g/L D-lactic acid exhibited a significant reduction in biofilm growth (adjusted P-value: 0.0003). Also addition of lactic acid after a biofilm was grown overnight (19 h), did not detach the biofilm completely. Since colleagues were in the timeframe of my PhD project able to show that lectin-like proteins (Llp) can inhibit the biofilm formation of Gram-negative urogenital and gastro-intestinal bacteria such as *Salmonella* and *E. coli* UTI (Petrova *et al.*, 2016a, 2016b), the impact on a starting and grown *Moraxella* biofilm of the major lectin Llp1 of both *L. rhamnosus* GG and GR-1, was investigated as well. A concentration of 50 µg/mL of Llp1 of *L. rhamnosus* GR-1's and 25 µg/mL of *L. rhamnosus GG*'s Llp1 reduced the biofilm formation of *M. catarrhalis* significantly (adjusted P-values: 0.0004 and 0.0003, respectively) (Figure 3.5A,B). Similar as for lactic acid,

no complete breakdown of a developed biofilm could be detected. Of note, combining lactic acid and the Llp1 proteins to explore possible synergistic interactions, did show the opposite: the presence of lactic acid seemed to decrease the ability of Llp1 to inhibit biofilm formation significantly (adjusted P-values: all 0.0001) (Figure 3.5C).

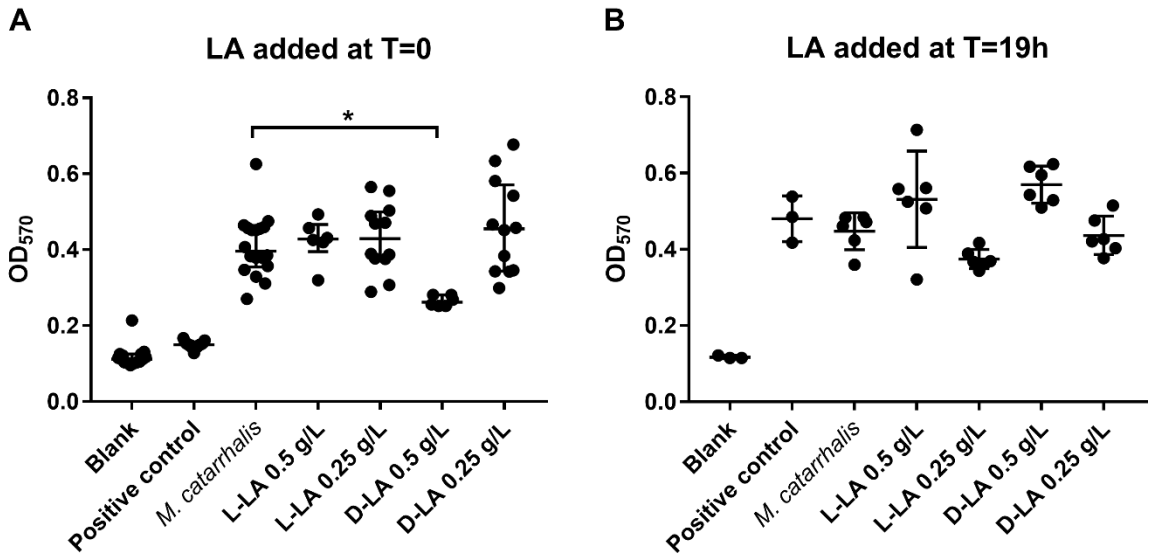


Figure 3.4: Impact of lactic acid on biofilm formation of M. catarrhalis. *The lactic acid was either (A) directly added or (B) after 19h of biofilm growth.*

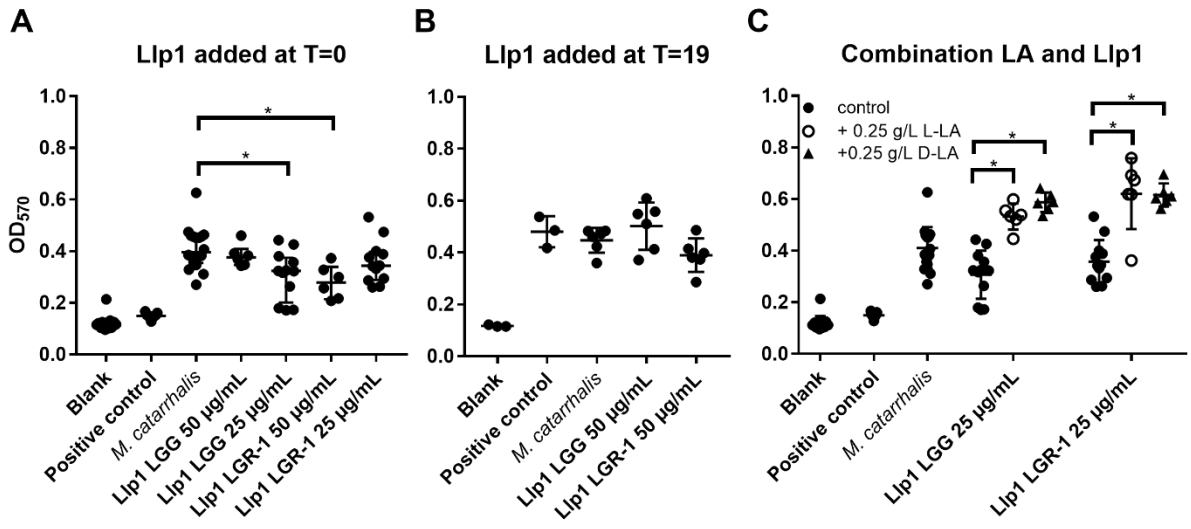


Figure 3.5: Impact of Llp1 separate or in combination with lactic acid on biofilm formation of M. catarrhalis. *The Llp1 was either (A) directly added or (B) after 19h of biofilm growth. (C) Impact of combination of lactic acid and llp1 on biofilm formation of* M. catarrhalis. *Both effector molecules were added at T=0.*

3.3.3 Influence of *Lactobacillus* SCS and lactic acid on the membrane and cell morphology of *M. catarrhalis*

Because the antimicrobial activity of lactic acid is thought to be based on membrane disruption in the target bacteria (Alakomi *et al.*, 2005), we wanted to subsequently investigate the impact of treatment with *Lactobacillus* SCS or lactic acid on the outer and cell membrane of *M. catarrhalis* via scanning electron microscopy (SEM). To optimise the in-house SEM staining procedure of our bacteria in collaboration with Sofie Thys from the Laboratory of Cell Biology and Histology (UAntwerpen), the effect of five different types of sterilisation of the cellulose acetate filter membrane was checked: no autoclavation (blank), wet autoclavation, dry autoclavation, 3h on 160°C and 30 min on 180°C. The mode of sterilization showed to have no clear impact on the staining membrane structure (data not shown). Also, no contamination was observed in the blank. Then, the influence of a poly-L-lysine coating and the amount of bacteria to spot on the membrane was optimised (Figure 3.6). The optimal amount to spot on the membrane was chosen between 10^6 and 10^7 CFU while there was no effect observed of the poly-L-lysine coating.

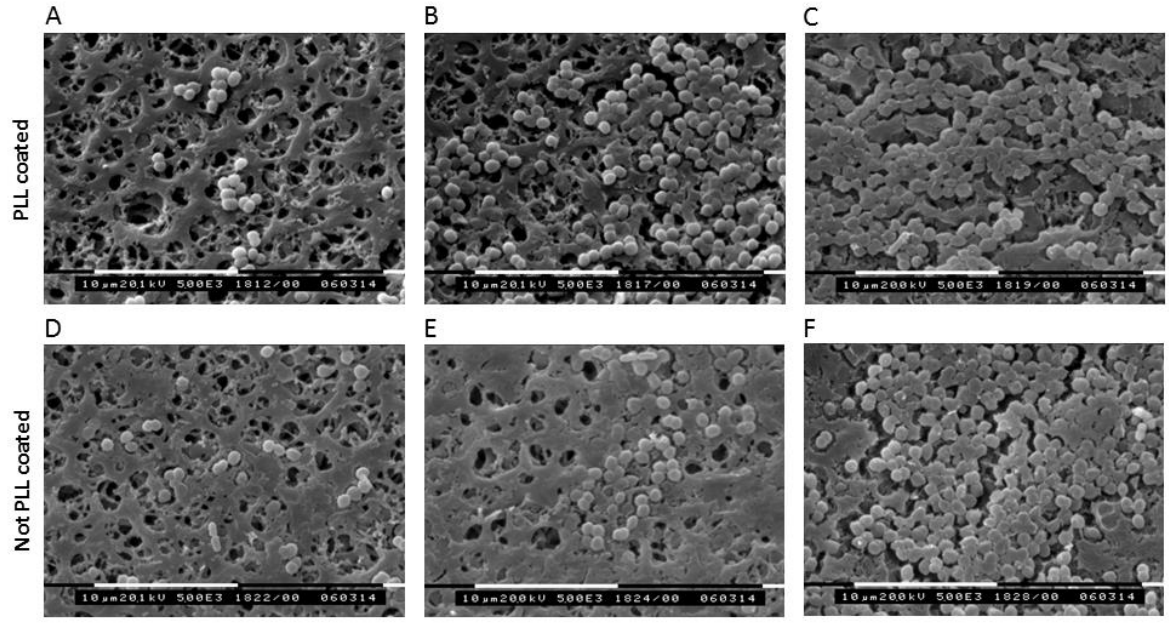


Figure 3.6: Influence of amount of bacteria and PLL coating on SEM visualisation. *(A and D) 10^5 CFU; (B and E) 10^6 CFU; (C and F) 10^7 CFU* M. catarrhalis*. The membrane in the upper row was coated with PLL while the lower row not. PLL: poly-L-lysine.*

In the quest for an adequate positive antimicrobial, membrane-disrupting control, we incubated *M. catarrhalis* for one hour with several active compounds: erythromycin, tetracyclin and hexetidine (all 0.05 mg/mL), EDTA (0.05 M) and zeocin (5 mg/mL). Afterwards, bacteria were spotted on a membrane for further SEM processing. Another sample of the same treated bacterial cultures was also by monitoring their growth via time-course analysis These bacteria were first washed twice with PBS to remove antibiotics as much as possible. Then, the pellet was resolved in MH broth and different dilutions were monitored for growth at 37°C (Figure 3.7A). The growth experiment showed the best results for zeocin and 0.1% hexetidine treatment in the damaging of *M. catarrhalis.* However, the SEM results were not conclusive for cell damage. Longer incubation times (overnight) with the active molecules did not disrupt all cells but good results were again seen after treatment with zeocin and 0.005% hexetidine (Figure 3.7B). Because of the price, the ease of use, the other data and the safety, hexetidine was used in further experiments as a positive control.

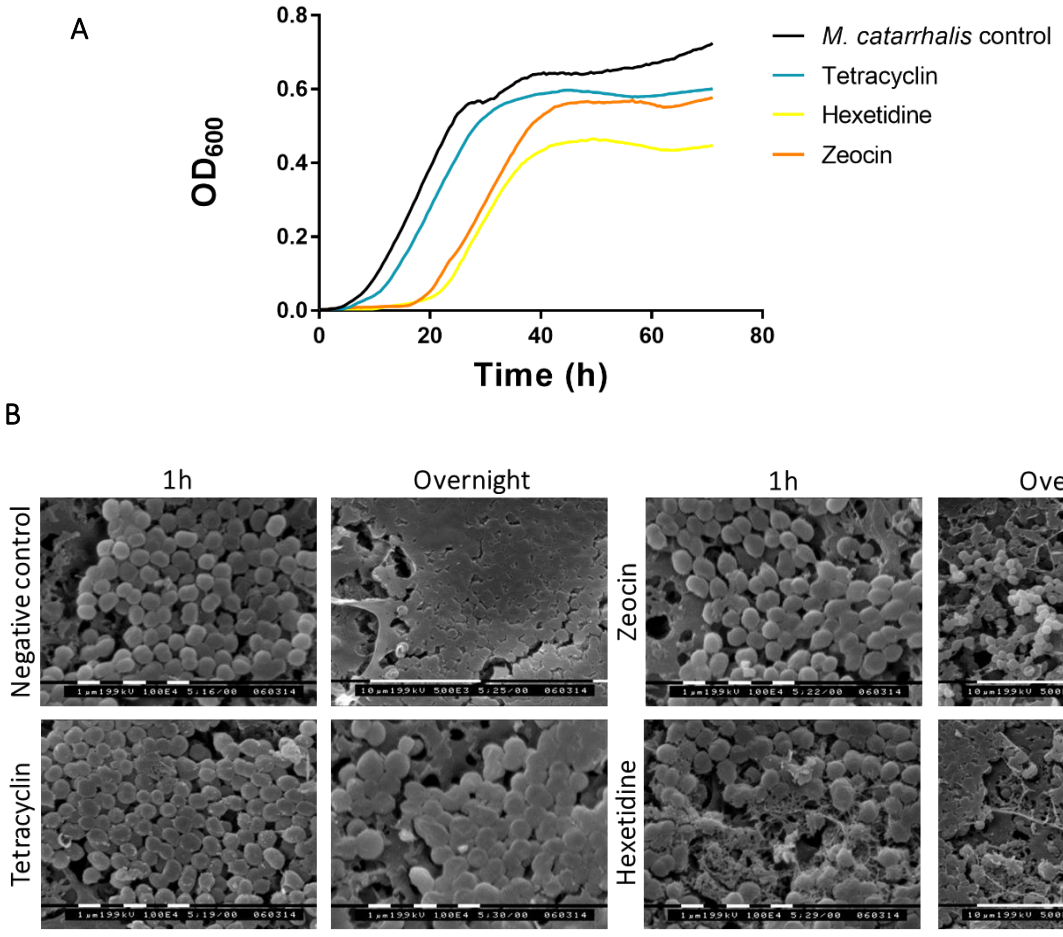


Figure 3.7: Optimisation for different positive controls for membrane damage. *(A) growth curves after 1h treatment with active compounds (B) SEM pictures of* M. catarrhalis *after incubation for 1h or overnight with the active compounds.*

Consequently, the impact of treatment with *Lactobacillus* SCS or lactic acid on the membrane of *M. catarrhalis* was investigated using SEM (Figure 3.8). Both stereoisomers of lactic acid at 0.5 g/L and their combination (total concentration of 1 g/L) clearly affected the membrane of *M. catarrhalis*, as indicated by the formation of possible small extramembrane vesicles or nanotubes on the membrane (Figure 3.8). No difference between D- and L-lactic acid was observed. Interestingly, after treatment with SCS of *L. rhamnosus GG* (produces almost only L-LA) or *L. pentosus* KCA1 (produces 50/50 L-LA and D-LA), the membrane of *M. catarrhalis* was dented and filament-like structures were formed between the cocci. No clear difference was observed between de two lactobacilli although more pili/nanotubes appeared to be formed after treatment with the SCS of *L. rhamnosus* GG.

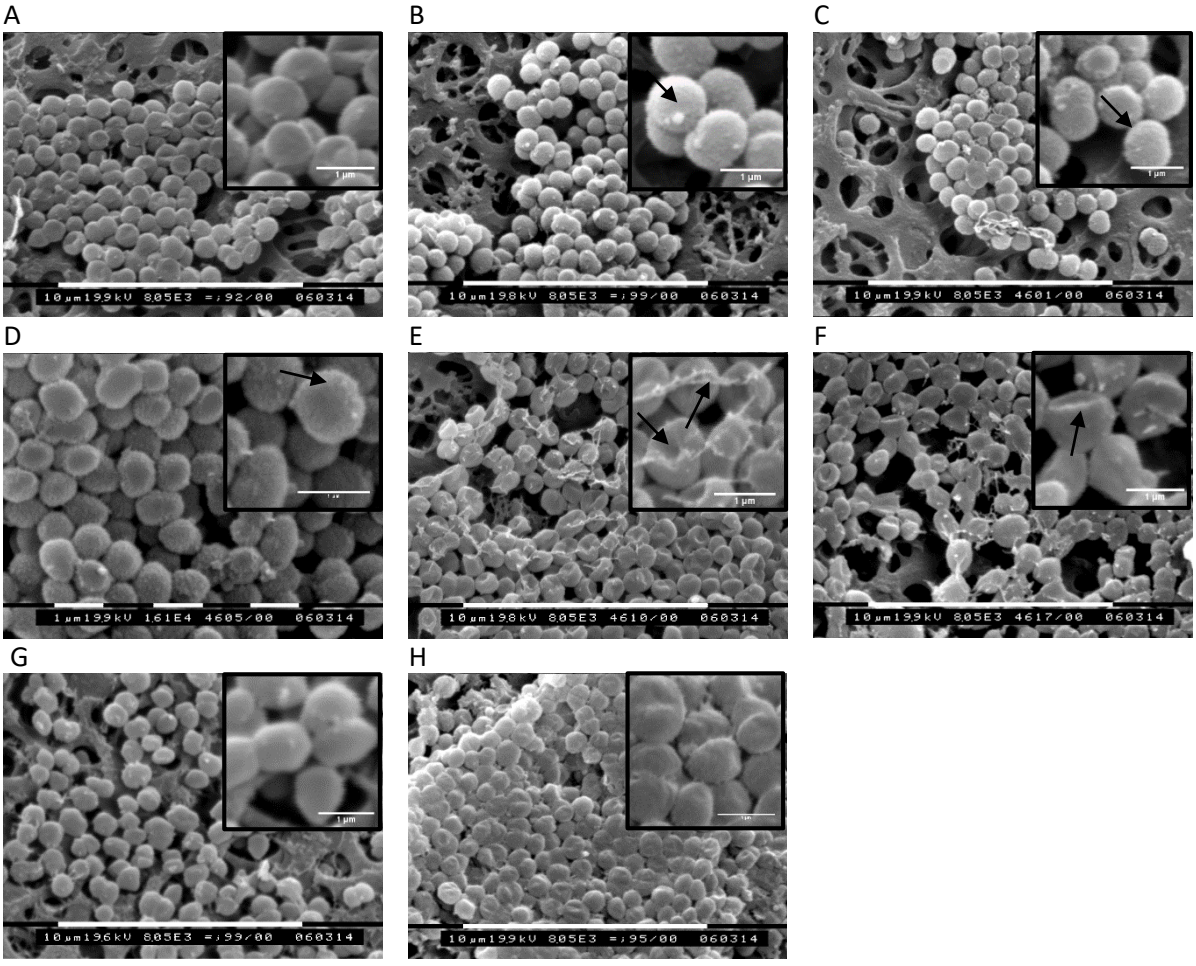


Figure 3.8: Impact of SCS of Lactobacillus strains and lactic acid on the membrane of M. catarrhalis*. SEM pictures were taken from* M. catarrhalis *bacteria after (A) no treatment; (B) treatment with 0.5 g/L L-lactic acid; (C) treatment with 0.5 g/L D-lactic acid; (D) treatment with 1 g/L D- and L-lactic acid mixture; (E) treatment with 5% SCS of* L. rhamnosus *GG; and (F) treatment with 5% SCS of* L. pentosus *KCA1 (G) treatment with 5% MRS pH 4.3 (H) treatment with 5% hexetidine.*

3.4 DISCUSSION

The work presented in this chapter has led to the optimisation of a number of in-house protocols such as MIC and checkerboard assays and the SEM procedure. As to the identification of active compounds, acids appeared to play a major role in the inhibition of the pathogen, since the activity against *M. catarrhalis* disappeared after neutralising the pH. Furthermore, a link was found between the concentration of lactic acid in the SCS and the inhibitory activity. It should be noted, however, that that the concentration of lactic acid in the supernatant of the lactobacilli in this study only was measured via an enzymatic kit. Although this enzymatic reaction is very specific and sensitive (Bergmeyer and Gawehn, 1974), star activity of the present enzymes, D/L-lactate- dehydrogenase, could have occurred. Confirmation of the concentration via other techniques such as HPLC would have been recommended as a control. 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), lactic acid represents a clear antimicrobial factor vs. *M. catarrhalis* as the inhibitory activity was also confirmed in broth-based assays. Most lactobacilli are homofermentative lactic acid bacteria that produce D- and/or L-lactic acid (Duar *et al.*, 2017). To investigate the importance of both isomers in the antimicrobial activity of the *Lactobacillus* species, MIC assays were performed. We observed a MIC of ca. 0.5 g/L for *M. catarrhalis* for both 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 (see Chapter 1) 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; De Boeck *et al.*, 2017; 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. Of course, collaboration with the immune system cannot be neglected as host immune factors can synergise with active antimicrobial molecules (Kumaraswamy *et al.*, 2016; Lin *et al.*, 2015). It is important to note that the MIC values of lactic

acid against URT pathogens were found here to significant increase with increasing pH. As previously ascertained for the pathogens *E. coli*, *Pseudomonas aeruginosa* and *S.* Typhimurium (Alakomi *et al.*, 2005), the undissociated form of lactic acid appears to be most active also against URT pathogens. For *S. pneumoniae* and *H. influenzae* similar results were observed with a slight but non- significant reduction in the MIC of D-lactic acid compared to L-lactic acid.

Although we could clearly substantiate an antimicrobial role for lactic acid against URT pathogens in our present work, some data, especially the SEM and biofilm results, point towards other active molecules present in the SCS of lactobacilli. These other molecules are also non-proteinaceous, heat-resistant and pH- dependent based on the agar-based antimicrobial and biofilm assays. Such molecules could be small antimicrobial peptides such as bacteriocin. Unfortunately, no sign of bacteriocin activity was detected, although we tried to induce its production with co-cultivation of the lactobacilli with *L. lactis* MG1363, as described by (Maldonado *et al.*, 2004) and we treated the SCS to select for bacteriocin activity. Larger screenings with a higher number of lactobacilli and better bacteriocin-inducing protocols would be needed to detect the presence of such bacteriocins. Nevertheless, comparative genomic analysis of *L. rhamnosus* GG, detected the presence of a putative bacteriocin gene (Kankainen *et al.*, 2009). It remains unclear, however, whether the strain also actually produces a bacteriocin.

Synergistic effects of possible effector molecules with lactic acid should also not be neglected in future research. However, for the Llp1 proteins of *L. rhamnosus* GG and GR-1, the presence of lactic acid decreased their biofilm inhibiting capacity. Neither did D- and L-lactic acid acted synergistically based on the checkerboard assays performed here. In contrast, 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%). Similar synergistic interactions were observed for reuterin and lactic acid against *E. coli* O157:H7 and *L. monocytogenes* (El-Ziney *et al.*, 1999). Such synergistic effects are likely secondary to the possible permeabilisation of the membrane by lactic acid. Future studies on the identification of antimicrobial *Lactobacillus* molecules that can be triggered to penetrate the membrane and affect the cytoplasm of the pathogenic bacteria is thus of high interest.

4 ANTIMICROBIAL ACTIVITY OF LACTOBACILLI AGAINST (A)OM PATHOGENS BY COMPETITIVE EXCLUSION AND CYTOKINE MODULATION IN EPITHELIAL CELLS

Part of this chapter is accepted for publication: **van den Broek, M. F. L.,** De Boeck, I., Claes, I. J. J., Nizet, V. and Lebeer, S. 2017. Multifactorial inhibition of lactobacilli against the respiratory tract pathogen Moraxella catarrhalis. Beneficial microbes. Accepted for publication (Reference: BM-2017-07-0101.R2).

4.1 INTRODUCTION

As mentioned in the first chapter, two of the main modes of action of probiotics are the inhibition of pathogen adhesion to host cells and the modulation of immune responses in different host cells. Much research on these activities has been done for pathogens and immune responses in the intestinal environment. However, the knowledge on similar activities in the nasopharynx is limited.

To be able to compete with (A)OM pathogen that can strongly adhere to their target epithelial cells (see overview in Chapter 1), it can be postulated that the lactobacilli must also be able to adhere strongly to the epithelial cells in the upper respiratory tract. Indeed, in many studies, a correlation between adhesion and the capacity to exclude pathogens is observed (O'Toole and Cooney, 2008). Documented adhesion factors of gastro-intestinal lactobacilli include proteins (*e.g.* in pili), exopolysaccharides (EPS) and TA (Lebeer *et al.*, 2008b), but the capacity of these adhesins to also mediate adhesion is not explored yet in nasopharyngeal study set-ups.

After adhesion, pathogens can invade and cross the epithelial barrier and cause antigen presenting cells (APC) to secrete different cytokines which play a pivotal role in the inflammation. So, in addition to blocking adhesion of the (A)OM pathogens, it can be postulated that it will be an added value if the applied lactobacilli can also counteract unwanted host responses induced by the (A)OM pathogens. As reviewed in Chapter 1, quantification on both protein and mRNA levels of several cytokines in MEF samples of OM children has shown that cytokines IL-1 β, IL-6 and IL-8, and TNF-α and the mucins MUC5AC and MUC1 probably play an important role in the disease (Kerschner, 2007; Krueger *et al.*, 2016; Si *et al.*, 2014; Val, 2015; Zielnik-Jurkiewicz and Stankiewicz-Szymczak, 2016). Of note, their expression was correlated with the amount of OM pathogens in the MEF (Shen *et al.*, 2008; Zielnik-Jurkiewicz and Stankiewicz-Szymczak, 2016). In gastro-intestinal diseases, probiotics have already shown to have immunomodulating capacities such as the downregulation of pro-inflammatory responses (Isolauri *et al.*, 2002). For the nasopharyngeal niche, however, the immunomodulating potential of probiotics is underexplored. Furthermore, decreasing the mucus production in OM would provide a potential therapy as it impairs mucociliary clearance and helps the pathogens to survive in the middle ear (Yazıcı, 2015).

Therefore, in this chapter, we investigated whether lactobacilli can adhere to nasopharyngeal cells and decrease the adhesion of OM pathogens. *L. rhamnosus* GG was further evaluated for its capacity to modulate the immune responses (IL-8, IL-1β, TNF-α, MUC1 and MUC5AC) of *M. catarrhalis* and *H. influenzae* in respiratory epithelial cells.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains and growth conditions

Bacterial strains used in this chapter were cultured as described previously.

Table 4.1: Inventory of bacterial strains used in this chapter.

Species	Strain	Relevant genotype or description	Reference and/or source
		LACTOBACILLI	
Lactobacillus bulgaricus	AMB-1	Single colony isolate from Colombian yoghurt	Yoghurt
Lactobacillus casei*	AMBR-2	Single colony URT isolate from healthy adult	(Wuyts *et al.*, 2017)
Lactobacillus casei	ATCC334	Single colony isolate obtained from a stock culture of ATCC334	ATCC
Lactobacillus casei*	Shirota	Single colony isolate obtained from a commercially available fermented drink containing *L. casei* Shirota (Yakult®), confirmed by sequencing	Commercial probiotic product
Lactobacillus 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	Commercial probiotic product
Lactobacillus casei *	MCJ	Single colony isolate from spontaneously fermented carrot juice	Fermented carrot juice
Lactobacillus parabuchneri	AB17	Single colony isolate from spontaneously fermented beet juice	Fermented beet juice
Lactobacillus parabuchneri	NM63-3	Single colony isolate from spontaneously fermented carrot juice	Fermented carrot juice
Lactobacillus paracasei	LMG12586	Single colony isolate obtained from a stock culture of LMG12586	BCCM/LMG
Lactobacillus pentosus	KCA1	Single colony isolate from KCA1 (vaginal origin)	(Anukam *et al.*, 2013)
Lactobacillus plantarum	LMG1284	Single colony isolate from *L. plantarum* ATCC8014 or LMG1284	BCCM/LMG
Lactobacillus plantarum	CMPG5300	Single colony isolate (vaginal origin)	(Malik *et al.*, 2014)
Lactobacillus plantarum	5057	Single colony isolate of *L. plantarum* 5057	(Danielsen, 2002)
Lactobacillus plantarum	WCFS1	Single colony isolate of *L. plantarum* WCFS1	(Kleerebezem *et al.*, 2003)

Lactobacillus reuteri	RC14	Single colony isolate from a commercially available probiotic supplement containing *L. reuteri* RC14, 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 (urethra origin)	(Chan *et al.*, 1984, 1985; Reid and Bruce, 2001; Reid and Reid, 1999), ATCC
Lactobacillus rhamnosus	GG	Single colony isolate of wild-type strain, isolated from human faeces	(Kankainen *et al.*, 2009)
Lactobacillus rhamnosus	CMPG5357	*spaCBA* pili mutant of *L. rhamnosus* GG, Tc^R	(Lebeer *et al.*, 2012)
Lactobacillus rhamnosus	CMPG5351	*welE* mutant of of *L. rhamnosus* GG, Tet^R	(Lebeer *et al.*, 2009)
Lactobacillus rhamnosus	C11	Pili mutant of *L. rhamnosus* GG	Willem de Vos, personal communication

*** According to a recent large-scale comparative genomics study in our lab by Wuyts *et al.* (2017), these strains classify actually in the *L. paracasei* clade of the *L. casei* group.**

PATHOGENS

Haemophilus influenzae	ATCC49247	Single colony isolated from expectorated sputum from a 76 year-old male with pneumonia	ATCC
Moraxella catarrhalis	ATCC25238	Type strain, nasal cavity	ATCC
Streptococcus pneumoniae	ATCC49619	Single colony isolated from sputum of 75-year-old male	ATCC
Staphylococcus aureus	MI/1310/4731	Methicillin resistant *Staphylococcus aureus* isolate	Vaxinfectio (UAntwerpen)

4.2.2 Cell culture

The human bronchial epithelial cell line Calu-3 ATCC® HTB-55™ (purchased from ATCC) was cultured in 75 cm² flasks containing 20 mL Minimum Essential Medium (MEM) (Life Technologies) supplemented (10%) with heat inactivated foetal calf serum and a penicillin-streptomycin mix (100 U/mL and 100 µg/mL, respectively) (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 once per week at a 1:2 split ratio using 0.25% trypsin-EDTA solution. For adhesion and immunomodulation experiments, Calu-3 cells were seeded in 12-well culture plates at a density of 1.85×10^6 cells/ml. Approximately a week after seeding, confluent monolayers were obtained.

4.2.3 Adherence of bacteria to Calu-3 cells

The adherence capacity of lactobacilli to bronchial epithelial Calu-3 cells was investigated as described previously for adherence to intestinal epithelial cells (Lebeer *et al.*, 2012) with minor modifications. Adhesion tests were carried out by adding a volume of 0.5 ml containing lactobacilli (5 x 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 bacterial cells to the Calu-3 cells was determined by macrodilution method on MRS agar. Each condition was carried out at least in triplicate.

4.2.4 Inhibition of *M. catarrhalis, S. pneumoniae* and *H. influenzae* adherence to epithelial cells by *Lactobacillus* species.

The influence of lactobacilli on the adherence of *M. catarrhalis, S. pneumoniae* and *H. influenzae* to bronchial 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 x 10^7 or 5 x 10^6 CFU) and *L. rhamnosus* GG (5 x 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.

4.2.5 Phagocytosis assay

Bacterial uptake was measured by phagocytosis assays as described earlier (Vargas García *et al.*, 2015). Bacteria were prepared as described for the adhesion assay. The initial steps of the experiment were similar to those in the protocol of adhesion experiments. Bacteria (5 x 10^7 CFU) were incubated with the cells at 37°C, incubated for 1h and washed trice with ice cold Dulbecco's PBS. Afterwards, penicillin-streptomycin (100 U/mL and 100 µg/mL, respectively) was added and incubated for 1 h to kill extracellular bacteria. Then, cells were washed again 3 times to remove antibiotic residues and 500 µL Triton X-100 (0.2%) was added and incubated in shaking conditions to lyse the cells. The cellular suspension was mixed by pipetting up and down and dilution series were prepared before plating out. The amount of phagocytosed

bacteria was quantified as the ratio between the number of bacteria after phagocytosis and the amount of originally added bacteria.

4.2.6 Induction of cytokine gene expression in CaLu-3 epithelial cells.

The cytokine response on co-incubation of CaLu-3 cells with *M. catarrhalis* and lactobacilli was determined at mRNA level. Strains were grown overnight and subsequently centrifuged at 2,000 × g at 4°C for 10 min, washed with PBS and resuspended in MEM medium in a final concentration of 2×10^8 CFU/ml. The pathogens were added to the CaLu-3 epithelial cells, alone or in presence of lactobacilli in a 1:1 ratio. After 2 or 4 hour of incubation, the cells were rinsed three times with PBS. Next, 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 ReadyScript® reverse transcriptase (Sigma Aldrich). The cytokine gene expression was measurement by qRT-PCR.

4.2.7 qRT-PCR

To determine the best reference genes to use in immunomodulation assays, Calu-3 cells were incubated for 2h with cell culture medium MEM, 0.26×10^6 CFU/cm² *L. rhamnosus* GG, *Staphylococcus aureus* or *M. catarrhalis.* RNA was extracted (RNeasy mini kit, Qiagen) and cDNA was prepared as described above. Expression of interleukin (IL)-8, IL-1β, mucin MUC5AC and tumor necrosis factor-α (TNFα) was analysed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each PCR amplification contained 10 µL of Power SYBR Green PCR Master Mix, 0.3 µL of both primers (20 µM) and 5.4 µL of MilliQ water and 4 µL 1 to 10 diluted DNA of the sample in a total volume of 20 µL. A no-template control (NTC) was included on each plate. The amplification program consisted of 1 cycle of 50°C for 10 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. Data were quantified with the ΔΔCt method, relative to the reference genes (RGs) Cytochrome c1 (CYC1) and ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B). Primer sequences can be found in Table 4.2 and Table 4.3. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were strictly followed (Bustin *et al.*, 2009). Primer efficiencies were calculated by running a qPCR experiment with 7 dilutions of a known amount of DNA (sample 1 was diluted

4 times to get sample 2, sample 2 4 times to sample 3 etc.). Then, the Ct values were plotted against the log(DNA) and the slope of the graph was determined. The primer efficiency should lie between 90 and 110% and is calculated as follows:

$$primer\ efficiency = 10^{\frac{-1}{slope}} - 1$$

Table 4.2: Primers used for qRT-PCR of possible reference genes.

Target mRNA	Primer	Oligonucleotide sequence (5'-3')	Reference
CYC1	Forward	CATGTCCCAGATAGCCAAGGA	Own lab
	Reverse	CTTGTGCCGCTTTATGGTGTAG	
ATP5B	Forward	GCAGGAAAGAATTACCACTACCAAG	Own lab
	Reverse	TGGTAGCATCCAAATGGGCAA	
GAPDH	Forward	ATTTGGCTACAGCAACAGGG	(Jacobsen, 2013)
	Reverse	TCAAGGGGTCTACATGGCA	
b-ACT	Forward	GeNorm primer	
	Reverse	GeNorm primer	
B2M	Forward	CTGAAGCTGACAGCATTCGG	(Serrano *et al.*, 2001)
	Reverse	CTTTGGAGTACGCTGGATAGCC	
GNBL1	Forward	CACTGTCCAGGATGAGAGCCA	Ellen Dilissen (KULeuven)
	Reverse	CATACCTTGACCAGCTTGTCCC	

Table 4.3: Primers used for cytokine and mucin gene expression.

Target mRNA	Primer	Oligonucleotide sequence (5'-3')	Reference
IL-1β	Forward	TTGCTCAAGTGTCTGAAGCAGC	Own lab
	Reverse	CAAGTCATCCTCATTGCCACTG	
IL-8	Forward	TGGCAGCCTTCCTGATTTCT	(Tsaryk *et al.*, 2013)
	Reverse	TTAGCACTCCTTGGCAAAACTG	
IL-10	Forward	GTGATGCCCCAAGCTGAGA	(Sen *et al.*, 2004)
	Reverse	CACGGCCTTGCTCTTGTTTT	
TNF-α	Forward	CCTCTGATGGCACCACCAG	(Popa *et al.*, 2007)
	Reverse	TCTTCTCGAACCCCGAGTGA	
MUC1	Forward	AGAGAAGTTCAGTGCCCAGC	(Dharmaraj *et al.*, 2009)
	Reverse	TGACATCCTGTCCCTGAGTG	
MUC2	Forward	GGGGACAGTGGCTGCGTTCC	(Dharmani *et al.*, 2011)

	Reverse	CGGGGCAGGGCAGGTCTTTG	
MUC3	Forward	GCAGCTGTTGGGGCTCCTCG	(Dharmani *et al.*, 2011)
	Reverse	GAGAGGCGAGCTGGGGGACA	
MUC4	Forward	TTCTAAGAACCACCAGACTCAGAGC	(Kim *et al.*, 2012)
	Reverse	GAGACACACCTGGAGAGAATGAGC	
MUC5AC	Forward	GGGACTTCTCCTACCAAT	(Sim *et al.*, 2017)
	Reverse	TATATGGTGGATCCTGCAGGGTAG	
MUC5B	Forward	CACATCCACCCTTCCAAC	(Sim *et al.*, 2017)
	Reverse	GGCTCATTGTCGTCTCTG	

4.2.8 Cell viability assay

The viability of human cells was determined via an XTT assay, in which the oxidised (colourless) form of XTT is reduced to an orange formazan in the presence of living eukaryotic cells. The absorbance at 450 nm then correlates with the amount of living eukaryotic cells. First, Calu-3 cells were seeded in a 96 well plate in a concentration varying from 1000-100000 cells/well and incubated for 4 days in 5% CO_2 and 37°C environment. The medium used for the culture of the seeded cells was MEM supplemented with 10% foetal calf serum and was changed daily. The total volume in the wells was 100 µL. After 4 days, the cells were treated with 100 µL of testing solution/bacteria for 2 h. Triton X-100 (0.5%) was used as a positive control, MEM medium as a negative control and wells without cells as blank. Afterwards, 50 µL of detection solution (Table 4.4) was added and incubated for 2 h in 5% CO_2 and 37°C environment. The experiment was done in triplicate. Finally, the plate was put on a shaker for a short period and the absorbance of the wells was measured at 450 nm with the Synergy HTX multimode plate reader (BioTek, Vermont, USA). In the case when bacteria were added to the wells, the adequate amount of bacteria was estimated based on the OD_{600} of the overnight culture. This volume was centrifuged for 10 min. at 2000 x g, washed with PBS and centrifuged again at the same conditions. The pellet was then suspended in an adequate volume of MEM cell culture medium.

Table 4.4: Ingredients for detection solution in XTT assay.

	Ingredients
PMS solution	0.3% (wt/v) 5-Methylphenazinium methyl sulfate (PMS) in PBS
XTT solution	0.1% (wt/v) 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) in MEM cell culture medium
Detection solution	0.25% (v/v) PMS solution in XTT solution

4.2.9 Statistics

Data are represented as mean values ± standard deviation. One-way ANOVA and t-tests were used to determine statistical significance in GraphPad Prism. Differences were considered statistically significant at $P<0.05$.

4.3 RESULTS

4.3.1 Adhesion of lactobacilli to respiratory epithelium and competition with pathogens

4.3.1.1 Large variation in adhesion capacity to respiratory epithelial cells between different lactobacilli

First, adhesion assays were done with the most important lactobacilli on Calu-3 epithelial cells. Most lactobacilli showed an adhesion ratio between 1 and 10% (Figure 4.1). However, large variation was observed between different repetitions of the experiment. The lactobacilli of the *plantarum* and *pentosus* species tested, tended to adhere better to the cells than the others. It should be noted, however, that *L. plantarum* CMPG5300 shows high aggregation and the determination of the total CFUs is often difficult to interpret as depending on the degree of aggregation different CFU numbers can originate from the same amount of bacteria (Malik *et al.*, 2016). This probably explains- at least partially- the large variation and the large adherence percentages. Because SpaCBA pili have been previous shown by our group to play a key role in adhesion of *L. rhamnosus* GG to intestinal epithelial cells (Lebeer *et al.*, 2012), we aimed to investigate here whether these pili also play a role in adhesion to respiratory epithelial cells, by comparing the pili mutant *L. rhamnosus* C11 with the wild-type (Figure 4.2). In contrast to intestinal epithelial cells were the pili mutant shows a 10-fold reduction in adherence capacity (Lebeer *et al.*, 2012), here we could only observe a decreasing, but non-significant reduction in adhesion of the pili mutant compared to wild-type (Figure 4.2).

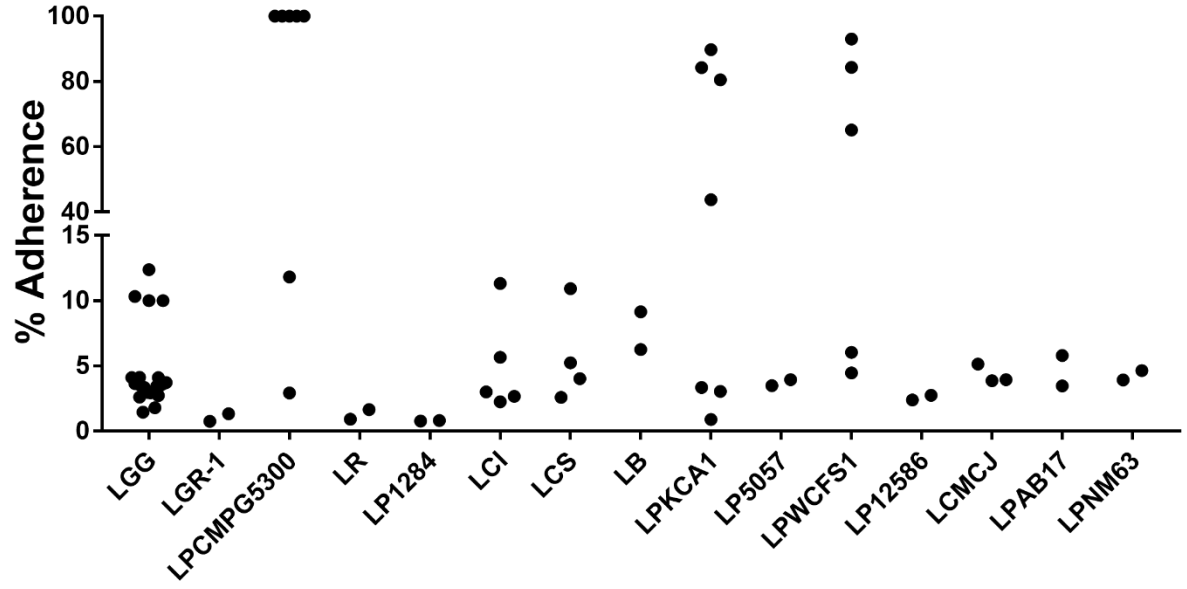


Figure 4.1: Adherence capacity of different Lactobacillus *strains to Calu-3 respiratory epithelial cells. This adherence capacity is determined based on plating (CFU determination) before and after incubation for 1h with the epithelial cells. All dots represent biological repetitions. Because adherence percentages above 100% are not biologically meaningful, the maximum was set at 100%. Names of the lactobacilli can be found in Table 4.1.*

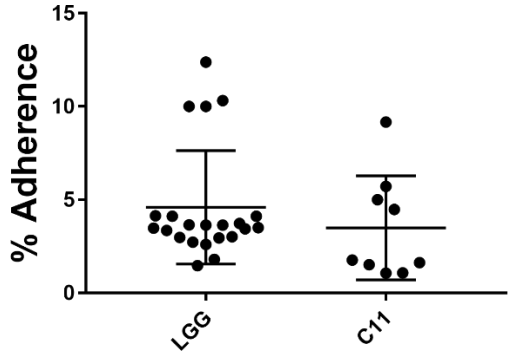


Figure 4.2: Comparison of the adherence capacity of L. rhamnosus *GG wild-type and its pili mutant* L. rhamnosus *C11 to Calu-3 respiratory epithelial cells.*

4.3.1.2 L. rhamnosus GG decreases pathogen adhesion

Subsequently, we aimed to investigate whether lactobacilli can interfere with pathogen adhesion to the respiratory epithelial cells under the tested conditions. We started with adhesion competition assays with our model probiotic strain *L. rhamnosus* GG and added the probiotic in different ratios compared to the pathogens, namely ratio 1:1 and 10:1. *L. rhamnosus* GG showed significant competition for the adhesion of both *M. catarrhalis* and *S.*

pneumoniae with a decrease in adhesion up to 61 (adjusted P-values: 0.012) and 68% (adjusted P-value: 0.0037), respectively (Figure 4.3). Competition assays were not performed with *H. influenzae* because of its low adhesion to the cells. Instead, a phagocytosis assay was performed to explore the possible phagocytosis of this pathogen by the Calu-3 cells. However, no significant phagocytosis was observed (data not shown).

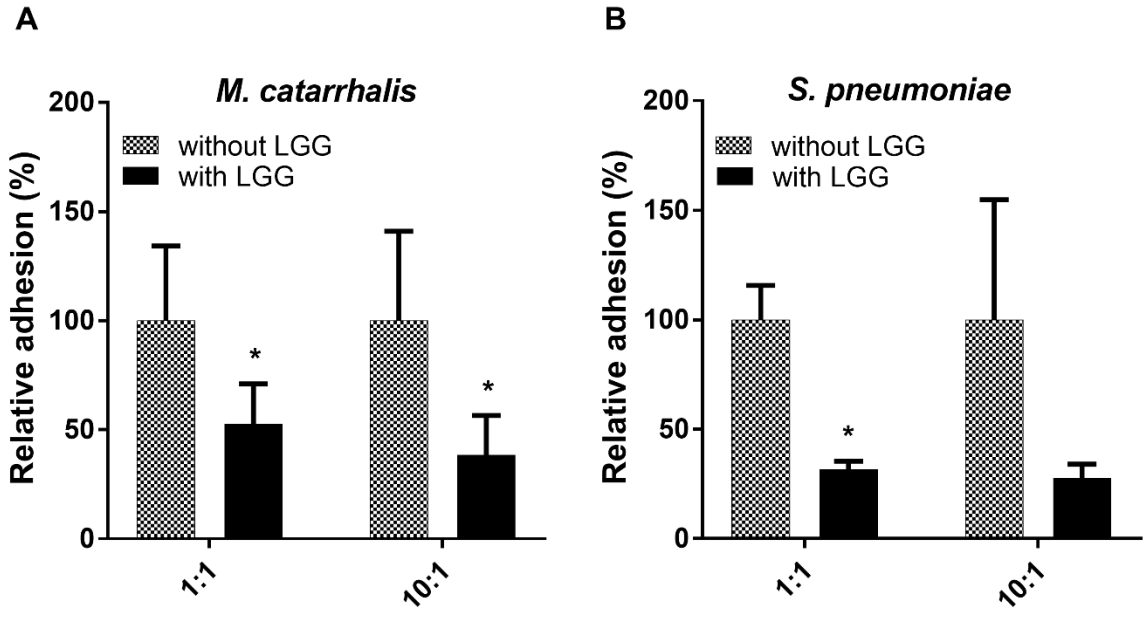


Figure 4.3: Adhesion competition of L. rhamnosus *GG against (A)* M. catarrhalis *and (B)* S. pneumoniae*. The ratio* L. rhamnosus *GG (LGG):pathogen is depicted on the x-axis. LGG was added in a concentration of 2,6 x* 10^7 *CFU/cm².*

To investigate a plausible mechanism of action of this competitive exclusion of the pathogens by *L. rhamnosus GG*, the same experiment was repeated with several *L. rhamnosus* GG surface mutants available in-house: the previously mentioned pili mutant *L. rhamnosus* C11 and gene deletion mutants having respectively mutations in the *spaCBA* and *welE* genes, encoding SpaCBA pili and exopolysaccharides (*L. rhamnosus* CMPG5357 and CMPG5351, respectively) as shown in Figure 4.4. Interestingly, these data suggest a potential role of pili in the competitive exclusion of *M. catarrhalis* by *L. rhamnosus* GG.

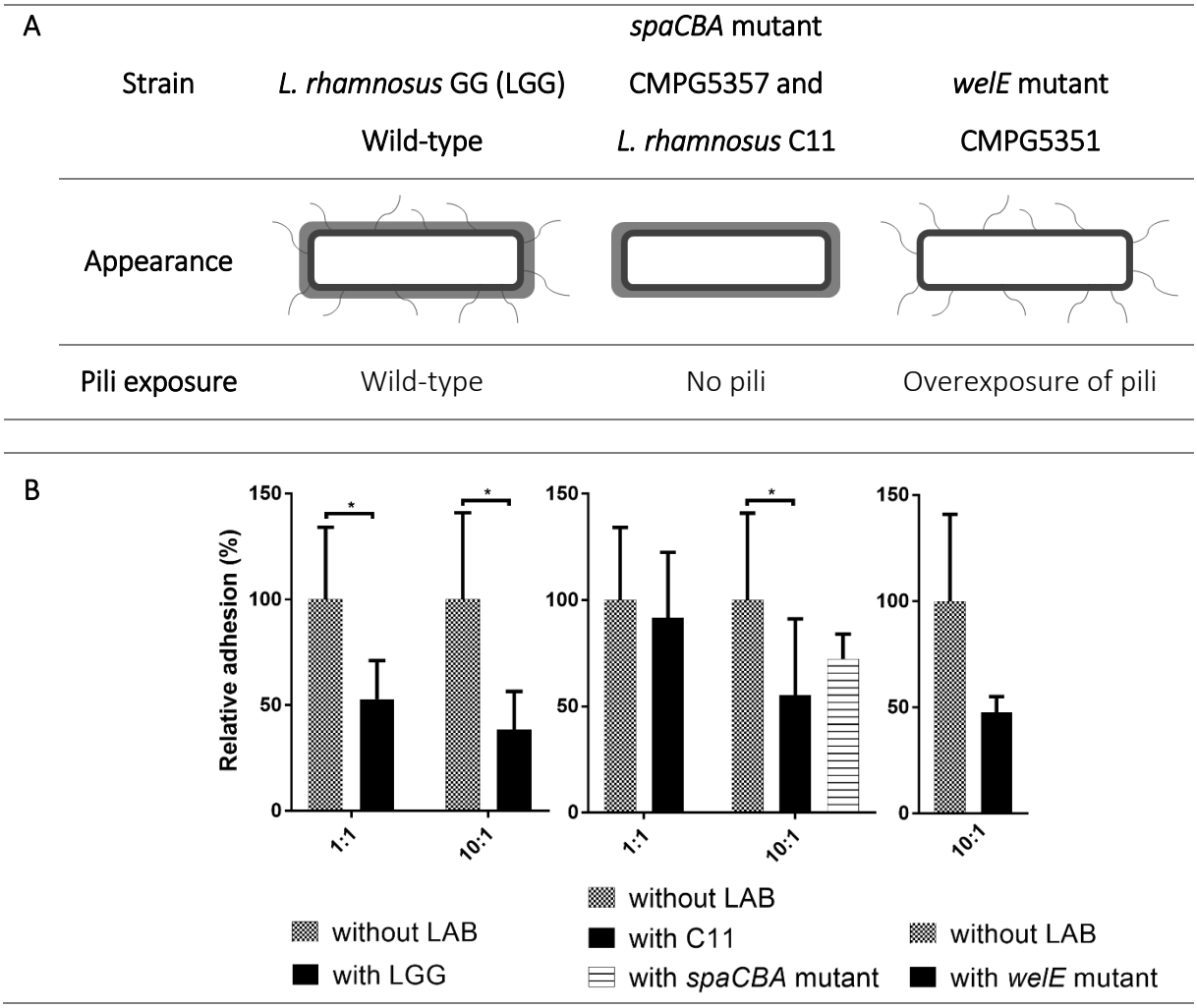


Figure 4.4: Adhesion competition between M. catarrhalis *and* L. rhamnosus *GG wild-type and pili-related mutants. (A) Schematic representation of* L. rhamnosus *GG wild-type and mutants (B) Lactobacilli were added in a concentration of 2,6 x 10^7 CFU/cm² while the concentration of* M. catarrhalis *varied and is depicted in the graph.*

4.3.2 Immunomodulating activity of lactobacilli in respiratory epithelial cells in response to (A)OM pathogens

4.3.2.1 CYC1 and ATP5B are suitable reference genes for interaction studies in Calu-3 cells

Because co-incubation experiments with lactobacilli and Calu-3 cells were not yet performed, we explored first six potential reference genes with qPCR: B2M, GAPDH, GNBL1, b-ACT, CYC1 and ATP5B. The GeNorm M (M) and Coefficient of Variation (CV) values which represent expression stability were calculated via Qbase+ software (Table 4.5 and Figure 4.5). Adequate reference genes should have M and CV values lower than 0.5 and 0.2, respectively. Only b-ACT did not meet this standard. We then plotted the average gene stability for these references

genes via GeNorm analysis from unstable to stable (left to right). Based on these results, CYC1 and ATP5B were chosen to use as reference genes. However, GAPDH, GNBL1 and B2M can be used as well, based on their M and CV values.

Table 4.5: GeNorm M (M) and Coefficient of Variation (CV) values of Calu-3 cell experiment.

Reference gene	M	CV
ATP5B	0,350	0,172
B2M	0,336	0,144
CYC1	0,315	0,127
GAPDH	0,323	0,126
GNBL1	0,332	0,140
b-Act	0,424	0,236

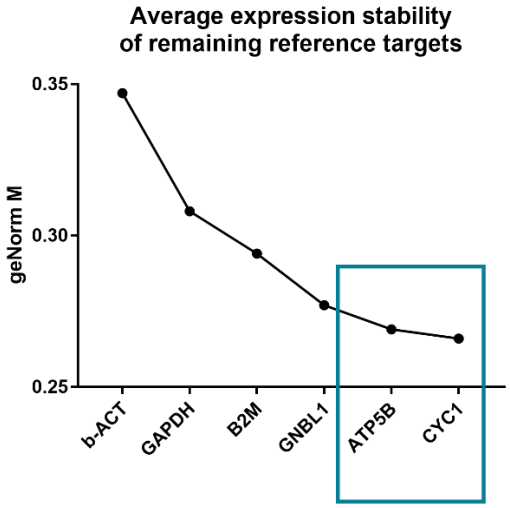


Figure 4.5: Average expression stability of reference genes b-Act, GAPDB, B2M, GNBL1, ATP5B and CYC1. *Lower GeNorm M values indicate more stable genes.*

4.3.2.2 Impact of incubation time and pathogen concentration on cytokine expression

To control for the optimal incubation time of bacteria on Calu-3 cells, we tested two time points: 2 h and 4 h. The Calu-3 cells were incubated with three bacterial strains: *L. casei* AMBR-2, *M. catarrhalis* and *S. aureus.* This latter strain is known to have a large inflammatory effect on Calu-3 cells (Mitchell *et al.*, 2011) and was thus tested as a positive control. As shown in Figure 4.6, incubation of 4 h induced generally a higher mRNA of the tested cytokines under the tested conditions. As a consequence, this incubation time was chosen for further experiments.

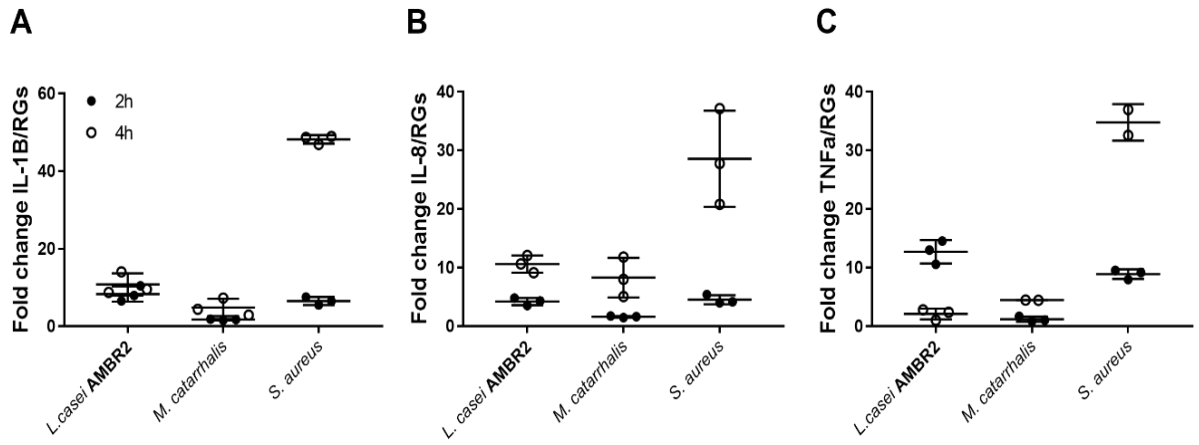


Figure 4.6: Time assay for incubation of bacteria on Calu-3 epithelial cells. The bacteria indicated on the x-axis were incubated on the cells for 2h (black dots) or 4h (open dots). Three cytokines were analysed: (A) IL-1B, (B) IL-8 and (C) TNF-a.

4.3.2.3 Calu-3 cells express MUC1 and MUC5AC

As mucus production is an important physiological characteristic of URT infections, we screened the Calu-3 cells for MUC mRNA upregulation. The primer efficiencies for several MUC genes (MUC1-4, MUC5AC and MUC5B) were monitored as described in §4.2.7. As shown in Figure 4.7, only MUC1 and MUC5AC were expressed by Calu-3 epithelial cells which in agreement with the literature (Berger *et al.*, 1999)

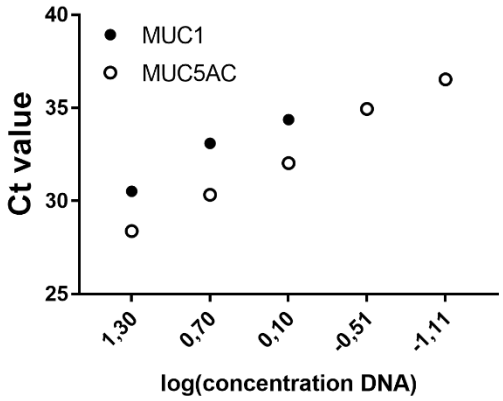


Figure 4.7: mRNA expression of MUC1 and MUC5AC on Calu-3 epithelial cells. To calculate the primer efficiency, the slope of these graphs are used.

4.3.2.4 LGG decreases inflammatory responses induced by M. catarrhalis

Subsequently, we investigated the effect of *L. rhamnosus* GG on the immune responses induced by *M. catarrhalis*. A significant reduction between 1.2 and 1.9-fold in IL-1β, IL-8, MUC5AC and

TNF-α gene expression in the Calu-3 cell line was observed (adjusted P-values: 0.0276, 0.0270, 0.0109 and 0.0298) upon 1:1 co-incubation of *L. rhamnosus* GG with *M. catarrhalis* only (Figure 4.8).

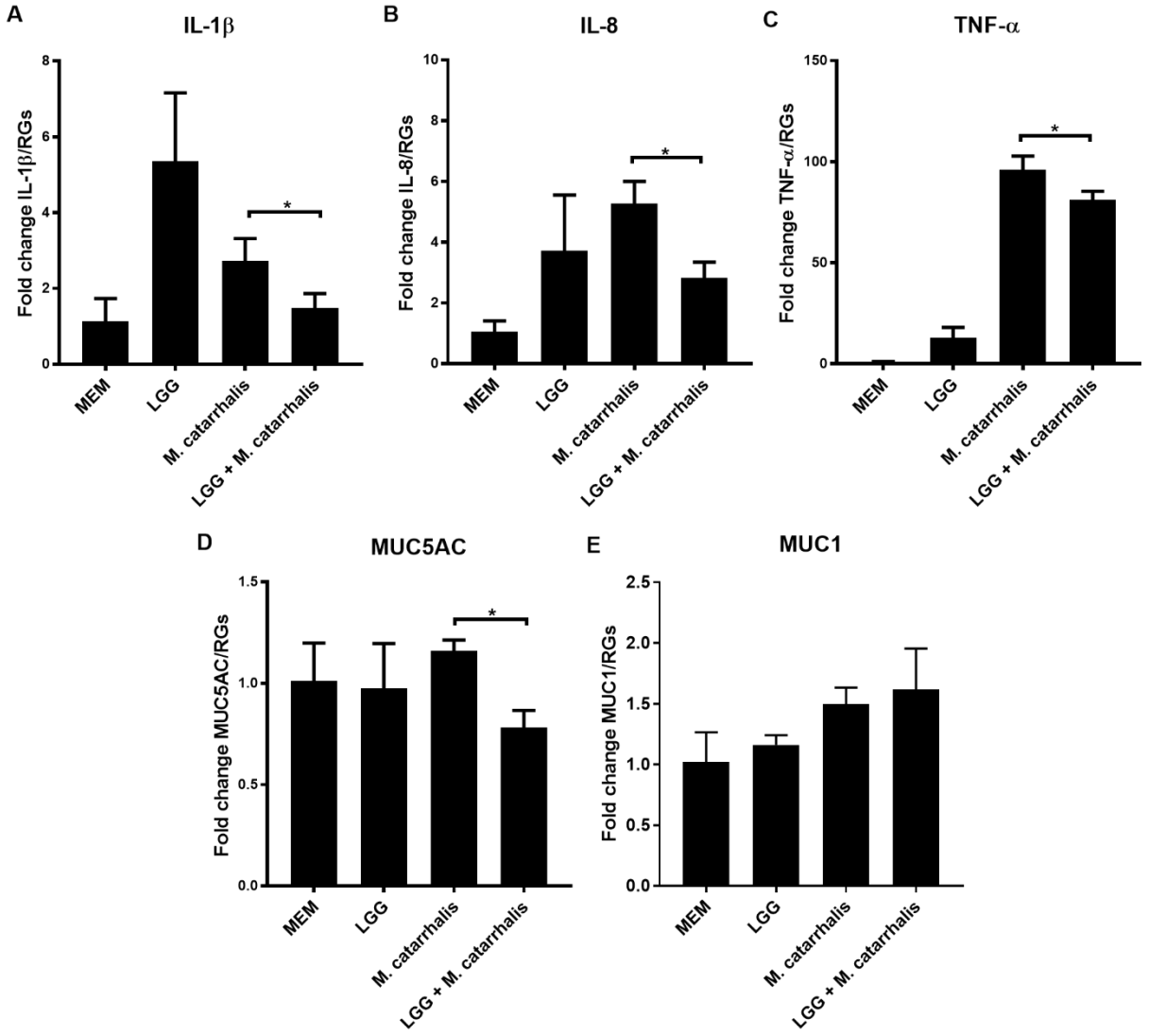


Figure 4.8: Immunomodulatory activity of L. rhamnosus *GG (LGG) vs* M. catarrhalis *in Calu-3 epithelial cells. Three cytokines: IL-1β, IL-8 and TNF-α (A, B and C, respectively) and two mucins MUC5AC and MUC1 (D and E, respectively) were tested. In co-cultures, bacteria were added in a 1:1 ratio. Four hours of co-incubation were tested. MEM: Minimal Essential Medium, used in this experiment as the negative control. RGs: reference genes.*

A similar assay was performed for *H. influenzae* and *S. pneumoniae.* The first pathogen increased the expression of IL-1β, IL-8 and especially TNF-α (Figure 4.9). A slight decrease of this expression was observed for IL-1β and IL-8 after co-incubation with *L. rhamnosus* GG. However, in contrast to previous results with *M. catarrhalis*, this decrease was not significant due to too much variation in the results. On the other hand, a tendency towards an elevated

TNF-α induction was observed after exposure to *L. rhamnosus* GG and *H. influenzae* compared to *L. rhamnosus* GG alone. This increase was, however, not-significant. Also the secreted mucin MUC5AC and the membrane-tethered mucin MUC1, did not show a significant change after co-incubation with *L. rhamnosus* GG.

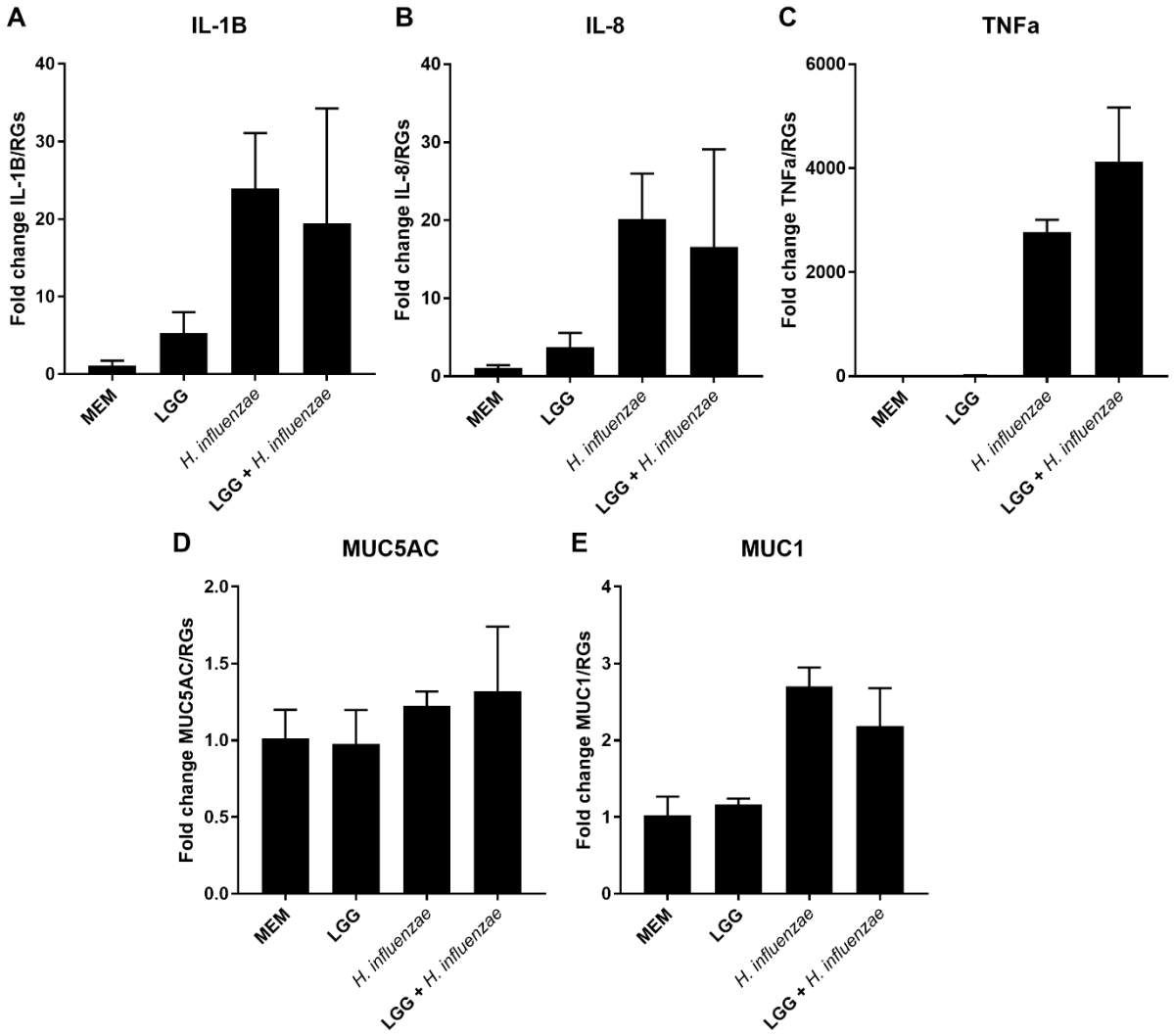


Figure 4.9: Immunomodulatory activity of L. rhamnosus *GG (LGG) vs* H. influenzae *in Calu-3 epithelial cells. Three cytokines: IL-1β, IL-8 and TNF-α (A, B and C, respectively) and two mucins MUC5AC and MUC1 (D and E, respectively) were tested. In co-cultures, bacteria were added in a 1:1 ratio. Four hours of co-incubation were tested. No significant differences between addition of* H. influenzae *alone or in combination with LGG were observed. MEM: Minimal Essential Medium, used in this experiment as the negative control.*

After incubation of the Calu-3 cells with *S. pneumoniae*, no RNA could be isolated due to the death of the epithelial cells. Incubation with the combination of *S. pneumoniae* and *L. rhamnosus* GG, however, appeared not to harm the cells that much, suggesting a protective

role for *L. rhamnosus* GG. To investigate the impact of bacteria on the viability of the Calu-3 cells, an XTT assay was optimised as discussed in the next paragraph.

4.3.3 Cytotoxicity of Calu-3 epithelial cells in after exposure to OM pathogens

To test the impact of OM pathogens on the viability of nasopharyngeal epithelial cells, XTT assays were optimised for use in Calu-3 epithelial cells. A range of cell concentrations (0, 1000, 2000, 5000, 10000, 15000, 25000, 50000, 75000 and 100000 cells/well) were tested in the XTT assay to test the viability of the Calu-3 cells. The absorbance at 450 nm was plotted against the cell density to determine the optimal density to use in further assays. A cell density of 5000 cells/well showed the best absorbance and was chosen for further experiments (Figure 4.10).

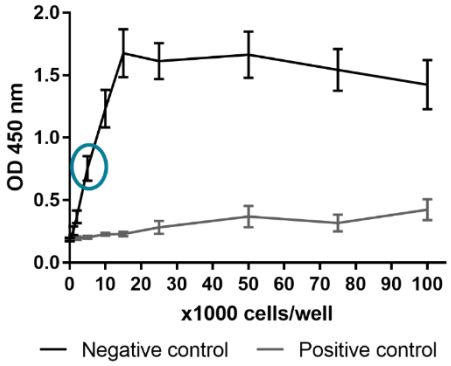


Figure 4.10: Optimisation graph for optimal cell density determination for XTT assay. *This optimum falls on the most linear part of the plot and needs to have an absorbance below 1.0.*

To test possible interference of the bacteria with the assay, wells with and without Calu-3 cells were treated with bacteria or their supernatant, and this for different pathogens and *Lactobacillus* strains (*L. rhamnosus* GG and the closely related, but less immunomodulatory *L. rhamnosus* GR-1 (Petrova *et al.*, in preparation)). The supernatant was added in a 1:20 ratio with MEM and bacteria at 10^6 and 10^8 CFU/well respectively (Figure 4.11). A significant distinction between living (treated with MEM) and dead (treated with Triton X-100) cells was observed. The OD_{450} of the cells treated with Triton X-100 resembled the OD_{450} of the empty wells (Figure 4.11A). When adding 10^6 CFU bacteria to the wells, no increase in absorbance was detected in the wells without seeded Calu-3 cells. This was not the case when adding 10^8 CFU/well, pointing to interference with the assay at this concentration of bacteria. Especially *H. influenzae*, *L. rhamnosus* GG and *L. rhamnosus* GR-1 seemed to interfere with the XTT assay when 10^8 CFU was added (Figure 4.11B-C). Interestingly, *S. pneumoniae* showed different

cytotoxicity when different CFUs were added: 10^8 CFU of this pathogen harmed the Calu-3 cells more severe than 10^6 CFU. This confirmed earlier findings in the cytokine induction assay where no RNA could be isolated form the (dead) Calu-3 cells after exposure with 5 x 10^7 CFU of *S. pneumoniae*. The addition of supernatant of the three (A)OM pathogens, *L. rhamnosus* GG and *L. rhamnosus* GR-1 (5 % v/v) did not interfere with the assay (Figure 4.11D).

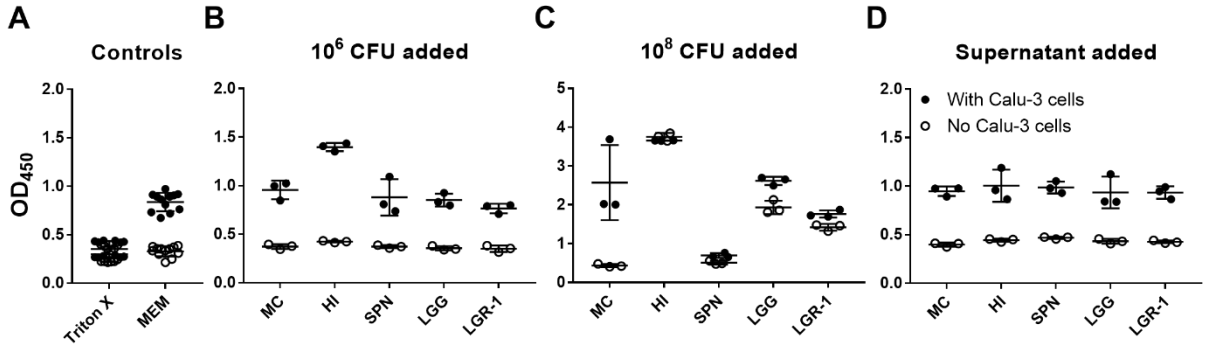


Figure 4.11: Optimisation of optimal amount of CFU to add in XTT assay to prevent interference*. Cell culture plates with (filled circle) and without (open circle) seeded Calu-3 cells were compared (A) Controls 0.5% Triton X-100 and MEM added; (B) 10^6 CFU of the tested bacteria added; (C) 10^8 CFU of the tested bacteria added (D) Spent culture supernatant (5 %)of the tested bacteria added. MEM: Minimal Essential Medium, used in this experiment as the negative control.*

4.4 DISCUSSION

As reviewed in Chapter 1, indirect activities such as competition for adhesion sites and nutrients (competitive exclusion) and modulation of the immune system are also postulated to be key factors in the antimicrobial potential of probiotics. In this chapter, we provided *in vitro* evidence that the model probiotic *L. rhamnosus* GG, can compete with OM pathogens for adhesion and cytokine induction in respiratory epithelial cells. More specifically, we investigated the effect of *L. rhamnosus* GG on adhesion and pro-inflammatory gene expression in Calu-3 cells by *M. catarrhalis, S. pneumoniae* and *H. influenzae. L. rhamnosus* GG significantly dampened the gene expression of IL-1β, IL-8 and TNF-α after incubation with *Moraxella*. However, a 5.35-, 3.7- and 12.8-fold upregulation, respectively, of these cytokines was also observed after exposure to *L. rhamnosus* GG only. It is not uncommon that lactobacilli upregulate both pro-and anti-inflammatory cytokines (for a review see *e.g.* Lebeer *et al.*, 2010). This can have important immune signalling effects to increase the immune responses against pathogens. For *L. rhamnosus* GG, the lipoteichoic acid (LTA) is an important immunostimulatory cell wall molecule, as it induces TLR2-activated IL-8 mRNA induction (Claes *et al.*, 2012a) while the

presence of pili has been shown to downregulate IL-8 mRNA induction by *L. rhamnosus* GG (Lebeer *et al.*, 2012) in intestinal Caco-2 epithelial cell lines. Roselli and colleagues (Roselli *et al.*, 2006) have also investigated the immune response of *L. rhamnosus GG* alone and in combination with enterotoxigenic *Escherichia coli* (ETEC) and observed a similar trend: *L. rhamnosus GG* alone upregulated IL-8 and IL-1β mRNA levels, while lower expression of the mRNA was observed in combination with ETEC. So the immunomodulation of *L. rhamnosus GG* appears different in the presence or absence of a pathogenic stimulus, which is an important consideration in the view of bacterial communities.

In addition, the gene expression of MUC5AC, a secreted mucin commonly correlated with OM (Kerschner *et al.*, 2010), was significantly reduced after co-incubation of *L. rhamnosus GG* with *M. catarrhalis*. 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*) can significantly increase mucin secretion in the host, which could be attenuated in the presence of a *Lactobacillus* strain, namely *L. sakei.* The importance and exact molecular signalling events in the activity of lactobacilli against mucin hypersection remains however to be further explored. The expression of the membrane-tethered mucin MUC1, which is also commonly found in the human Eustachian tube and middle ear epithelium and upregulated in chronic OM (Kerschner, 2007), did not show significant changes after co-incubation of a pathogen with *L. rhamnosus GG*. Literature suggests that this mucin is regulated by TNF-α (Choi *et al.*, 2011; Koga *et al.*, 2007) and might prevent infection with *H. influenzae* and *S. pneumoniae* (Dhar *et al.*, 2017; Kyo *et al.*, 2012). In mice, MUC1 was found to promote phagocytosis of *S. pneumoniae* by alveolar macrophages leading to increased bacterial clearance and protection against pneumonia (Dhar *et al.*, 2017). Furthermore, *Muc1*$^{-/-}$ mice harboured significantly increased levels of pro-inflammatory cytokines in the lung compared to the wild-type mice, pointing to a protective role of MUC1 in the inflammatory response induced by pneumococci. Interestingly, such an anti-inflammatory role of MUC1 was also observed in A549 cells after infection with *H. influenzae.* Here, MUC1 decreased the IL-8 induction in a negative feedback loop manner by suppression of TLR2 signalling (Kyo *et al.*, 2012).

The changes in cytokine induction comparing exposure to a pathogen or a combination of pathogen and *L. rhamnosus* GG are, although often significant, quite subtle. Although this remains to be further investigated, these subtle changes could nevertheless be biological relevant. Although not a direct health parameter/read-out, cytokine expression plays a key signalling role in immune responses towards bacteria and some changes in expression could certainly result in biologically relevant outcomes for the host. Similarly, we previously found that *L. rhamnosus GG* could also inhibit pro-inflammatory cytokine expression by *Salmonella* (Lebeer *et al.*, 2012) and this has been further validated in more complex *ex vivo* models by other groups (Ardita *et al.*, 2014; Ganguli *et al.*, 2015). Similarly, although with another *Lactobacillus* strain, we could show with our collaborators that lactobacilli can also reduce *Salmonella* infections in rat models, at least partially mediated by reducing pro-inflammatory cytokine expression (Borges Acurcio *et al.*, 2017)

Interestingly, our results also suggest that SpaCBA pili of *L. rhamnosus* GG might play an important role in the competition with the pathogen *M. catarrhalis* but not in adhesion to Calu-3 airway epithelial cells (Figure 4.12). Earlier research showed that SpaCBA pili are key in the adherence of *L. rhamnosus* GG to the Caco-2 intestinal epithelial cell (IEC) line (Lebeer *et al.*, 2012). Probably, different surface sugars and receptors on the two different cell lines play a pivotal role in adhesion and mediate tissue-specific adherence as is also observed by Petrova *et al.* (2016b) for *L. rhamnosus* GR-1 and endocervical cell lines.

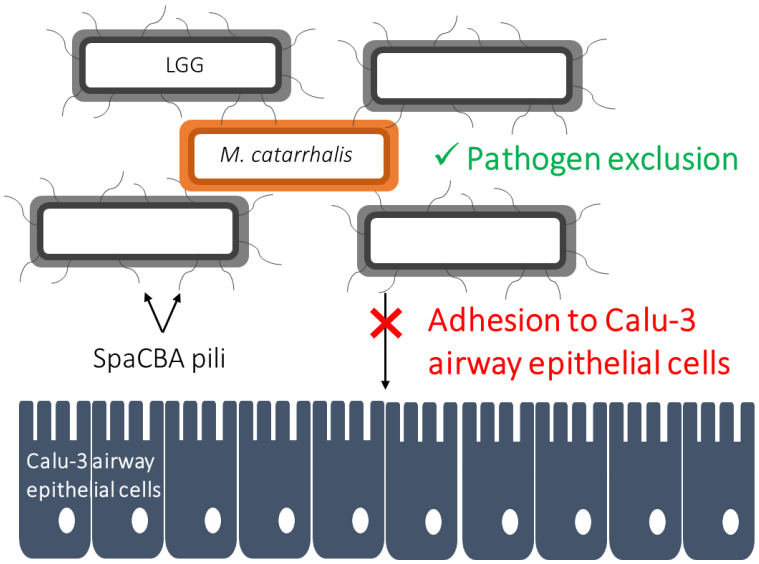


***Figure 4.12: Role of SpaCBA pili of* L. rhamnosus *GG**. Our results suggest a role of the SpaCBA pili in binding with pathogen* M. catarrhalis *and preventing its adhesion to Calu-3 epithelial cells (pathogen exclusion). No role in adhesion to Calu-3 airway epithelial cells was observed.*

As can be derived from its name, the *L. rhamnosus GG* pilus contains three distinct pilin monomers which are covalently linked in a sortase-dependent manner. SpaA forms the pilus backbone and thus has a more structural function while the smaller pilin SpaB is thought to act as a molecular switch responsible for pilus termination and initiation of peptidoglycan binding by the housekeeping sortase (Claes *et al.*, 2012b; von Ossowski *et al.*, 2010). The SpaC pilin, on the other hand, is a key molecule in the adhesion to mucus (Segers and Lebeer, 2014). Former PhD student Tripathi *et al.* (2013) gave more insight in the mechanisms behind interaction of the long flexible pili of *L. rhamnosus* GG with substrates. A zipper-like mechanism involving the SpaC pilin subunits distributed along the pilus length and nanospring properties enable pili to resist high forces such as shear flow or peristalsis. However, in-house experiments to compare the adherence capacity to Calu-3 cells of the *L. rhamnosus* GG wild-type and its pili mutant did not give conclusive results on the importance of the pili in Calu-3 cell adhesion. The role of SpaCBA pili in the competition with other pathogens, on the other hand, has also recently been explored for the pathogen *Enterococcus faecium* E1165 (Tytgat *et al.*, 2016). Their results indicated that the pili of *L. rhamnosus* GG and the pathogen competed for the same binding site on intestinal mucus and that the SpaC pilin played a key role in this activity. Moreover, the SpaCBA pili of *L. rhamnosus* GG have a functional role in balancing IL-8 mRNA expression as mentioned earlier (Lebeer *et al.*, 2012). Follow-up work from von Ossowski *et al.* (2013) used recombinant lactococcal constructs to investigate the contribution of the SpaCBA pilus in the modulation of pro-an anti-inflammatory cytokines. They observed that the SpaCBA pilus influenced the cytokine modulation in human monocyte-derived dendritic cell and that it is a contributory factor in the activation of TLR2-dependent signalling in HEK cells. Whether this is also the case in the nasopharyngeal niche still needs to be evaluated. Future experiments with pili- (or other surface molecule-) mutants to investigate their effect on competition with pathogens and modulation of the immune response would thus be interesting.

We also tried to investigate the influence of *L. rhamnosus* GG on the viability of Calu-3 cells that are exposed to OM pathogens. For this research question, an XTT assay was optimised for the epithelial cells. The oxidised form of XTT is a colourless or slightly yellow compound which does not enter eukaryotic cells due to its negative charge and which, when reduced, becomes a bright orange formazan (Riss *et al.*, 2016). This reduction is mediated by an intermediate electron acceptor, PMS, which transfers electrons from the plasma membrane to XTT to get

the pigmented formazan product. Viable cells with an active metabolism can convert XTT to its reduced form while dead cells cannot. The exact mechanism for this XTT reduction, however, is not fully understood but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT (Marshall *et al.*, 1995). It is assumed that XTT assays are measuring mitochondrial activity (Berridge *et al.*, 2005). The use of the XTT format to investigate cytotoxicity in cells after exposure to bacteria should be discussed as well as a lot of interference between the bacteria and the assay was observed in our experiments. Especially, *H. influenzae* showed high absorbance without presence of eukaryotic Calu-3 cells. Since the supernatant of *H. influenzae* did not interfere with the assay, the living bacteria themselves probably produce molecules that can reduce XTT after exposure to XTT. However, since the exact mechanism behind the XTT assay is not fully understood, it is difficult to screen bacteria beforehand to prevent interference. Several options are described in literature though: chemical compounds such as ascorbic acid, or sulfhydryl-containing compounds including reduced glutathione, coenzyme A, and dithiothreitol are able to reduce tetrazolium compounds such as XTT non-enzymatically (Bernas and Dobrucki, 2002; Chakrabarti *et al.*, 2001; Collier and Pritsos, 2003; Pagliacci *et al.*, 1993; Ulukaya *et al.*, 2004) leading to increased absorbance values. As far as we know, only coenzyme A, is produced by all our bacteria of interest (Bremus *et al.*, 2006), while only *L. rhamnosus* GG is able to produce glutathione (Lebeer *et al.*, 2008a). Further investigations and follow-up experiments necessary to get more insight in the cytotoxicity protection of *L. rhamnosus* GG.

Since these were the first experiments in our lab with Calu-3 cells, a lot of optimisation needed to be done before results could be obtained. In addition, Calu-3 cells are slow growing cells which makes the experiment really time consuming and slow evolving. Furthermore, MIQE guidelines (Bustin *et al.*, 2009) for the qPCR experiments were strictly implemented. A critical view on primers and methods in literature was necessary as many researchers neglect these guidelines (Bustin *et al.*, 2009). Nevertheless, thanks to this research and that of our Calu-3-collaborating colleague, Ilke De Boeck, we could enhance our in-house knowledge about these cells. For instance, we found that an interesting anti-inflammatory cytokine, IL-10, was not significantly produced by Calu-3 cells Furthermore, the experiments were made more high throughput by a transition from 12-well to 24-well plate. Another obstacle, which occurred in

a crucial phase of this research was the emergence of contamination with *Burkholderia contaminans* in the water bath of cell culture room.

5 *IN VIVO* ACTIVITY OF ORALLY ADMINISTERED LACTIC ACID BACTERIA IN THE UPPER RESPIRATORY TRACT OF OM PATIENTS

5.1 INTRODUCTION

As reviewed in Chapter 1, only a limited number of clinical trials have been conducted with potential probiotics and the URT as target human body site. Furthermore, data on the clinical efficiency of probiotics against OM show variable results. Both oral and topical intake of the probiotics has been explored in recent years for possible therapeutic effects (as reviewed in Marom *et al.*, 2016; Niittynen *et al.*, 2012). However, many aspects remain unexplored. For example, studies on the possible transfer of oral administered probiotics to different nasopharyngeal human body sites are currently lacking. Furthermore, the direct influence of the administered probiotic on the presence or relative abundance of OM pathogens and other microbiome members is not yet understood. As shown in the previous chapters, many *Lactobacillus* strains have an *in vitro* antimicrobial and immunomodulatory activity against especially *M. catarrhalis*, including the commercially available and well-documented probiotic strain *L. rhamnosus* GG (LGG).

In this chapter, we designed an *in vivo* study to explore the *in vivo* activity of the *in vitro* selected probiotic *L. rhamnosus GG* in the upper respiratory tract of OME patients needing transtympanic drainage surgery. To obtain more insights in both the more technical and biological questions related to probiotic and (A)OM pathogen colonisation, we designed an intervention study in which children with OM administered orally a commercially available mixture of *L. rhamnosus* GG and *Bifidobacterium lactis* BB-12 (Probactiol® Mini) via liquid oil droplets daily during 4 weeks. The nasopharyngeal microbiome of these children and the concentrations of pathogens and the applied probiotics in their URT was monitored and compared with a control group that did not receive probiotic supplements. More specifically, presence of both probiotic bacteria and (A)OM pathogens was explored used DNA-based strategies, such as dedicated qRT-PCRs and *16S rRNA* gene amplicon sequencing via Illumina MiSeq. In order to perform these DNA-based microbiome interaction studies in the URT of these children, several methodological aspects were explored and optimised.

We especially aimed to obtain data that could provide answers to the following questions:

(1) Can we detect orally administered probiotic strains to be active in the URT and transfer from the oral cavity to the URT, including the nasopharynx, adenoids and middle ear?

(2) Can orally administered probiotics have an impact on the URT microbiome of the nasopharynx, adenoids and middle ear?

5.2 MATERIALS AND METHODS

5.2.1 Subjects

Participants between 2 and 5 years old who had to undergo transtympanic drainage surgery (*i.e.* insertion of ventilation tubes in ear drum to drain fluid from the middle ear) because of OME were recruited between April 2017 and November 2017 via the University Hospital of Antwerp, after approval of the study by the Ethical Committee of the Antwerp University Hospital / University of Antwerp (registration number B300201731908). A written informed consent was obtained from all participants, as well as a general information on their medical history. Potential candidates for the study with trisomy 21 were excluded

5.2.2 Experimental design of the probiotic treatment

A randomised study was performed to investigate the impact on the nasopharyngeal microbiome of a commercially available probiotic product (Probactiol® Mini) containing *L. rhamnosus* GG and *Bifidobacterium animalis* subsp*. lactis* BB-12 in liquid oil drops (Figure 5.1). Nine children were recruited in the hospital, ca. half of the group received the probiotic product and the other half did not, but this was blinded to the people doing the microbial analyses. The probiotic administration had a duration of four weeks just before the planned surgery. The probiotic group had to administer 6 droplets/day (total volume of 270 µL) during 4 weeks containing 0.67 x 10^9 CFU *L. rhamnosus* GG and 0.33 x 10^9 CFU *B. animalis* subsp*. lactis* BB-12 per intake and was asked to fill in a dairy daily for compliance and possible adverse effects. The volunteers were asked to shake the vessel containing the probiotics very, since it was observed to be crucial for an optimal mixture of the bacteria in the suspension.

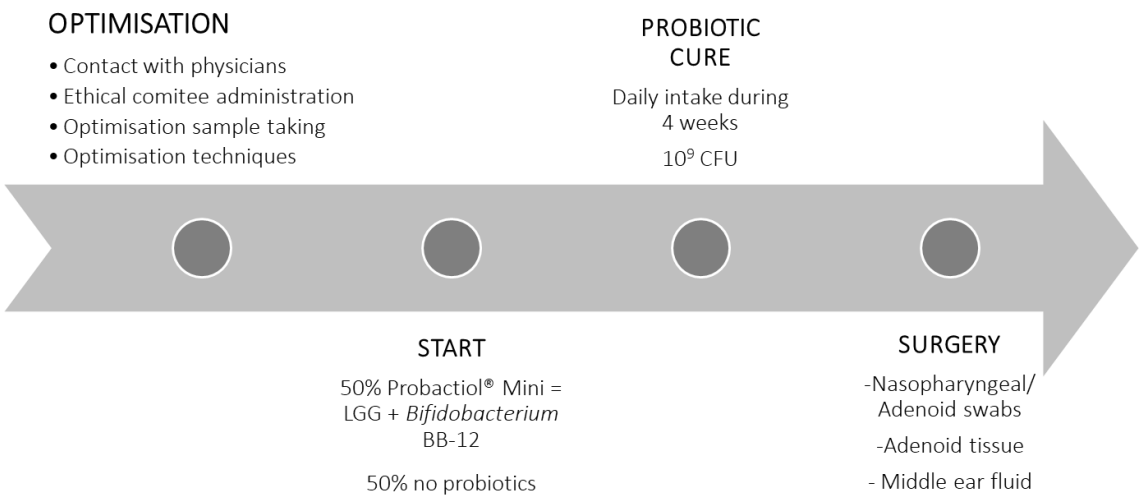


Figure 5.1: Experimental design of intervention study. *Half of the group received the probiotic product (Probactiol® Mini) and the other half did not. The probiotic group had to administer 6 droplets/day during 4 weeks containing 0.67 x 10^9 CFU* L. rhamnosus *GG and 0.33 x 10^9 CFU* B. animalis *subsp.* lactis *BB-12 per intake. During the surgery, following nasopharyngeal samples were taken if possible: nasopharyngeal/adenoid swabs, adenoid tissue and middle ear fluid.*

5.2.3 Sample collection

During the transtympanic drainage surgery combined with adenotomy/adenotonsillectomy, samples were taken when the patients were anesthetised. Middle ear fluid (MEF) was collected via sterile suction in an Argyle Specimen Trap (Covidien). Nasopharyngeal and adenoid swab samples were collected from the patients with flocked swabs (Copan, 503CS01 and 511CS01, respectively). Adenoid tissue samples were collected afterwards in a sterile reservoir. All the sample collections were done in a standardised way by the responsible ENT (ear, nose and throat) specialist. Samples were immediately suspended in 750 µl MoBio bead solution (PowerFecal® DNA isolation kit; MO BIO Laboratories Inc., CA, USA) and placed on ice prior to DNA extraction. DNA extraction took place within 4 hours after sample collection. DNA samples were stored at -20°C until further use.

5.2.4 DNA extraction

The MoBio PowerFecal® DNA isolation kit (lot PF16J31) was used according to the instructions of the manufacturer. DNA concentrations were measured with a Qubit® 3.0 fluorometer (Life Technologies, Ledeberg, Belgium). DNA extractions were performed in a laboratory room

dedicated for DNA/RNA extraction, physically separated from the microbiology room to minimise contamination. DNA was stored at -20°C until further use.

5.2.5 Reference strains and culture conditions

L. rhamnosus GG and *B. animalis* subsp. *lactis* BB-12 were grown overnight at 37°C in de Man, Rogosa and Sharpe (MRS) liquid broth (Difco). Solid media containing 1.5% (w/v) agar was used for bacterial enumeration (48h, 37°C). *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB-12 were grown in a microaerobic and anaerobic environment, respectively.

5.2.6 Bacteria quantification by quantitative PCR (qPCR)

5.2.6.1 Quantitative PCR

The number of total bacteria, *L. rhamnosus* GG, *B. animalis* subsp. *lactis* BB-12 and the (A)OM pathogens, was estimated using primers and probes described in Table 5.1. qPCR was performed in triplicate using Step One Plus RT-PCR system 4376592 (Applied Biosystems, ThermoFisher) with software version 2.3. For testing the probiotics, the Taqman probes contained the minor groove binder (MGB) and a non-fluorescent quencher in combination with a FAM reporter dye. Each PCR amplification contained 15 µL of 2 X Taqman Gene Expression Master Mix (ThermoFischer), 1.33 µL of both primers (10 µM), 1.33 µL of the probe (all IDT, Belgium), 7 µL of MilliQ water and 4 µL 1 to 10 diluted DNA of the sample in a total volume of 30 µL. A no-template control (NTC) was included on each plate. The amplification program consisted of 1 cycle of 50°C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60°C for 1 min for *L. rhamnosus GG* accordingly to Sybesma *et al.* (2013). For *B. animalis subsp. lactis* BB-12 the amplification program consisted of 1 cycle of 50°C for 2 min and 95 °C for 5 min, followed by 50 cycles of 95 °C for 15 s and 60°C for 1 min and 72°C for 30 s accordingly to (Solano-Aguilar *et al.*, 2008). qPCR for the (A)OM pathogens, was performed using Power SYBR Green PCR Master Mix according to manufacturer's instructions. Each PCR amplification contained 10 µL of Power SYBR Green PCR Master Mix, 0.3 µL of both primers (20 µM) and 5.4 µL of MilliQ water and 4 µL 1 to 10 diluted DNA of the sample in a total volume of 20 µL. A no-template control (NTC) was included on each plate. The amplification program consisted of 1 cycle of 50°C for 10 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were strictly followed (Bustin *et al.*, 2009).

Table 5.1: Sequences of primers and probes.

Microorganism	Primers and probes	Sequence 5'-3'	Reference
L. rhamnosus GG	mapF	CCGATCAACAGGCTCAGTGA	(Sybesma *et al.*, 2013)
	mapR	CATGTTGTGCGCTTGGAAAA	
	MGB-probe map	TTGCACTTGATTGTTTCG	
B. animalis subsp *lactis* BB-12	tufF	GTGTCGAGCGCGGCAA	(Solano-Aguilar *et al.*, 2008)
	tufR	CTCGCACTCATCCATCTGCTT	
	MGB-probe tuf	ATCAACACGAACGTCGAGA	
M. catarrhalis	copBF	GTGAGTGCCGCTTTACAACC	(Bootsma *et al.*, 2000)
	copBR	TGTATCGCCTGCCAAGACAA	
H. influenzae	fucKF	TCAATGCTCACTSAACGCTTAAC	(Latham *et al.*, 2015)
	fucKR	ACGCATAGGAGGGAAATGGT	
S. pneumoniae	plyF	AGCGATAGCTTTCTCCAAGTGG	(Greiner *et al.*, 2001)
	plyR	CTTAGCCAACAAATCGTTTACCG	

The cycle threshold of each sample was then compared to standard curves made by diluting genomic DNA (10-fold serial dilution) from cultures of *L. rhamnosus* GG, *B. animalis* subsp. *lactis* BB-12 and the (A)OM pathogens. Bacterial CFU enumeration of these strains was determined via the plating method prior to DNA extraction. When plate count correlated with the estimated CFU/mL, DNA of the technical repetitions was pooled and used as a reference.

5.2.6.2 Sensitivity and performance of the qPCR assays

To evaluate possible inhibition of DNA extraction by biological material, two dilutions (10^5 and 10^4 CFU) of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* BB-12 were or were not spiked with MEF, obtained from a different study (ClinicalTrials.gov Identifier: NCT03109496) and DNA was extracted. After DNA extraction, as described above, qPCR was performed and the results were compared.

5.2.6.3 Spiking method to test for PCR inhibition

To test for possible PCR inhibition, 18 µL of master mix (consisting of 10 µL Power SYBR Green PCR Master Mix, 0,3 µL of 515F-806R primers (Caporaso *et al.*, 2011) and 7,4 µL of MilliQ water) was mixed with 2 µL of a sample containing DNA. 3 mixtures were tested: (mixA) 1 µL of a high

concentration foreign DNA sample (not from this study) + 1 µL milliQ water; (mixB) 1 µL nasopharyngeal DNA sample + 1 µL of milliQ water; and (mixC) 1 µL of nasopharyngeal DNA sample + 1 µL of a high concentration foreign DNA sample (not from this study). A no-template control (NTC) was included. The amplification program consisted of 1 cycle of 50°C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 55°C for 30 s and 60°C for 1 min. MIQE guidelines were strictly followed (Bustin *et al.*, 2009).

5.2.7 Illumina MiSeq *16S rRNA* amplicon sequencing

The primers used for Illumina MiSeq sequencing were based on the previously described 515F-806R primers (Caporaso *et al.*, 2011) and altered for dual-index paired-end sequencing, as described by (Kozich *et al.*, 2013). Briefly, each DNA sample was subjected to dual barcoded PCR, amplifying the V4 region of the *16S rRNA* gene using Phusion High-Fidelity DNA polymerase (New England Biolabs, USA), which were then sequenced. PCR products were purified by the Agencourt AMPure XP magnetic bead capture kit (Beckman Coulter, Suarlee, Belgium), and quantified using the Qubit® 3.0 fluorometer. The library was prepared by pooling all PCR samples in equimolar concentration and loaded onto a 0.8% agarose gel. The product was purified by gel extraction using the Nucleospin® Gel and PCR clean-up (Machery-Nagel). The final library concentration was determined with the Qubit® 3.0 fluorometer. The library was denatured with 0.2 N NaOH (Illumina), diluted to 7 pM and spiked with 10% PhiX control DNA (Illumina). The library was loaded onto the flow cell of the v2 chemistry MiSeq reagent kit (paired-end dual indexing sequencing; 2 x 250 bp kit; Illumina, San Diego, California, USA) on the MiSeq Desktop sequencer (M00984, Illumina) at the Centre of Medical Genetics, University of Antwerp, Belgium.

5.2.8 Sequence processing and biostatistical analysis

Raw sequencing reads were filtered and denoised using the DADA2 pipeline (v 1.1.6), as described in (Callahan *et al.*, 2016). The DADA2 method is a denoising algorithm that infers the set of most specific biological variants (called amplicon sequence variants or ASVs abbreviated here) that are not the result of sequencing errors. Of note, we prefer the abbreviation ASVs throughout our manuscript (Callahan *et al.*, 2017) and not ribosomal sequence variants (RSVs) such as in the original manuscript (Callahan *et al.*, 2016), because of the possible confusion with RSV being an important virus in the URT. The algorithm does this by modelling the sequencing

error rate as a function of quality score and error type (e.g. A being sequenced as C). It alternates between two steps until convergence: i) inferring the 'real' underlying sequences given the error rates, and ii) estimating the error rates given the real sequences.

Briefly, in the used DADA2 pipeline, paired reads were filtered by excluding reads with more than two expected errors and reads that contained undetermined bases. Based on a visual inspection of the quality score profiles, trimming was performed by removing nucleotides outside the position interval 13 to 240 for forward reads and 13 to 220 for reverse reads. Next, DADA error correction was applied using alternation of sample inference and error rate estimation until convergence. Forward and reverse reads were then merged into contigs, and contigs longer than 230 nucleotides were removed. At this point, chimeras were removed by the "removeBimeraDenovo" function. Taxonomic annotation from the kingdom to the genus level was then assigned to the remaining ASVs, making use of the Silva reference database (version 123 from the DADA2 website). Finally, ASVs classified as Archaea, Eukarya, chloroplasts or mitochondria were removed, as well as global singleton and doubleton DSVs.

The resulting ASV table was imported and analysed in R, using the phyloseq (v 1.16.2) (Mcmurdie and Holmes, 2013), ggplot2 (v 2.1.0) (Ginestet, 2011), and vegan package (v 2.3-5) (Oksanen *et al.*, 2016). Observed ASV-richness and the inverse Simpson index calculated on the non-normalised read count data were used as alpha-diversity indices. Bray-Curtis distance calculated on the relative abundance data was used as beta-diversity metric. Ordination techniques at genus level and clustering techniques at both genus and ASV level were performed.

5.2.9 Questionnaire and monitoring some quality-of-life aspects and adverse events

The parents of the patients were asked to fill in a dairy daily during the probiotic cure to indicate the health status of the child (ear ache/swollen adenoids) (see A. Appendix). The day of the surgery, a questionnaire was filled in about earlier OM events, the use of antibiotics or other drugs the 4 last weeks, the mode of delivery, breastfeeding duration, the amount of siblings, vaccination, exposure to cigarette smoke or pets, day-care attendance, other URT diseases and intake of fermented food.

5.2.10 Statistics

Data are represented as mean values ± standard deviation. One -way ANOVA and t-tests were used to determine statistical significance in GraphPad Prism. Differences were considered statistically significant at $P<0.05$.

5.3 RESULTS

5.3.1 Sample details of patients

Six children between 2 and 12 years old were included in the intervention study until now. Information of all the patients and their samples is given in Table 5.2. At the end of the intervention study, half of the study population will have received Probactiol® Mini, while the other half will not, but this is currently blinded to the microbiology researchers involved in the study.

Table 5.2: Information about patients and their samples. *NFS: nasopharyngeal swab; ADS: adenoid swab; ADT: adenoid tissue; MRA: aspirate MEF right middle ear; MLA: aspirate MEF left middle ear.*

Patient	Sample	Date Surgury	Sex	Age	Conc.DNA [ng/µL]
1	NFS	May 16, 2017	M	2	37.3
	ADS				148
	ADT				594
	MRA				36
2	NFS	May 16, 2017	V	3	38
	ADS				135
	ADT				434
	MRA				36.7
	MLA				38
3	NFS	June 6, 2017	M	2	7,47
	ADS				5,72
	MRA				3,74
4	NFS	June 15, 2017	V	3	43,2
	ADS				144
	MLA				7,08
5	NFS	November 16, 2017	V	2.5	9.530
	ADS				5.990
	ADT				too low
	MRA				0.595
	MLA				4.300

6	NFS	December 19, 2017	M	2.5	16.6
	ADS				5.480
	MRA				too low
	MLA				too low
7	NFS	November 16, 2017	M	4.5	3.63
	ADS				22.7
	MRA				0.84
	MLA				0.327
8	NFS	December 21, 2017	M	3	20.4
	ADS				18.0
	MRA				17.7
	MLA				18.9
9	NFS	January 4, 2018	M	4	14.5
	ADS				14.6
	ADT				14.8
	MRA				14.6
	MLA				13.6

5.3.2 Detection of specific probiotics and (A)OM pathogens via qPCR

DNA of clinical samples of OME patients was tested for PCR inhibition with a spiking method. Three mixtures were tested: (mixA) 1 µL of a high concentration foreign DNA sample (not from this study) + 1 µL milliQ water; (mixB) 1 µL nasopharyngeal DNA sample + 1 µL of milliQ water; and (mixC) 1 µL of nasopharyngeal DNA sample + 1 µL of a high concentration foreign DNA sample (not from this study). A no-template control (NTC) was included. When no inhibition occurs, the Ct values of the mixC samples should be lower or the same (*i.e.* the same or more DNA) than those of mixA. However, the spiking of the DNA of our nasopharyngeal samples with a high concentration of foreign DNA (see §5.2.6.3 for details) resulted in higher Ct values (except for sample 2), which indicates the presence of PCR inhibitors in the nasopharyngeal samples (Figure 5.2A). 1/10 dilutions of nasopharyngeal DNA samples were then checked with barcoded MiSeq primers for the V4 region, which showed to diminish the PCR inhibition as shown in Figure 5.2B. For this reason, we always used a 1/10 dilution of the clinical samples in further assays. Subsequently, the influence of the presence of MEF during DNA extraction was investigated as described in §5.2.6.2, because we hypothesised that the high amount of mucus and slime polysaccharides ('glue') in the MEF could interfere with the DNA extractions. However, MEF did not appear to significantly affect the DNA extraction efficiency (Figure 5.2C).

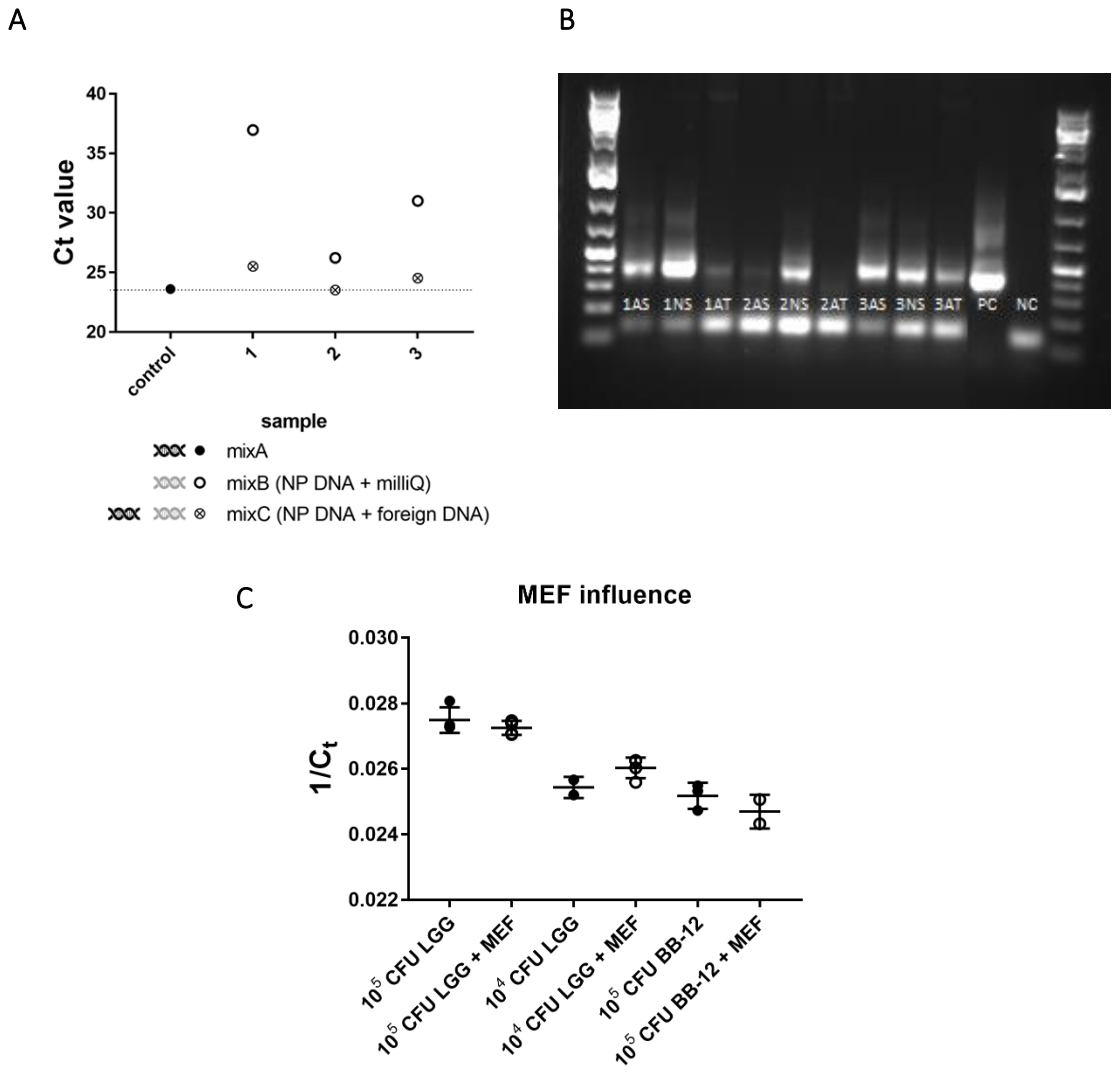


Figure 5.2: Possible PCR or extraction inhibition in nasopharyngeal samples.*(A) a qPCR experiment was performed to test for PCR inhibition. The spike control represents a high concentration of foreign DNA sample (see Materials and Methods for details) and is called mixA. Addition of other DNA samples to this spike control should normally decrease the Ct value of the mix. mixB is the combination of nasopharyngeal (NP) DNA and milliQ water; mixC is the combination of nasopharyngeal DNA and the high concentration of spike foreign DNA. (B) PCR gel of 1/10 diluted nasopharyngeal DNA samples. NS: nasopharyngeal swab; AS: adenoid swab; AT: adenoid tissue; PC: positive control; NC: negative control. (C) Influence of presence MEF during DNA extraction.*

After these control and optimisation steps, the DNA samples of the intervention study were analysed and compared with DNA of known amounts of *L. rhamnosus GG*, BB-12 and the (A)OM pathogens. As shown in Figure 5.3, we plotted the inverse of the C_t value for the different intervention study samples, A higher $1/C_t$ reflects a higher DNA concentration in the samples, allowing a more intuitive visualisation than mere Ct values. Interestingly, the qPCR data clearly showed the presence of *L. rhamnosus GG* in the upper respiratory tissues of patients 1 and 2, namely in the adenoid and nasopharynx of both patients and even in the middle ear of one

patient. No *L. rhamnosus GG* was detected in the other four patients. When the results are compared with the controls, ca. 10^4 CFU of LGG/sample were detected in the samples. A similar analysis was done for the detection of *B. lactis* BB-12. However, in none of the patient's samples, BB-12 could be detected.

Subsequently, we also explored by dedicated qPCR experiments whether we could observe a difference in DNA-concentration of the (A)OM pathogens. Amounts varying between 0 CFU and 10^7 CFU/sample were detected in the different samples and these data confirm that the OM pathogens studied *in vitro* in Chapters 2-4 also occur in relative high numbers in URT samples of OME patients (Figure 5.4). No clear difference was seen between different patients with or without probiotic intervention.

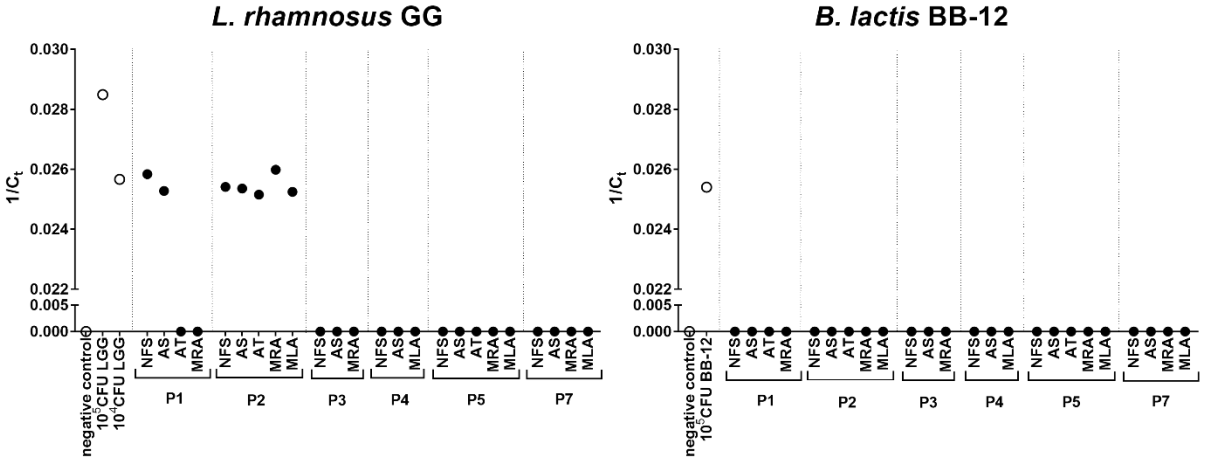


Figure 5.3: $1/C_t$ values of LGG and BB-12 DNA samples of intervention study. Control samples are depicted with open dots. NFS: nasopharyngeal swab; ADS: adenoid swab; ADT: adenoid tissue; MRA: middle ear aspirate right; MLA: middle ear aspirate left. Intervention study patients are identified with patient number (see Table 5.2).

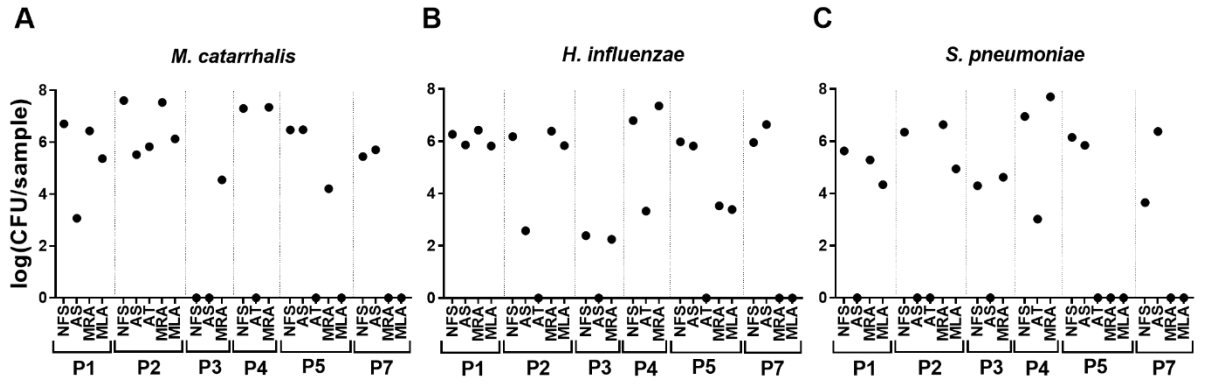


Figure 5.4: Estimated log (CFU/sample) values of (A)OM pathogen DNA samples of intervention study. (A) M. catarrhalis*, (B)* H. influenzae *and (C)* S. pneumoniae*. Intervention study patients are identified with patient number (see Table 5.2).*

5.3.3 Microbiome analysis of upper respiratory tract after the oral probiotic intervention

We then analysed the overall bacterial URT community by *16S rRNA* amplicon sequencing of the different patients and identified the sequences via EzBioCloud (Yoon *et al.*, 2017). We admit that due to the deep sequencing and barcode leakage, some spurious ASVs can be formed and errors can occur during identification. However, this analysis allowed a more high-throughput, detailed mapping of differences between patients and between samples from the same patient, as this analysis was unbiased towards the probiotics and pathogens under study (Figure 5.5 for patients 1-4, Figure 5.6 for patients 5-9). Generally, more similarity in the relative abundance profile was observed between the samples of one patient than between different patients but importantly the pathogens were present in all patients. *Moraxella* and *Haemophilus* seemed to be most abundant genera in all URT samples confirming also a key role for these (A)OM pathogens in OME. Interestingly, the microbial community of both middle ear aspirates (left and right) in patient 2 showed to be similar, with *M. catarrhalis* as most abundant ASV (Figure 5.5B and Table 5.3), suggesting that it is indeed an important pathogen in OME, which is highly relevant for our focus on *M. catarrhalis* in Chapters 2-4. Furthermore, this pathogen is also present in the other eight patients although in lower amounts (Figure 5.5B for patients 1-4 and Figure 5.6B patients 5-9). Different *Haemophilus* ASVs, the other most abundant ASVs, were identified as *H. aegyptius* and *H. influenzae / haemolyticus/ quentini*, respectively (Table 5.3 and Table 5.4). *H. aegyptius* is already known for its contribution in conjunctivitis and Brazilian purpuric fever (Kilian, 2015) and also seems to be present in higher relative abundances in the nasopharynx of chronic rhinosinusitis patients compared to healthy controls (De Boeck *et al.,* unpublished results). *H. influenzae,* on the other hand, is known to be one of the main pathogens in OM, hence also a focus of this thesis.

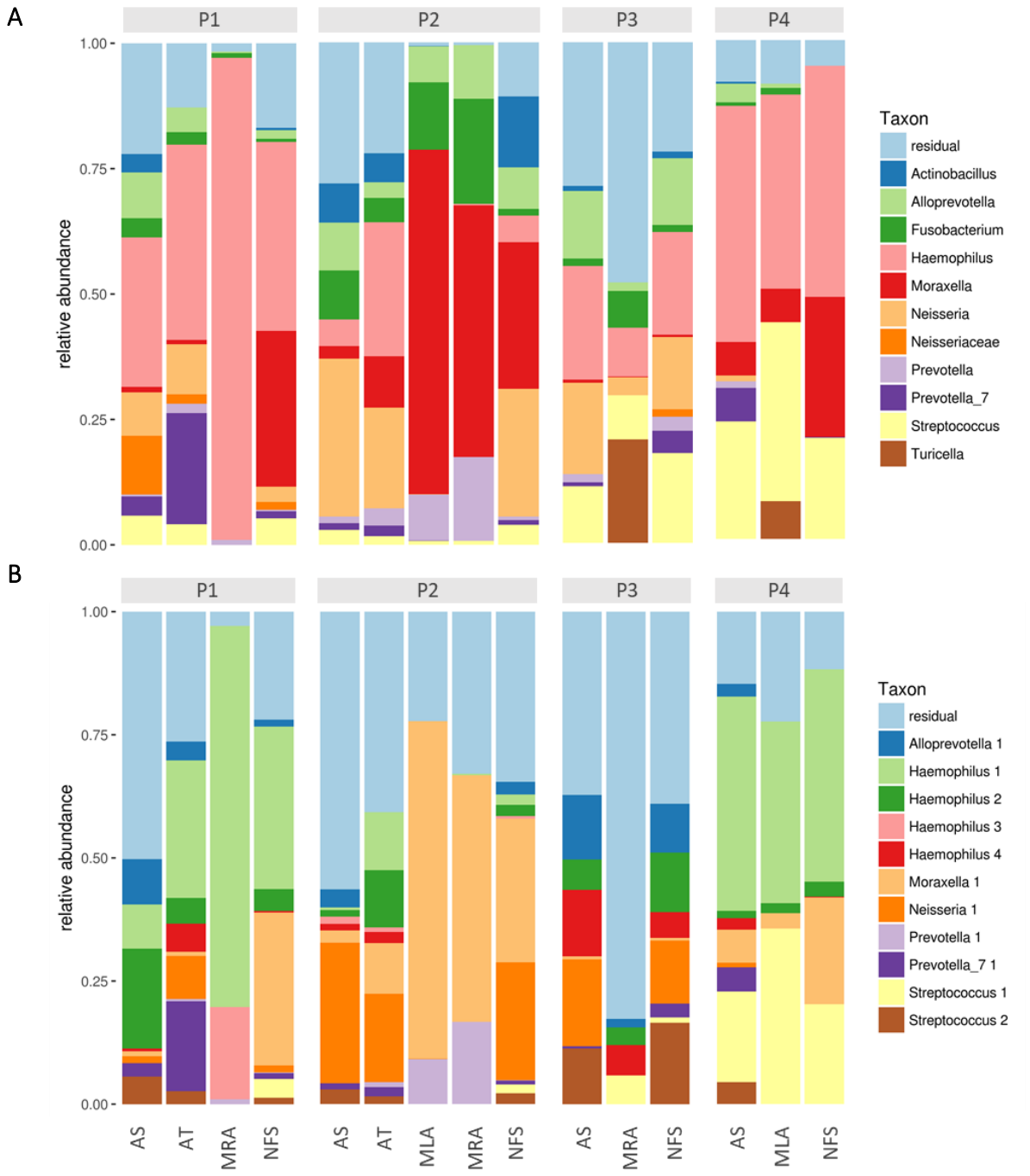


Figure 5.5: Relative abundance profile of intervention study patients 1-4*. (A) general microbiome, and (B) ASV microbiome. NFS: nasopharyngeal swab; AS: adenoid swab; AT: adenoid tissue; MRA: middle ear aspirate right; MLA: middle ear aspirate left. Intervention study patients are identified with patient number (see Table 5.2). The top 10 most abundant genera or ASV (amplicon sequence variants) are shown.*

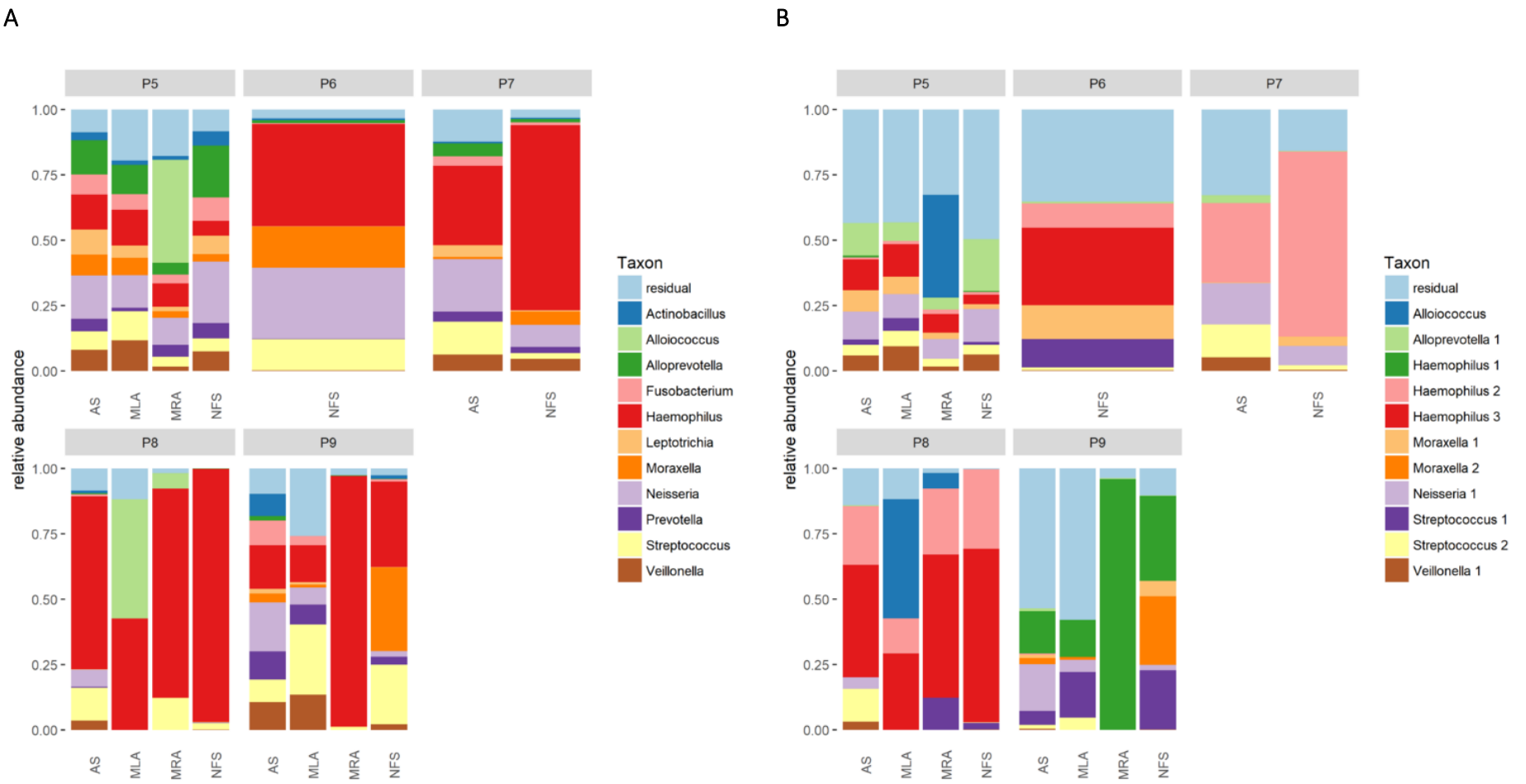


Figure 5.6: Relative abundance profile of intervention study patients 5-9*. (A) general microbiome, and (B) ASV microbiome. NFS: nasopharyngeal swab; AS: adenoid swab; AT: adenoid tissue; MRA: middle ear aspirate right; MLA: middle ear aspirate left. Intervention study patients are identified with their patient number (see Table 5.2). The top 10 most abundant genera or ASV (amplicon sequence variants) are shown.*

Table 5.3: Details specific ASVs for general overview of patients 1-4

Order	Family	Genus	Species	Taxon name
Bacteroidales	*Prevotellaceae*	*Alloprevotella*	*n.a.*	Alloprevotella 1
Pasteurellales	*Pasteurellaceae*	*Haemophilus*	*aegyptius*	Haemophilus 1
Pasteurellales	*Pasteurellaceae*	*Haemophilus*	*influenzae / haemolyticus/ quentini*	Haemophilus 2
Pasteurellales	*Pasteurellaceae*	*Haemophilus*	*influenzae/ parainfluenzae*	Haemophilus 3
Pasteurellales	*Pasteurellaceae*	*Haemophilus*	*influenzae/ parainfluenzae*	Haemophilus 4
Pseudomonadales	*Moraxellaceae*	*Moraxella*	*catarrhalis*	Moraxella 1
Neisseriales	*Neisseriaceae*	*Neisseria*	*perflava/ subflava/ mucosa/ cinerea*	Neisseria 1
Bacteroidales	*Prevotellaceae*	*Prevotella*	*oris*	Prevotella 1
Bacteroidales	*Prevotellaceae*	*Prevotella_7*	*melaninogenica*	Prevotella_7 1
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*pneumoniae / pseudopneumoniae*	Streptococcus 1
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*infantis/ oralis subsp. dentisani / oralis subsp. tigurinus/ mitis/ oralis subsp. oralis/ oralis*	Streptococcus 2

Table 5.4: Details specific ASVs for general overview of patients 5-9

Order	Family	Genus	Species	Taxon name
Carnobacteriaceae	*Lactobacillales*	*Alloiococcus*	*otitis*	Alloiococcus
Bacteroidales	*Prevotellaceae*	*Alloprevotella*	*n.a.*	Alloprevotella 1
Pasteurellales	*Pasteurellaceae*	*Haemophilus*	*n.a.*	Haemophilus 1
Pasteurellales	*Pasteurellaceae*	*Haemophilus*	*n.a.*	Haemophilus 2
Pasteurellales	*Pasteurellaceae*	*Haemophilus*	*aegyptius*	Haemophilus 3
Pseudomonadales	*Moraxellaceae*	*Moraxella*	*catarrhalis/ nonliquefaciens*	Moraxella 1
Pseudomonadales	*Moraxellaceae*	*Moraxella*	*canis/porci*	Moraxella 2
Neisseriales	*Neisseriaceae*	*Neisseria*	*perflava/ subflava/ mucosa/ cinerea*	Neisseria 1
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*pneumoniae / pseudopneumoniae*	Streptococcus 1
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*n.a.*	Streptococcus 2
Veillonellaceae	*Veillonellales*	*Veillonella*	*n.a.*	Veillonella 1

In addition to the global bacterial profiles based on relative abundances of the most abundant taxa, we also performed a dedicated analysis for the order of *Lactobacillales* (LAB) and

Bifidobacteriales as they are rarely found in the top 10 of most abundant ASVs. Although no *Bifidobacteriales* were detected*,* this in-depth analysis did show the presence of *Lactobacillales.* Of note, the order of *Lactobacillales* contains both potential pathogens such as *S. pneumoniae,* as well as potential probiotic *Lactobacillus* species with a qualified presumption of safety (QPS) status- in Europe. Of particular interest, *Dolosigranulum pigrum* (Table 5.5 and Table 5.6), a lactic acid bacterium which is commonly linked to a healthy development of the URT microbiome as discussed in the literature study (Chapter 1), was observed in all nasopharyngeal swabs. Furthermore, although a high number of *Streptococcus* ASVs was found in the patients, only Streptococcus 1 corresponded with an OM pathogen *S. pneumoniae*. Interestingly, this species was found in a higher abundance in patients 3 and 4, patients in who we could not detect *L. rhamnosus GG*. In patients 5-9, we cannot make such comparisons as only in patient 8 a *Lactobacillus* ASV was observed after *16S rRNA* amplicon sequencing (Table 5.8)and Streptococcus 1 is present in all patients, except for patient 7 (Figure 5.7B).

When further focusing only on *Lactobacillus*-genus specific ASVs, we detected in two aspirate samples of the middle ear of patients 2 and 4, each two ASVs of *Lactobacillus* (Figure 5.8A) and in two aspirates of patient 8 one ASV of *Lactobacillus*. As shown in Table 5.7 and in Table 5.8, the *Lactobacillus* 6 taxon, present in patient 2, and the *Lactobacillus* taxon in patient 8 include the *L. rhamnosus* ASV and thus possibly represents the presence of *L. rhamnosus* GG as described earlier in the qPCR data for patient 2. The *Lactobacillus* 7 taxon, in contrast, possibly represents *L. iners*. The lactobacilli in patient 4, on the other hand, were identified as *L. acidophilus/gallinarum/crispatus* for taxon 3 and *L. oris* for taxon 5.

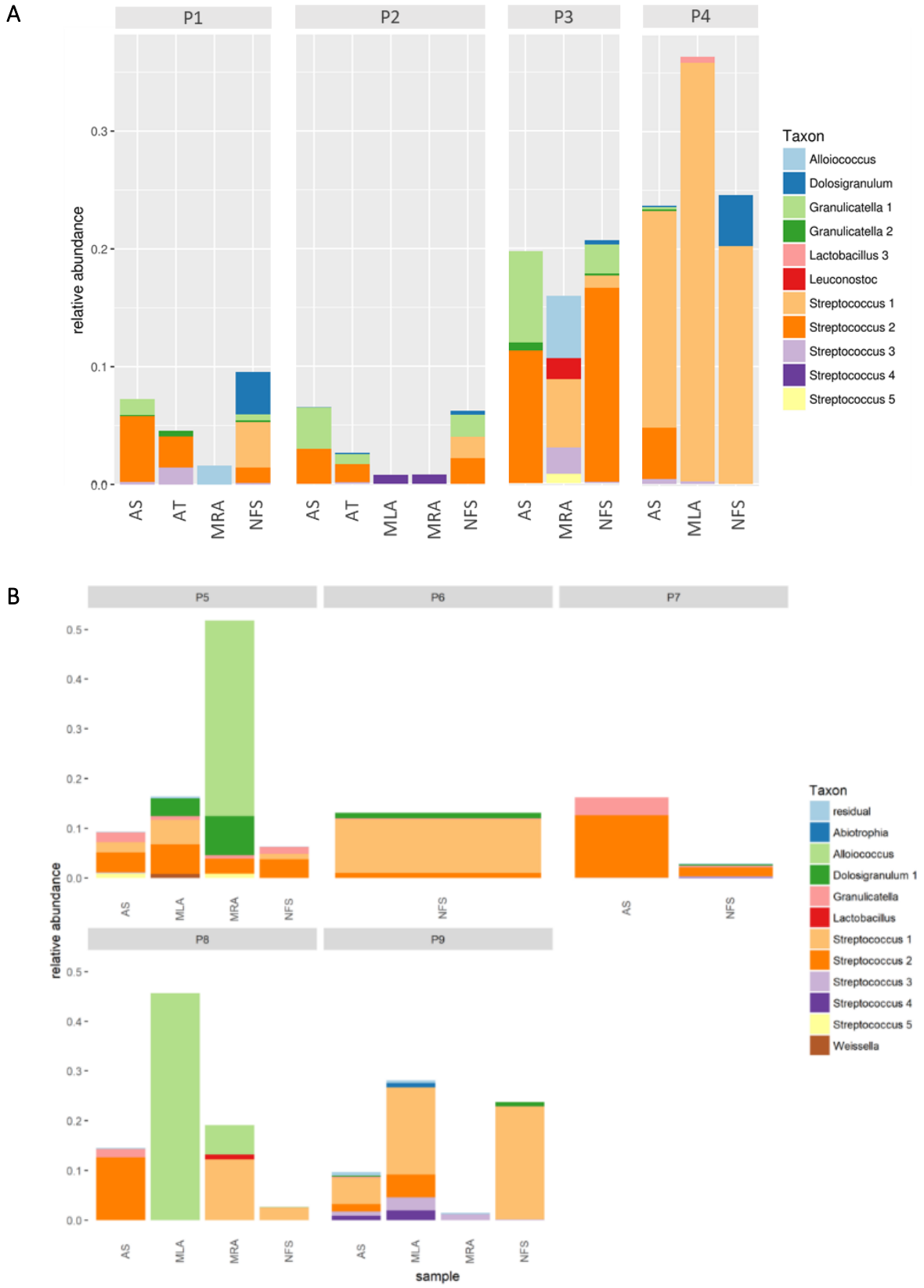


Figure 5.7: Lactobacillales ***focused relative abundance profile of intervention study patients (A) 1-4, and (B) 5-9.*** *NFS: nasopharyngeal swab; AS: adenoid swab; ADT: adenoid tissue; MRA: middle ear aspirate right; MLA: middle ear aspirate left. Intervention study patients are identified with their patient number (see Table 5.2). The top 10 most abundant genera or ASV (amplicon sequence variants) are shown.*

Table 5.5: Details specific ASVs for Lactobacillales *order of patient 1-4.*

Order	Family	Genus	Species	Taxon name
Lactobacillales	*Carnobacteriaceae*	*Alloiococcus*	*otitis*	Alloiococcus
Lactobacillales	*Carnobacteriaceae*	*Dolosigranulum*	*pigrum*	Dolosigranulum
Lactobacillales	*Carnobacteriaceae*	*Granulicatella*	*elegans*	Granulicatella 1
Lactobacillales	*Carnobacteriaceae*	*Granulicatella*	*adiacens*	Granulicatella 2
Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*acidophilus / gallinarum / crispatus*	Lactobacillus 1
Lactobacillales	*Leuconostocaceae*	*Leuconostoc*	*suionicum/ mesenteroides*	Leuconostoc
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*pneumoniae/ pseudopneumoniae*	Streptococcus 1
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*infantis / oralis subsp. dentisani / oralis subsp. tigurinus / mitis / oralis subsp. oralis/ oralis*	Streptococcus 2
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*hongkongenis/ vestibularis/ salivarius subsp. salivarius / thermophilus*	Streptococcus 3
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*constellatus subsp. viborgensis/ constellatus subsp. constellatus/ constellatus subsp. pharyngis*	Streptococcus 4
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*gordonii/ sinensis/ cristatus/ himalayensis/ oligofermentans/*	Streptococcus 5

Table 5.6: Details specific ASVs for Lactobacillales *order of patient 5--9.*

Order	Family	Genus	Species	Taxon name
Lactobacillales	*Aerococcaceae*	*Abiotrophia*	*defectiva*	Abiotrophia
Lactobacillales	*Carnobacteriaceae*	*Alloiococcus*	*otitis*	Alloiococcus
Lactobacillales	*Carnobacteriaceae*	*Dolosigranulum*	*pigrum*	Dolosigranulum 1
Lactobacillales	*Carnobacteriaceae*	*Granulicatella*	*elegans*	Granulicatella
Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*casei/paracasei/ zeae/ rhamnosus*	Lactobacillus
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*pneumoniae/ pseudopneumoniae*	Streptococcus 1
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*n.a.*	Streptococcus 2
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*n.a.*	Streptococcus 3
Lactobacillales	*Streptococcaceae*	*Streptococcus*	*n.a.*	Streptococcus 4

Lactobacillales	*Streptococcaceae*	*Streptococcus*	*pyogenes*	Streptococcus 5
Lactobacillales	*Leuconostocaceae*	*Weisella*	*bombi/cibaria/confusa/hellenica*	Weisella

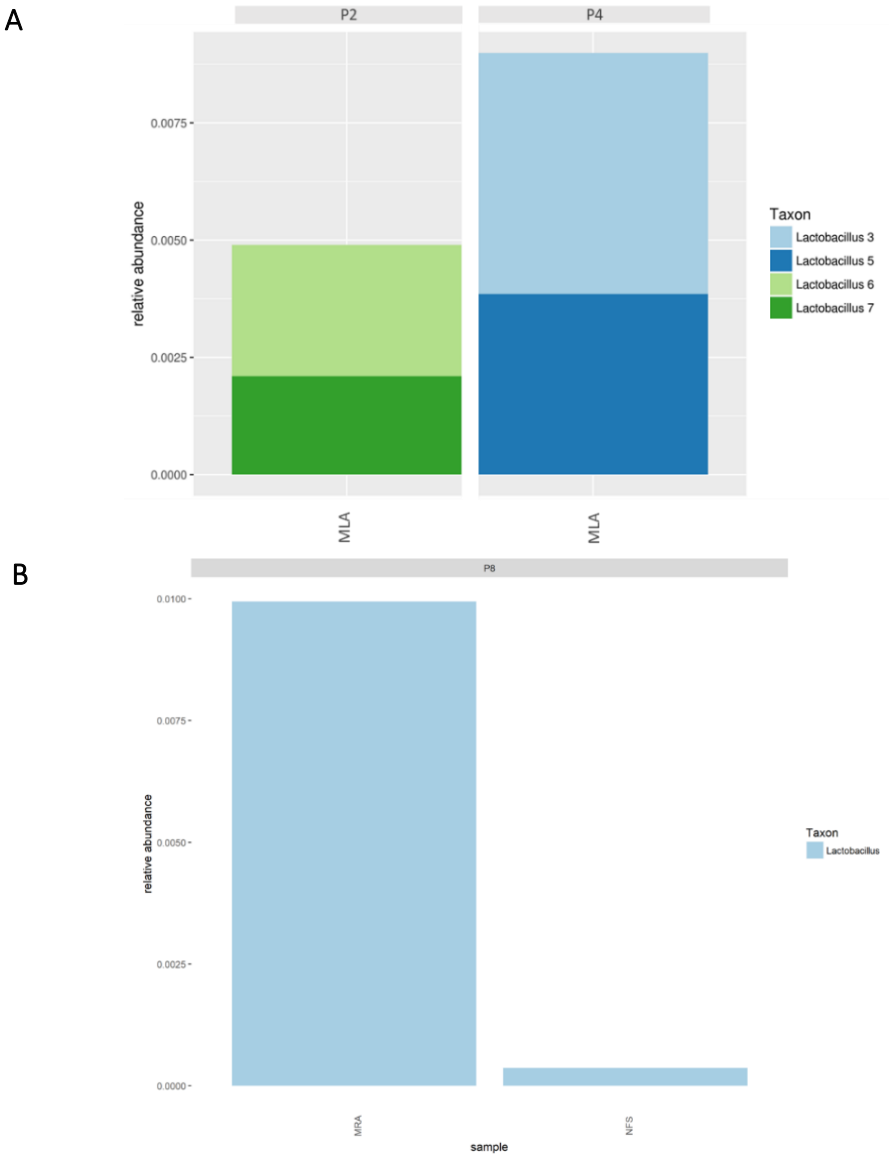


Figure 5.8: Lactobacillus ***focused relative abundance profile of intervention study patients (A) 1-4, and (B) 5-9.*** *MRA: middle ear aspirate right; MLA: middle ear aspirate left. Intervention study patients are identified with their patient number (see Table 5.2).*

Table 5.7: Details specific ASVs for Lactobacillus ***genus for patients 1-4.***

Order	Family	Genus	Species	Taxon name
Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*acidophilus/ gallinarum/ crispatus*	Lactobacillus 3
Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*oris*	Lactobacillus 5
Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*casei/ paracasei/ rhamnosus/ zeae*	Lactobacillus 6
Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*iners*	Lactobacillus 7

Table 5.8: Details specific ASV for Lactobacillus *genus for patient 8.*

Order	Family	Genus	Species	Taxon name
Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*casei/ paracasei/ rhamnosus/ zeae*	Lactobacillus

5.4 DISCUSSION

In this chapter we aimed to investigate the potential of orally administered probiotics (*L. rhamnosus* GG and *B. lactis* BB-12) to persist in the URT and to influence the URT microbiome and OM pathogen colonisation. Although orally applied probiotics can benefit the URT via systemic immune effects (as reviewed in Chapter 1), it is also not unlikely that orally ingested probiotics can actually also transfer to regions of the URT via the nasopharynx, since all these niches are interlinked (see Figure 5.9 for the anatomy). It can also be postulated that the matrix/formulation/delivery format by which the probiotics are administered will play a key role in this transfer. Therefore, a key finding in this chapter is that we could show that orally applied *L. rhamnosus* GG (in a small volume in droplets) can withstand in the nasopharynx, as detected by a dedicated and specific qPCR. In fact, the amount of DNA of *L rhamnosus* GG detected in both swap, tissue and aspirate samples correlated with DNA of 10^4 CFU. Furthermore, *L. rhamnosus GG* could maybe even be picked up in three samples via *16S rRNA* amplicon sequencing. Interestingly, also *L. iners* was detected via *16S rRNA* amplicon sequencing which was in agreement with other in-house microbiome studies of the URT (De Boeck *et al.*, 2017). This *Lactobacillus* species can often dominate the vagina and was shown to have evolved the highest degree of niche specialisation (towards the human host) observed among the currently known lactobacilli (Duar *et al.*, 2017). How this species is able to survive in the URT niche and how it might differ from the strains in the vagina would thus be an interesting topic for further research. The orally applied probiotic *B. lactis* BB-12, could, in contrast to *L. rhamnosus GG*, not be detected in the URT samples. Of note, the qPCR was less sensitive for this probiotic as detection of the strain was only possible from 10^5 CFU on. So whether our detection method was not sensitive enough, the DNA extraction not adequate or *B. lactis* BB-12 was not able to persist the URT environment since it is an anaerobic bacterium needs to be further investigated. However, no DNA of the order *Bifidobacteriales* was observed via *16S rRNA* amplicon sequencing either suggesting that *B. lactis* BB-12 is not an ideal URT probiotic.

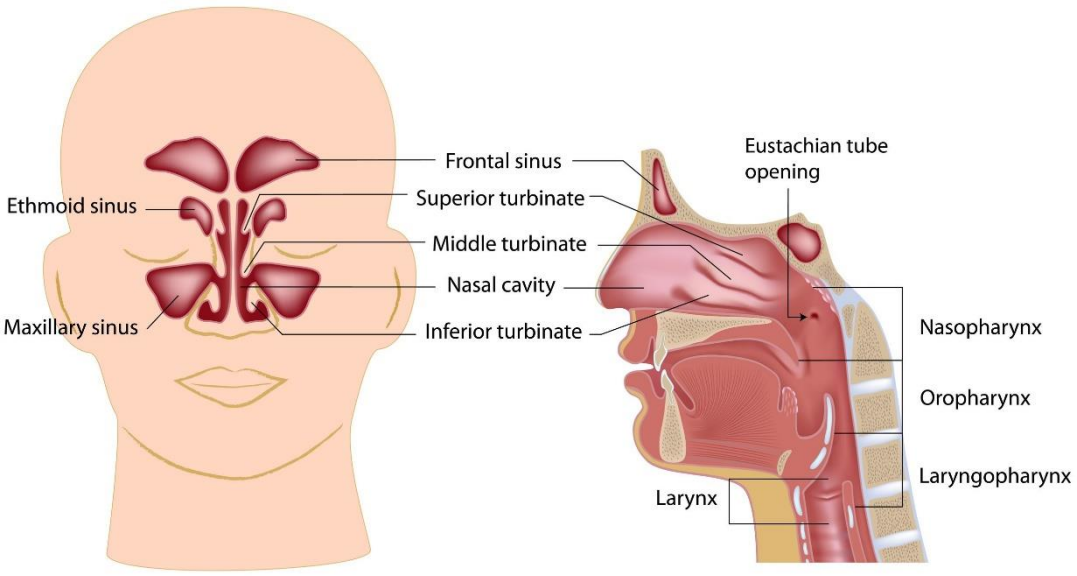


Figure 5.9: Anatomy nasopharyngeal niche*. Figure purchased from 123rf.com.*

As to the changes in the microbiome, the few human studies conducted which investigated the effect of orally administered probiotics on the pathogen load indicated that some specific *Lactobacillus* and *Lactococcus* species could protect the microbiota in the URT from colonisation with potentially pathogenic species (see Chapter 1). Application modes and formulation matrices, such as drinks, that result in longer contact times with the oral mucosa will probably result in a greater chance of transfer to the URT than tablets that are rapidly ingested and mainly released into the gastro-intestinal tract. Oil droplets, on the other hand, can be located in between these two. A probiotic fermented milk drink containing *L. rhamnosus* GG, *Bifidobacterium* sp B420, *Lactobacillus acidophilus* 145 and *Streptococcus thermophilus* was shown by cultivation to reduce potential pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, beta-hemolytic streptococci, and *Haemophilus influenzae*) in the URT (Glück and Gebbers, 2003). In contrast, other studies investigating oral administration of *L. rhamnosus* GG for 3 weeks did not observe any reduction in bacterial or viral pathogen carriage as monitored by qPCR (Swanljung *et al.*, 2015; Tapiovaara *et al.*, 2014). In our data presented here, the microbiome of the investigated samples did resemble the OME microbiome in literature as reviewed in Chapter 1. *Moraxella* and *Haemophilus* were the most abundant taxa together with *Streptococcus* and *Neisseria,* which is of high relevance for our *in vitro* screening assays presented in Chapter 2-4. Also *Turicella*, a pathogen on which there is currently little knowledge, appeared in the results. Including other pathogens such as *Neisseria*

and *Turicella* in the screening platform would thus be interesting for future research. Interestingly, *Turicella* was only detected in in high relative abundances in middle ear samples of patients in who's samples we did not detect *L. rhamnosus* GG while *Moraxella* dominated the microbiome profile of patient 2, where we did find *L. rhamnosus GG*. However, the amount of patients included is still too small to draw conclusions about possible protection against pathogens. In addition, no significant differences in pathogen carriage monitored by qPCR between the probiotic and non-probiotic group and large variation (0– 10^7 CFU) between the amount of pathogens present were observed. This needs to be further investigated.

Furthermore, the intervention study performed, was a first screening of possibilities of URT probiotics. Probactiol® Mini was chosen as it contains *L. rhamnosus* GG, our probiotic of interest. No ready-to-use, GMP produced probiotic product was available at that moment with *L. rhamnosus* GG as only probiotic strain. So the presence of *B. lactis* BB-12 was investigated as well and this probiotic was detected less than *L. rhamnosus* GG in the URT. Nevertheless, our study design had several limitations that need to be overcome in the future. First of all, the detection limits of the qPCR and *16S rRNA* amplicon sequencing do not allow for low-abundant bacteria. In addition, in this study we took samples at one time point, because of ethical constraints when working with paediatric samples, which made us decide to now only take invasive URT samples during surgery when the children are anaesthetised. However, including more time points, especially before the probiotic administration, would give more information about the changes in the microbiome profile by the probiotic administration. Therefore, other, less-invasive swab sampling could bring a solution as the microbiome of *e.g.* the tongue appears to comparable with the nasopharynx in adults (Segata *et al.*, 2012). A third suggestion for improvement of the intervention study would be the inclusion of a standardised questionnaire to get information about the therapeutic possibilities of the probiotic cure. A sinonasal-outcome test (SNOT-20) (Piccirillo, 2002), for example, could be used to evaluate URT problems of the patient. Finally, the isolation of DNA does not discriminate between DNA of living or dead bacteria. As a consequence, the relative abundance profiles are possibly based on DNA sequences of dead bacteria. Although we assume that nasal clearance clears a lot of dead bacteria, RNA approaches that target the active members of microbial communities as discussed by Emerson *et al.* (2017) can be an option for future research as well.

A. APPENDIX

A.1 Questionnaire intervention study

VRAGENLIJST

– – –

Onderzoek naar het effect van probiotica op het microbioom van adenoidweefsel en middenoorvocht bij kinderen met otitis media met effusie.

Beste,

Dankuwel dat u toestemming geeft om uw kind aan dit onderzoek te laten deelnemen. Welke bacteriën zich in het menselijk lichaam bevinden kan worden beïnvloed door vele factoren, waaronder voeding, medicatie, ziekten en sociale contacten. Om rekening te houden met deze factoren bij de analyse van de data verkregen uit dit onderzoek, vragen wij u de volgende vragenlijst zo goed mogelijk in te vullen. Sommige vragen lijken misschien moeilijk en het is geen probleem indien u niet alle vragen kan beantwoorden. Natuurlijk zullen de gegevens van uw kind anoniem opgeslagen en geëvalueerd worden. Sommige vragen hebben betrekking op de tijdsperiode onmiddellijk voorafgaand aan de operatie van uw kind. Daarom verzoeken wij u deze ingevulde vragenlijst op de dag van de operatie aan de arts te geven.

DEEL 1: IN TE VULLEN DOOR EEN OUDER OF WETTELIJKE VERTEGENWOORDIGER

Geboortedatum:..... (dd/mm/jjjj) Geslacht: M ☐ V ☐
Bloedgroep (indien gekend):.....

MEDISCHE GESCHIEDENIS

Heeft uw kind ooit last van vocht achter het trommelvlies gehad? Ja ☐ Neen ☐

Indien ja:
Hoe vaak? .....
Hoelang geleden was de laatste keer? .....
Duurde het ooit langer dan 3 maanden? Ja ☐ Neen ☐ Ik weet het niet ☐

Heeft uw kind ooit last van gehad van acute middenoorontsteking? Ja ☐ Neen ☐
Indien ja:
Hoe vaak ? .....
Hoe lang geleden was de laatste keer?.....

Heeft uw kind in de afgelopen 4 weken antibiotica ingenomen? Ja ☐ Neen ☐
Indien ja, welke en welke dosis?.....

Heeft uw kind in de afgelopen 4 weken andere medicatie ingenomen? Ja ☐ Neen ☐
Indien ja, welke: .....
Dosis: .....

Reden:.....

Is uw kind gevaccineerd tegen pneumokokken? Ja ☐ Neen ☐ Gedeeltelijk (1 of 2 dosissen) ☐

Heeft uw kind een allergie met symptomen van de bovenste luchtwegen? Ja ☐ Neen ☐

Heeft uw kind last van astma? Ja ☐ Neen ☐

Heeft uw kind in de afgelopen 2 weken enige symptomen van de bovenste luchtweginfecties vertoond? (bv. loopneus, koorts, oorpijn, hoofdpijn…)? Ja ☐ Neen ☐ Ik weet het niet ☐
Indien ja, welke?: .....

Lijdt uw kind aan bepaalde ziektes van de...
– Huid: ..... – Hart/bloedvaten: .....
– Maag/darmen:..... – Luchtwegen: .....
– Ogen: ..... – Neus-keel-oor: .....
– Schildklier:..... – Suikerziekte:.....
– Bloed:..... – Andere: .....

ANDERE FACTOREN

Gaat uw kind naar de kinderopvang of is hij/zij naar de kinderopvang geweest? Ja ☐ Neen ☐
Indien ja, hoe lang en vanaf welke leeftijd? .....

Gaat uw kind al naar school? Ja ☐ Neen ☐
Indien ja, sinds welke leeftijd? .....

Hoeveel broers en zussen heeft uw kind? .....

Is uw kind blootgesteld aan sigarettenrook thuis? Ja ☐ Neen ☐

Heeft uw kind contact met dieren thuis of op school? Ja ☐ Neen ☐
Indien ja, welke? .....

Hoe is uw kind geboren? Vaginale geboorte ☐ Keizersnede ☐
Heeft de moeder antibiotica gekregen tijdens de bevalling? Ja ☐ Neen ☐ Ik weet het niet ☐
Heeft uw kind borstvoeding gekregen? Ja ☐ Neen ☐
Indien ja, hoe lang ongeveer? .....

PROBIOTISCHE PRODUCTEN IN HET DAGELIJKS LEVEN

Vermeld alle probiotische en gefermenteerde voedingsproducten die uw kind regelmatig gebruikt, bv.
– Zuivelproducten zoals (drink-)yoghurt, kaas .....
.....
– Andere voedselproducten zoals olijven, salami, zuurkool, zuurdesembrood, augurken.....
.....
– Pharmaceutische producten die probiotische micro-organismen bevatten .....
.....

OPMERKINGEN

Als u nog andere opmerkingen voor ons hebt, kunt u deze hier schrijven:

DEEL 2: IN TE VULLEN DOOR DE ARTS

Datum ingreep (dd/mm/jjjj): .....

Type ingreep:.....

Indicatie:
- ☐ recidiverende otitiden
- ☐ persisterende lijmoor
- ☐ chronische rhinitis
- ☐ obstructieve ademhaling en/of OSAS
- ☐ ander: .....

Gebruik antibiotica 4 weken voorafgaand aan ingreep (welke)?: Ja ☐ :..... Neen ☐

Gebruik neusspray 4 weken voorafgaand aan ingreep (welke)? Ja ☐ :..... Neen ☐

NKO antecedenten: .....

Comorbiditeit: .....

Plaatsing van een **trommelvliesbuisje** in het verleden? Ja ☐ Neen ☐

<u>Peroperatieve bevindingen</u>

Linker oor	Rechter oor
☐ verlucht middenoor	☐ verlucht middenoor
☐ purulent vocht	☐ purulent vocht
☐ glue	☐ glue
☐ helder vocht	☐ helder vocht

Rhinopharynx: aanwezigheid van purulente postnasale drip Ja ☐ Neen ☐

<u>Andere opmerkingen</u>

Datum van invullen (dd/mm/jjj): .....

Ingevuld door: .....

6 DISCUSSION

6.1 MAIN FINDINGS AND IMPORTANCE OF THIS PHD THESIS

This PhD thesis explored the potential of probiotics against otitis media (OM), because it is one of the most important problems of the URT in children. As reviewed in Chapter 1, the pathogenesis of OM is not yet fully understood as many factors are involved. However, the common cause of all forms of OM is dysfunction of the Eustachian tube which is mostly due to physical obstruction or by infections with pathogens such as *S. pneumoniae, H. influenzae*, and *M. catarrhalis* (Schilder *et al.*, 2016) and thus these pathogens formed the major target pathogens in this PhD thesis. Thanks to next generation sequencing approaches, also other pathogens pop up as being important for the infections (see paragraph §1.2), and this was also suggested in this PhD thesis for OME with *Neisseria, (Allo)Prevotella, Turicella, Alloiococcus, Fusobacterium and Granulicatella* being among the most abundant potential pathogens in the URT samples by Illumina MiSeq amplicon sequencing of the V4 region of the *16S rRNA* gene (Chapter 5). Thus future screenings might also have to include these pathogens to also include OME as disease conditions to be targeted by the probiotics.

In this PhD thesis, we focused on LAB as potential URT probiotics, because of their long history of use as gastro-intestinal probiotics. Bacteria from the genera *Lactobacillus* are generally considered as the safest choice of probiotics due to their history in fermented foods. Furthermore, *Lactobacillus rhamnosus* received a GRAS ("Generally Recognized As Safe")-status by the Food and Drug Administration (FDA) and a QPS ("Qualified Presumption of Safety)-status by the EFSA which means that it is been adequately shown to be safe under the conditions of its intended use. In addition, as discussed in Chapter 1, recent microbiome studies indicate that lactic acid bacteria can also be endogenous members of the nasopharyngeal niche. An inverse correlation has even been observed between the occurrence of lactobacilli and pathogens. Protecting characteristics and molecular mechanisms of action of nasopharyngeal probiotics have, however, been barely explored until now. Probiotics are defined as ""live micro-organisms that, when administered in adequate amounts, confer a health benefit to the host" (Hill *et al.*, 2014). The beneficial activity of probiotics can be categorised in –at least- three major groups (i) enhancing the epithelial barrier function; (2) having antimicrobial or other beneficial microbe-microbe interactions and (3) inducing immunomodulating effects (Figure 6.1) (Lebeer *et al.*, 2008b). The objective of this PhD thesis

was to investigate whether *Lactobacillus* species, among which well-documented probiotic strains, can have (direct and indirect) antimicrobial effects against the main (A)OM pathogens *in vitro* and whether this can be translated to *in vivo* strategies.

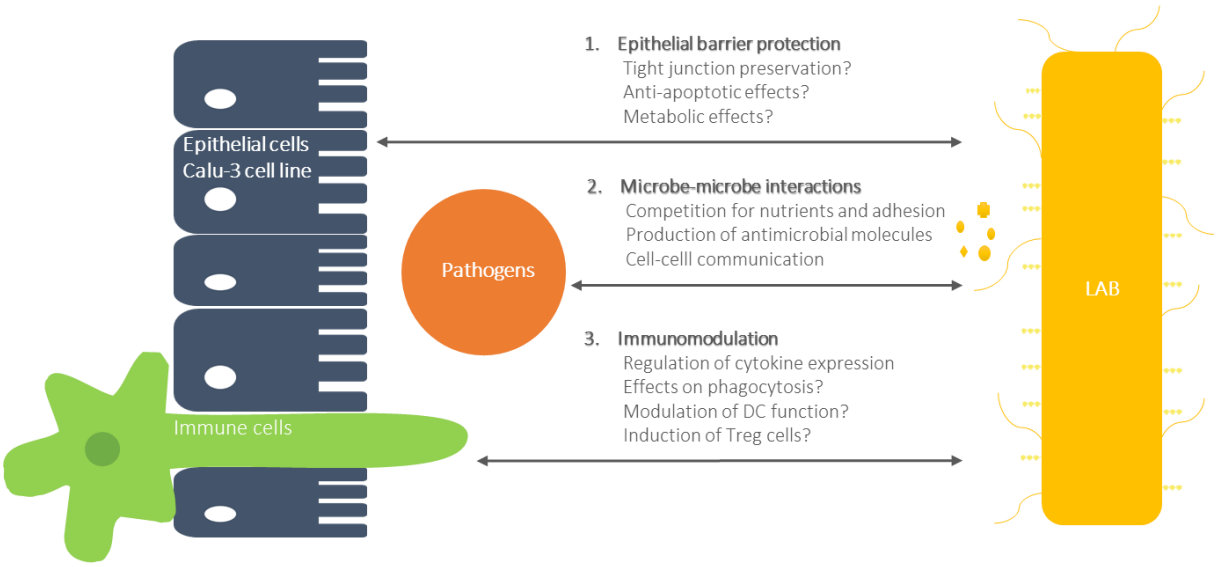


***Figure 6.1. Overview of health- promoting mechanisms of probiotics**, which were translated here towards the upper respiratory tract based on similar overview pictures for intestinal probiotics (Based on Lebeer et al., 2008).*

As the nasopharyngeal niche is quite unexplored in probiotic research, little is known about the potential of well-documented lactobacilli in this niche. Chapter 2 described the development of a screening platform in this PhD project, to test the antimicrobial activity of lactobacilli against the three main (A)OM pathogens. Such screening platforms are valuable in industrial settings where they can help to screen, in a high-throughput manner, for potential probiotic strains. In our study, almost all lactobacilli tested showed a clear antimicrobial activity against *M. catarrhalis* while activity against *H. influenzae* and *S. pneumoniae* was less pronounced. Both supernatant and living lactobacilli were able to inhibit the growth of *M. catarrhalis* (Figure 6.2). Furthermore, both planktonic growth and biofilm formation of the pathogen was affected by the (supernatant of) the lactobacilli tested. Chapter 3 described the search for the major antimicrobial compounds in the supernatant responsible for this antimicrobial activity. The assays developed in Chapter 2 were repeated with supernatant after treatment with proteinase K or heat or with pH neutral supernatant to investigate whether respectively proteins/peptides and/or acids could be involved. The activity against the pathogens remained after these treatments, except for the neutral supernatant pointing to a heat-stable, proteinase K-

resistant, pH-dependent, secreted molecule. As lactic acid is already known for its permeabilising abilities in Gram-negative intestinal pathogens such as *Salmonella* (Alakomi *et al.*, 2005; De Keersmaecker *et al.*, 2006), we investigated this important metabolite of lactobacilli in further detail. Indeed, lactobacilli which produced more lactic acid (D and/or L) showed more activity against the OM pathogens. Although it was perhaps not that surprising that lactic acid is an important antimicrobial molecule, it is the first time that this antimicrobial activity was shown against (A)OM pathogens. Investigations towards other antimicrobial compounds, such as bacteriocins, is more complex and did not (yet) reveal the presence of such molecules. However, larger screenings with a higher number of lactobacilli and better bacteriocin-inducing protocols would probably be needed to detect their presence.

Lactic acid bacteria and lactobacilli can also influence local inflammation and nutrient/receptor availability for pathogens in the host (see Figure 6.1) which was explored in Chapter 4 as potential probiotic mechanisms of action. In that chapter, we described the ability of *L. rhamnosus* GG to inhibit the adhesion of (A)OM pathogens to the nasopharyngeal epithelial Calu-3 cell line. Moreover, *L. rhamnosus* GG showed to be able to reduce the induction of pro-inflammatory cytokines by *M. catarrhalis*. These experiments were performed with a 10:1 and 1:1 LAB:pathogen ratio which can be a point of discussion. The exact *in situ* range/amount of *Moraxella* and *Streptococcus* in the nasopharyngeal tract is indeed not known, although these amounts will definitely vary in different individuals. Of note, based on the results obtained in Chapter 5, we could make some estimations as this chapter investigated the persistence of *L. rhamnosus* GG *in vivo*. Therefore, an intervention study was started where children with OM were asked to administer an oil-suspension containing *L. rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 daily for 4 weeks. After this period, swabs, aspirates and tissue samples were collected and investigated. We observed that the amount of *M. catarrhalis* and *S. pneumoniae* varies between 10^3 - 10^6 and between 10^4 - 10^6 CFU/sample, respectively, depending on the child and sampling site. Furthermore, we investigated whether a daily oral dose of *L. rhamnosus GG* ($6*10^8$ CFU) could – at least temporarily - colonise different niches of the URT of these children. Interestingly, so far, we could already detect generally ca. 10^4 CFU of LGG/ sample of different sites of the URT (adenoids, nasopharynx, tonsils, middle ear effluent). Therefore, ratios of 10:1 and 1:1 as tested in our *in vitro* competition experiments appear to be in agreement with the *in situ* environment of our intervention study.

Furthermore, we could also substantiate the presence of *L. rhamnosus GG* with the *in vivo* study by the microbial *16S rRNA* amplicon analysis. This is a very interesting finding, because it indicates that orally applied bacteria can naturally migrate to the upper respiratory tract, which is of huge importance for further applications. Moreover, the *16S rRNA* amplicon sequencing data suggest that *L. rhamnosus* GG application could also have some beneficial impacts on the microbiome such as a decreased *S. pneumoniae* load, although we should highlight that we only observed this trend in the first four patients.

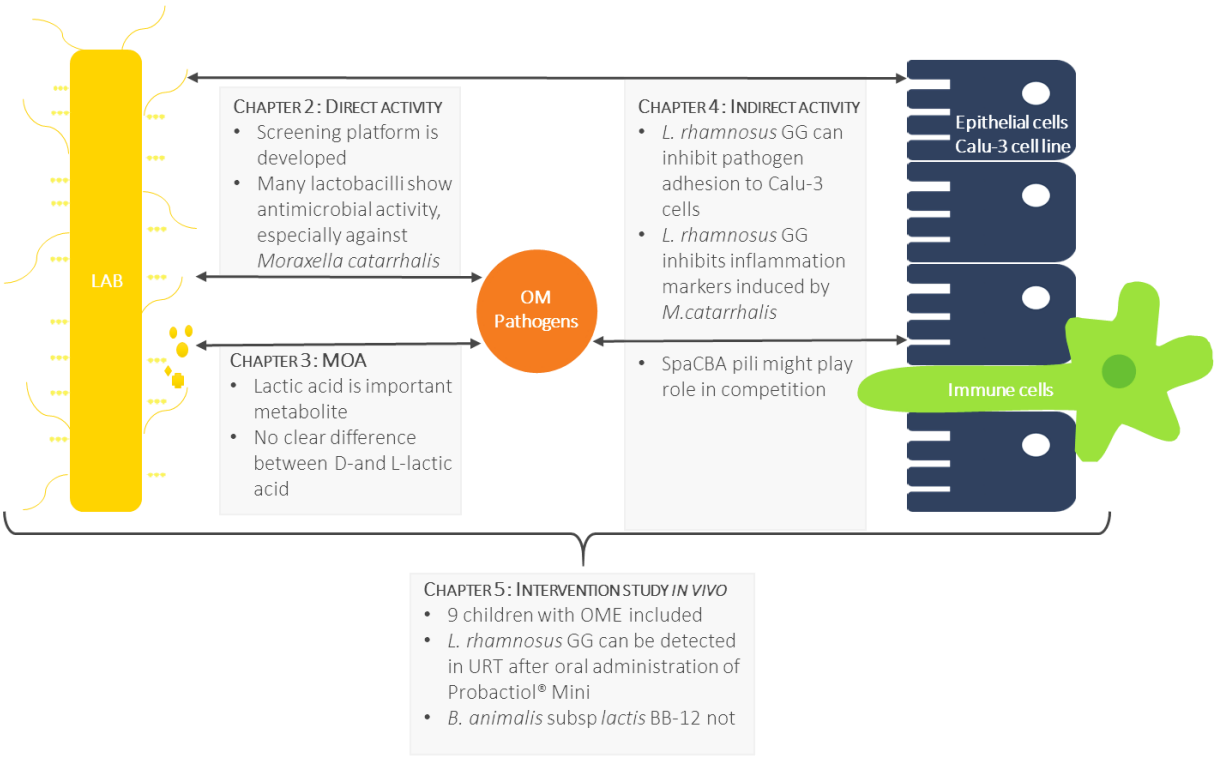


Figure 6.2: Overview of scientific outcome of this thesis.

6.2 THE POTENTIAL OF URT PROBIOTICS IN THE SEARCH FOR ALTERNATIVES FOR ANTIBIOTICS

Although only the first steps were made towards the application of URT probiotics in this PhD thesis, from the viewpoint that alternatives for antibiotics are urgently needed, every step is important. Indeed, both acute and chronic infections are still too often treated with antibiotics, which have several side effects and a high cost related to the widespread use and associated antibiotic resistance (Anthierens *et al.*, 2015; Mauldin *et al.*, 2010; Wollein Waldetoft and Brown, 2017). Despite the fact that the regulation about antibiotics is more clear and that the MIC values are lower than those of lactic acid produced by lactobacilli as observed in this PhD

(Chapter 3), the use of antibiotics includes many side-effects (Figure 6.3). Wide-spread and unnecessary prescription of antibiotics, for instance, is associated with the major health-care problem of bacterial resistance (Sandora and Goldmann, 2012). It is even stated as the number one problem in health care by the British House of Commons (O'Neill, 2017). Interestingly, especially children attending day-care centres often carry antibiotic-resistant organisms in their nasopharynx due to intensive antibiotic usage and these day-care centres provide an ideal environment for transmission of such organisms (Greenberg *et al.*, 2008). Moreover, an important common side effect of antibiotics is antibiotic-associated diarrhoea, which occurs in around 15-39% of people taking antibiotics. This leads to missed school/work days or prolonged hospital stays and contributes to additional healthcare cost. It should be noted, however, that the prescription rate is decreasing as a consequence of the increased awareness of the health threat. So, although it is clear that antibiotics are (still) necessary in serious clinical settings, and clinicians and patients are more aware about the disadvantages of their use, complementary therapies are needed.

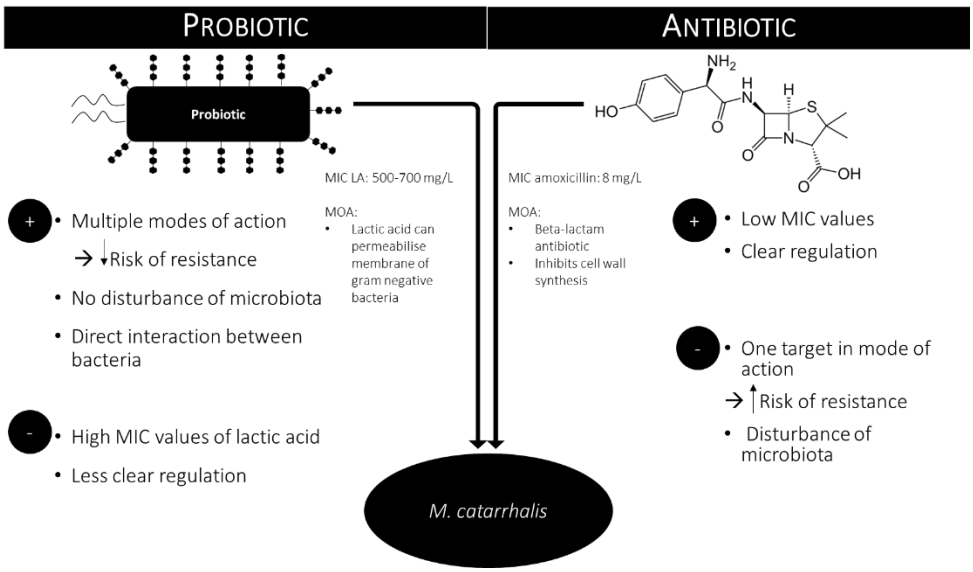


Figure 6.3: Comparison between pro- and antibiotic mechanism for the pathogen M. catarrhalis.

Based on the data presented in this PhD and the available literature, probiotics could thus be an alternative or complementary therapy for antibiotics for certain URT infections. In fact, studies have shown that administration of probiotics could lead to a decreased antibiotic intake and a reduction of the duration/occurrence of URT infections. However, these clinical trials do not always concur with each other, as both positive and neutral results occur (as shown in Table

6.1). Whether these varying results are due to the therapy duration, the amount of bacteria administered or the selected probiotic strain, is not clear. Most studies are conducted with oral administered probiotics but, as shown in the Table 6.1, also more direct applications, such as sprays, have been tested. Currently, in Belgium, no probiotic products for direct topical application in the nose and URT are available on the market. In contrast, in Italy and Poland, a spray called Rhinogermina (DMG Italia SRL) is available on the market containing lyophilised *S. salivarius* and *S. oralis* strains.

Table 6.1: Previous intervention studies with different lactobacilli targeted against URT infections.

URT Disease	Strain	Delivery mode (DM) and duration	Outcome	reference
URT infections in children	*L. rhamnosus* GG (LGG)	DM: Oral (daily 2 capsules containing 8-9 x 10^9 CFU mixed in dairy product of preference) Duration: 3 weeks	No reduction in bacterial or viral pathogen carriage as monitored by qPCR	(Swanljung *et al.*, 2015; Tapiovaara *et al.*, 2014)
URT infections in children	*L. fermentum*	DM: Oral (infant formula supplemented with 2 x 10^8 CFU and galactooligosaccharide (0.4 g/100 ml) Duration: 6 months	27% reduction in URT infections such as common cold, laryngitis, pharyngitis/tonsillitis, laringotracheitis, acute rhinitis, and acute rhinosinusitis.	(Maldonado *et al.*, 2012)
Respiratory infection with common pathogens	*L. rhamnosus* GG, *B. sp.* B420, *L. acidophilus 145* and *S. thermophiles*	DM: Oral (fermented drink supplemented with 7.1 x 10^9, 27 x 10^9, 3.2 x 10^9 and 8.4 x 10^9 CFU, respectively) Duration: 3 weeks	19% reduction of potential pathogenic bacteria in the URT (*Staphylococcus aureus*, *Streptococcus pneumoniae*, beta-hemolytic streptococci, and *Haemophilus influenzae*) as detected by cultivation	(Glück and Gebbers, 2003)
Respiratory infections in children	*L. rhamnosus* GG	DM: Oral (fermented drink, supplemented with 10^9 CFU) Duration: hospitalization period	Reduction in URT infections such as nasal discharge, sore throat, erythema of pharynx, cough, fever, wheezing, and dyspnea (3.17 times more infections in placebo group)	(Hojsak *et al.*, 2010a)
Daycare-associated respiratory infections in children	*L. rhamnosus* GG	DM: Oral (fermented drink, supplemented with 10^9 CFU) Duration: 3 months	Reduction in risk of URT infections, respiratory tract infections lasting longer than 3 days, and reduced number of days with respiratory symptoms	(Hojsak *et al.*, 2010b)

Common infections in the elderly	*L. casei* DN-114 001	DM: Oral (daily 2 fermented drinks, supplemented with 10^9 CFU) - Actimel Duration: 4.5 months	Reduced URT disease, *e.g.* rhinopharyngitis	(Guillemard *et al.*, 2010)
Children with secretory OM	*L. rhamnosus* GG	DM: Intranasal (two sprays (50 µL) per nostril, 2x /day of saline supension $5x10^9$ CFU/ml) Duration: 10 days	Half of the OM patients treated with *L. rhamnosus* GG showed some clinical improvement (*i.e.* slightly less fluid in at least one middle ear)	(Skovbjerg *et al.*, 2008)

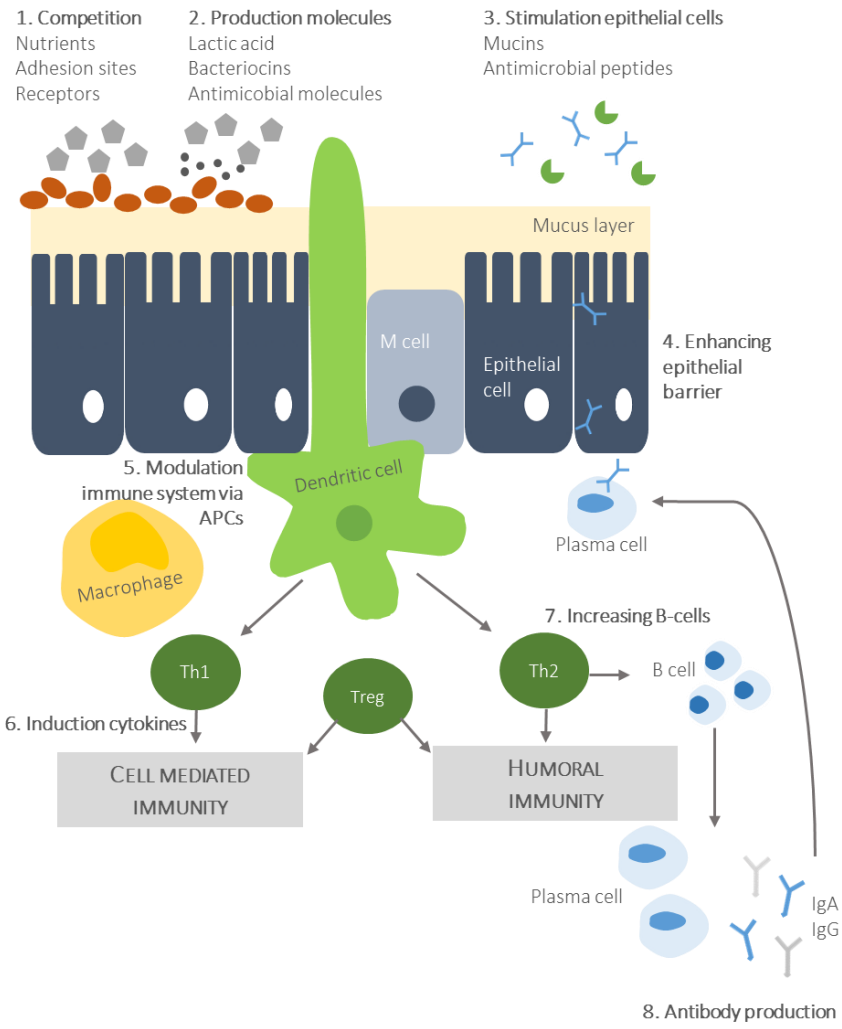
Probiotics for the URT have not only potential against AOM, but also for other URT diseases. Interestingly, Abreu *et al.* (2012) and Cope *et al.* (2017) showed that chronic rhinosinusitis patients have an altered bacterial community composition in the sinuses, with specific depletion of beneficial lactic acid bacteria (LAB) (including *Lactobacillus sakei* and *Dolosigranulum*). Similar results are observed in the currently running microbiome study of PhD student-colleague Ilke De Boeck. Also in our Flemish cohort, lactic acid bacteria such as *Dolosigranulum, Leuconostoc* and several *Lactobacillus* sequences seem to be reduced in the chronic rhinosinusitis patients (De Boeck *et al.*, 2017 + unpublished data).

6.3 LACTATE IS A CORE BENEFIT OF PROBIOTIC LAB, BUT WHAT ELSE?:

Several meta-analyses have been performed that bundle the available information on clinical trials that tested probiotics in the treatment or prevention of acute URT infections (Amaral *et al.*, 2017; Hao *et al.*, 2015; Wang *et al.*, 2016a). That different probiotic strains, mainly LAB show a benefit against URT diseases, suggest that these probiotics share a high number of mechanisms and effector molecules, which are commonly called their "core benefits". While strain-specificity of probiotic effects has been a cornerstone principle of probiotic science for decades, the search for "core benefits" of probiotics currently receives increasing attention (Hill *et al.*, 2014; Sanders *et al.*, 2018). The search for "core benefits" is currently mainly focused on orally applied probiotics, but could be translated to the URT. The effector molecules can be situated both intra- and extracellularly on the probiotic bacteria. The most important and well-known metabolites are short chain fatty acids (SCFAs) and lactic acid, which both have antimicrobial (Alakomi *et al.*, 2005; De Keersmaecker *et al.*, 2006; Sun and O'Riordan, 2013) and immunostimulatory effects (Maslowski *et al.*, 2009; Park *et al.*, 2007; Vinolo *et al.*, 2011a,

2011b). Furthermore, lactic acid can –at least in the gut - be converted to other health-promoting SCFAs, such as butyrate (Hamer *et al.*, 2008). The group of effector molecules can be further exemplified by the well-documented activity of antimicrobial bacteriocins and the immunostimulating bacterial-specific unmethylated cytosine-guanine (CpG)-containing DNA (interacting with TLR9), both produced by a selected group of lactobacilli including *L. rhamnosus* GG (Iliev *et al.*, 2008). Examples of extracellular molecules with documented probiotic effects include SpaCBA pili of *L. rhamnosus* GG that promote intestinal mucus adhesion (Kankainen *et al.*, 2009) an pathogen exclusion (Tytgat *et al.*, 2016) and EPS structures that promote survival in the intestinal tract (Lebeer *et al.*, 2009 for *L. rhamnosus GG*) and can mediate immune modulatory effects, etc. (see Lebeer *et al.*, 2010, 2018; Sanders *et al.*, 2018 for reviews on these molecules). All these structures come into in contact with the host cells leading to several physiological responses. In the gut, mucus-binding proteins (MUBs) such as SpaCBA pili can serve as anchor for the lactobacilli to remain attached to the surface they prefer by the close interaction with the mucosa and mucins (Call *et al.*, 2015; Lebeer *et al.*, 2012; Vargas García *et al.*, 2015), but whether this is also relevant for nasopharyngeal epithelium is not yet clear. Nevertheless, the results presented in this PhD thesis (Chapter 4) suggest that SpaCBA pili are less important for adhesion of *L. rhamnosus* GG to the nasopharyngeal epithelial Calu-3 cell line, but they do play a role in pathogen exclusion of *Moraxella* by *L. rhamnosus* GG. Of note, Tytgat *et al.* (2016) have recently shown that the SpaCBA pili of *L. rhamnosus* GG promote pathogen exclusion of *Enterococcus faecium* to intestinal mucus by the expression of similar pili. However, as discussed in Chapter 1, *Moraxella* expresses another type of pili (type IV pili), so detailed competitive binding assays will have to be performed to further unravel the exact details of this competitive exclusion capacity. This PhD thesis substantiated lactic acid as important mechanism of action while bacteriocins, SpaCBA pili, lectin-like proteins and TLR ligands also showed to play a role (Figure 6.4). Some of these factors are shared mechanism in probiotics as the table in the right panel of Figure 6.4 gives an overview of their taxonomic distribution. However, more detailed investigations towards these mechanisms and their relevancy to the upper respiratory tract, is a topic for further research. Although the core benefits of probiotics give some suggestions on potentially suitable probiotics, a thorough investigation towards the most adequate probiotic for certain disorders or individual is still indispensable. More detailed insights in the specific probiotic effector molecules per strain

would thus be the first step to personalised or disease specific probiotic applications. However, the road to such a product is complex and full of obstacles.



Mechanism	Gram +	Lactobacillus - genus wide	Lactobacillus - some species	Beneficial function (cfr. Figure)
Lactic acid		+		2. Production of (antimicrobial) molecules 6. Induction cytokines
Bacteriocins			+	2. Production of (antimicrobial) molecules
Sortase-dependent pilin proteins			+	1. Competition 6. Induction cytokines
Lectins/lectin-like proteins			+	2. Production of (antimicrobial) molecules
TLR ligands	+			3. Stimulation epithelial cells 6. Induction cytokines 7. Increasing B-cell 8. Antibody production

Figure 6.4: Overview of suggested probiotic mechanisms of action in this PhD thesis and their taxonomic distribution. Based on (Sanders et al., 2018).

6.4 OTHER OBSTACLES TOWARDS CLINICAL PROBIOTIC APPLICATIONS

In addition to the difficulty in understanding molecular mechanisms of action, other obstacles that that can be encountered when in probiotic applications are related to (i) safety and (ii) regulation for health claims, which will be discussed in this paragraph. With regard to safety, we refer to a report released by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), which defined four types of side-effects where probiotics may theoretically be responsible for: (1) systemic infections; (2) deleterious metabolic activities; (3) excessive immune stimulation in susceptible individuals; and (4) gene transfer (leading to antibiotic resistance) (FAO/WHO, 2002). Doron and Snydman (2015) evaluated these risks in probiotic clinical settings and observed that the latter two theoretical risks, are not yet detected in research. Metabolic activities by lactobacilli such as the production of an excessive amount of D-lactic acid, in contrast, can lead to D-lactic acidosis, a rare complication of short bowel syndrome (Ku *et al.*, 2006; Munakata *et al.*, 2010; Oh *et al.*, 1979). The selection of probiotic bacteria which do not produce a high amount of D-lactic acid, as discussed in Chapter 3, should be a solution to prevent these kinds of side-effects. Moreover, the effect of administration route will probably influence such metabolic activities as well. In addition, occasional systemic infections (at least nine cases are reported) such as *Lactobacillus* bacteremia and sepsis are reported in clinical trials with, often, immunocomprised persons (De Groote *et al.*, 2005; Kunz *et al.*, 2004; Land *et al.*, 2005; LeDoux *et al.*, 2006; Tommasi *et al.*, 2008; Vahabnezhad *et al.*, 2013; Zein *et al.*, 2008). However, in both Finland and Sweden, a longitudinal study was performed to check whether an increased *Lactobacillus* bacteremia was correlated with the increase in the probiotic ingestion during that period (Salminen *et al.*, 2002; Sullivan and Erik Nord, 2006). An absence of any change in the prevalence of *Lactobacillus* bacteremia was observed suggesting a very low risk for the general population. Therefore, only in patients with a decreased immune system, probiotic use might be discouraged, while their use in “healthy” persons should not be considered as a risk. Prebiotics, substrates that are selectively utilised by host microorganisms conferring a health benefit (Gibson *et al.*, 2017), can be an alternative for persons where the use of probiotics would be discouraged. The advantage of prebiotics compared to probiotics is the fact that they are non-viable and thus involve less of the above mentioned risks. Furthermore, stability is not a concern. On the other hand, prebiotics are less specific and can thus stimulate a broad range of (possible pathogenic) bacteria (Bindels *et al.*, 2015).

The second obstacle, regulation for health claims, can be attributed to the fact that the documentation of health benefits by living microbes/probiotic bacteria is not straightforward. It is rather common to 'criticise' probiotics because clinical trials do not produce univocal results as mentioned above. Moreover, behind these health-promoting effects are plenty mechanisms of actions from a host perspective, as discussed in Chapter 1 and above in paragraphs §6.2 and §6.3. Effects such as modulation of immune responses and pathogen exclusion are complex to document. For this reason, scientists and food companies have difficulties to substantiate the beneficial effects of probiotic functional foods and food supplements, which would result in health claims approved by the EFSA (European Food and Safety Authority). In 2016, they published a new guidance document on health claims that could clarify the assessments of probiotics (EFSA Panel on Dietetic Products, 2016). Despite the accumulation of scientific information about probiotic health effects, there are thus a lot of difficulties to obtain an approved health claim for them by EFSA. Moreover, as long as health benefits are not fully substantiated for particular probiotics strains or probiotics foods, in Europe, at this moment, the use of the word 'probiotic' is prohibited on most foods. In March 2017, however, a first probiotic (*Propionibacterium freudenreichii* W200) with an EU health claim was brought to the market by Winclove Probiotics. This probiotic was optimised in such a way that that it produces substantial amounts of vitamin B12, which has several beneficial effects on the host (Winclove Probiotics, 2017). So, due to the strict implementation of the regulation for the use of health claims on functional foods by Europe, the food market and food applications of probiotics experience difficulties, resulting in the shift towards more pharmaceutical and other applications of probiotics.

6.5 FUTURE PERSPECTIVES FOR URT PROBIOTIC PRODUCTS

In contrast to probiotic functional foods, it can be anticipated that nasopharyngeal probiotics will rather be regulated and marketed as 'over the counter' pharmaceuticals, medical devices (nose spray) or drugs by the European Medicine Agency (EMA) in Europe. Various terms exist for these probiotics such as 'pharmabiotics', 'microbiotic medicinal products' or 'live biotherapeutic products'. The pathway to develop a live therapeutic product (or probiotic) is strictly described by regulatory authorities such as the EMA in Europe and the Food and Drug Administration (FDA) in the USA and organisations such as the 'Pharmabiotic Research Institute' (PRI) (www.pharmabiotic.org) are now working together with EMA to formulate guidelines

suited for living microorganisms (*e.g.* on quality control testing of microbial viability). As depicted in Figure 6.5, a live biotherapeutic product should be first identified, characterised and produced, after which clinical trials can be conducted. The advantage of the implementation of *L. rhamnosus* GG in a live biotherapeutic product is that this bacterium was already identified by Goldin and Gorbach,(Doron *et al.*, 2005), characterised by whole genome sequencing and antibiogram analysis (Kankainen *et al.*, 2009), as well as produced at industrial settings. The latter was mainly done as freeze-dried products or fermented foods, and not in formulations that are more suited for nasopharyngeal applications. Nevertheless, in collaboration with Prof. F. Kiekens and PhD student Géraldine Broeckx (dept. Pharmaceutical Sciences, UAntwerpen), we could recently show that *L. rhamnosus* GG can also be efficiently spray dried with preservation of bacterial viability if appropriate process parameters are used and excipients such as trehalose added (Broeckx *et al.*, 2017).

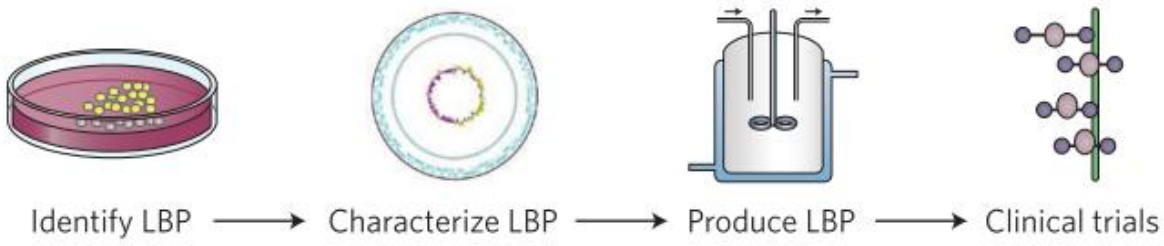


Figure 6.5: The pathway to regulatory approval for live biotherapeutic products (LBP) *(O'Toole et al., 2017).*

Potential probiotic bacteria must withstand the process of growth, enrichment, drying or product incorporation, and retain viability during product shelf-life (Burgain *et al.*, 2013). Robustness to industrial settings and processes thus influences the selection of a successful probiotic tremendously. But how do we define a successful probiotic and how do we see the future of such live therapeutic products? One thing is sure, their complexity is higher than a 'classic' pharmaceutic product (such as antibiotics) as probiotics are living organisms expressing a multitude of bioactive molecules and exhibiting a high number of interactions with their host. But although we have not unravelled every single mechanism of action of probiotic bacteria yet, we should value their overall health-promoting effects and embrace their potential.

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2. Allonsius, C. N., van den Broek, M. F. L., De Boeck, I., Kiekens, S., Oerlemans, E. F., Kiekens, F., Foubert, K., Vandenheuvel, D., Cos, P., Delputte, P. and Lebeer, S. 2017. Interplay between *Lactobacillus rhamnosus* GG and *Candida* and the involvement of exopolysaccharides. Microbial biotechnology, 10(6), 1753-1763.

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1. van den Broek, M., Lebeer, S., Kiekens, F., Claes, I.J. and Broeckx, G. Nasopharyngeal probiotics. Patent No. 14160852.1 – 1456

2. van den Broek M., Lebeer, S., Claes, I. and Oerlemans, E. Vaginal preparations for maintaining and/or restoring healthy female microbiota. Patent No. BE2016/5201

8 CURRICULUM VITAE

PERSONAL DETAILS

Marianne van den Broek

Mobile: +32 494 61 75 69

Date of birth: 5/07/1989

vandenbroekmarianne@hotmail.com

Marianne van den Broek

MariannevdB5

JOB EXPERIENCE

PhD FELLOWSHIP

University:	University of Antwerp, Belgium
Research group:	Environmental Ecology and Applied Microbiology (ENdEMIC)
Lab:	Lab of Applied Microbiology and Biotechnology
Supervisor:	prof. dr. ir. Sarah Lebeer
Start and end Date:	October, 1st 2013 – February, 16th 2018
Title of project:	*In vitro* and *in vivo* probiotic potential of *Lactobacillus* spp. for otitis media

RESEARCH STAY

University:	University of California, San Diego (UCSD)
Research group:	Bacterial Pathogenesis and Innate Immunity
Lab:	Nizet Lab
Supervisor:	prof. MD. Victor Nizet
Start and end Date:	November, 2nd – November, 29th 2015
Title of project:	Synergistic activity between supernatant of lactobacilli and host immune factors

CONFERENCES

2017	12th International Symposium on Lactic Acid Bacteria, Egmond Aan Zee, NL. Poster presentation.
	MELiSSA Mini-symposium, Antwerp, BE. Poster presentation.
	Pharmabiotics Global Conference, Paris, FR. Poster presentation.
2016	6th ASM Conference on Beneficial Microbes, Seattle, USA. Poster presentation.
	7th International Scientific Association for Probiotics and Prebiotics/ Students and Fellows Association (ISAPP/SFA) Annual Meeting, Turku, FIN. Poster presentation.

2015	20th National Symposium on Applied Biological Sciences (NSABS2015)' Louvain la Neuve, BE. Poster presentation.
	3rd Workshop on Bacterial and fungal biofilms, Antwerp, BE.
	FEMS Microbiology Conference, Maastricht, NL.
2014	11th International Symposium on Lactic Acid Bacteria, Egmond Aan Zee, NL. Poster presentation.
	Knowledge for growth ICC Gent, BE. Poster presentation
	Exploring Human Host-Microbiome Interactions in Health and Disease Wellcome Trust Genome Campus, Hinxton, Cambridge, UK. Poster presentation.
	19th National Symposium on Applied Biological Sciences (NSABS2014) Gembloux. Poster presentation.

COURSES

- Writing Academic English (Antwerp Doctoral School, ADS) 2014
- Time and Project Management (ADS) 2014
- Good Clinical Practice (UZA) 2015
- Good Manufacturing Practice (ie-net) 2017
- Leadership and Team Working (ADS) 2017
- Deep Dive Into Business: Business workshops (UAntwerpen) 2017

PAST STUDIES

University: KU Leuven, Belgium

Universidad de Salamanca, Spain (Erasmus project 1st semester of 3rd bachelor)

Degree	Graduation date	Diploma *Grade*
Master	July, 6th 2012	Master of Science in Bioscience Engineering: Cell and Gene Technology Minor: Industrial microbiology *Degree with great distinction*
Bachelor	July, 2nd 2010	Bioscience engineering: Cell and Gene Technology *Degree of satisfaction*

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