The Safety of Transtympanic Application of Probiotic *Lactobacillus plantarum*, a Candidate for Treatment of Chronic Suppurative Otitis Media

Carol Nhan, BSc (Physiology), MD, FRCSC

Department of Otolaryngology – Head and Neck Surgery,
McGill University, Montreal

December 2021

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science in Otolaryngology – Head and Neck Surgery

TABLE OF CONTENTS

List of Abbreviations 3

Abstract 4

Résumé 5

Acknowledgements 6

Preface 7

PART ONE: Introduction 8

Chapter 1.1. Rationale 8

Chapter 1.2. Objectives and Organization 9

PART TWO: Background & Literature Review 10

Chapter 2.1. Chronic Suppurative Otitis Media 10

Microbiology 10

Antimicrobial Resistance and Otomycosis 11

Chapter 2.2 Therapeutic Use of Probiotics 11

Chapter 2.3. Probiotics and Otitis Media 12

Chapter 2.4 Probiotics Against Organisms of Chronic Suppurative Otitis Media 15

Chapter 2.5. Ototoxicity of Microbacteria. 16

PART THREE: Manuscript 18

Chapter 3.1 Abstract. 19

Chapter 3.2. Introduction 20

Chapter 3.3. Materials and Methods 21

Chapter 3.4. Results 23

Chapter 3.5. Discussion 25

Chapter 3.6. Conclusion 27

PART FOUR: Overall Conclusion 28

Chapter 4.1. Conclusion 28

Chapter 4.2. Future Studies 28

References 30

List of Abbreviations

°C, degrees celcius

ABR- Auditory Brainstem Response

CFU – Colony forming units

CSOM – Chronic suppurative otitis media

dB- decibel

Hz- Hertz; kHz- kiloHertz

L. plantarum – Lactobacillus plantarum

L - liter

mL – milliliter

mmol - milimole

OHC- Outer hair cells

PBS - phosphate buffered saline

p. aeruginosa - *Pseudomas aeruginosa*

s. aureus - *Staphyloccus aureus*

SEM - scanning electron microscopy

v/v, volume/volume

Abstract

Background: Chronic suppurative otitis media, a cause of hearing loss and decreased quality of life, can be recalcitrant and difficult to treat particularly with the increasing occurrence of antibiotic resistance. Probiotics have been effectively used to treat infections and may be helpful in the treatment of chronic suppurative otitis media. However, before a probiotic can be applied in the ear, its ototoxicity potential must be evaluated.

Objective: This thesis aimed 1. To examine the literature supporting the use probiotics to potentially treat chronic suppurative otitis media, and 2. To test the ototoxicity of *Lactobacillus plantarum* applied topically to the middle ear in a validated animal model.

Methods: The literature on the use of probiotics in the treatment of otitis media, and their use against organisms commonly found in chronic suppurative otitis media was examined. An appropriate probiotic candidate for the potential treatment of chronic suppurative otitis media was selected based on the literature. A prospective controlled trial was conducted in a chinchilla animal model at the Animal Care Research facilities of the Montreal Children's Hospital Research Institute to determine whether *Lactobacillus plantarum* at 10^9 CFU/mL is ototoxic when applied transtympanically.

Results: 1. *Lactobacillus plantarum* is a probiotic that has been shown to decrease *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth in wounds, making it a candidate for the treatment of chronic suppurative otitis media. 2. There were no statistically significant differences in hearing thresholds between control and experimental ears at 28 days after transtympanic application of *Lactobacillus plantarum*. A difference of 11 dB was noted in the 25 kHz range at day 7-10, but resolved by day 28. No animals receiving probiotics developed vestibular nerve dysfunction based on behavioural observation. There was no histologic evidence of auditory hair cell damaged as verified by scanning electron microscopy.

Conclusion: This thesis study suggests that *Lactobacillus plantarum* at 10^9 CFU/mL does not cause ototoxicity in a chinchilla animal model. These preliminary auditory safety evaluations, and the pathogen inhibitory effects of *Lactobacillus plantarum* demonstrated by previous studies, suggest this probiotic as a candidate for further investigation for the treatment of chronic suppurative otitis media.

Résumé

Avant-propos : L'otite moyenne chronique suppurée, une cause de perte auditive et de diminution de la qualité de vie, peut être récalcitrante et difficile à traiter, en particulier avec l'augmentation de la résistance aux antibiotiques. Les probiotiques ont été utilisés efficacement pour traiter les infections et peuvent être utiles dans le traitement de l'otite moyenne chronique suppurée. Cependant, avant qu'un probiotique puisse être appliqué dans l'oreille, son potentiel d'ototoxicité doit être évalué. Cependant, avant de pouvoir être appliqué dans l'oreille, le potentiel d'ototoxicité d'un probiotique doit être évalué.

Objectif : Cette thèse visait 1. À examiner la littérature soutenant l'utilisation des probiotiques pour traiter l'otite moyenne chronique suppurée, et 2. À tester l'ototoxicité de *L. plantarum* appliqué topiquement à l'oreille moyenne dans un modèle animal validé.

Méthodologie : La littérature sur l'utilisation des probiotiques dans le traitement de l'otite moyenne et leur utilisation contre les organismes couramment trouvés dans l'otite moyenne chronique suppurée a été examinée. Un candidat probiotique approprié pour le traitement potentiel de l'otite moyenne chronique suppurée a été sélectionné sur la base de la littérature. Un essai contrôlé prospectif a été mené sur un modèle animal chinchilla dans les installations de recherche en soins des animaux de l'Institut de recherche de l'Hôpital de Montréal pour enfants afin de déterminer si *L. plantarum* à 10^9 UFC/mL est ototoxique lorsqu'il est appliqué par voie transtympanique.

Résultats : 1. *L. plantarum* est un probiotique qui réduit la croissance de *S. aureus* et de *P. aeruginosa* dans les plaies, ce qui en fait un candidat pour le traitement de l'otite moyenne chronique suppurée. 2. Il n'y avait aucune différence statistiquement significative dans les seuils auditifs entre les oreilles contrôle et expérimentales à 28 jours après l'application transtympanique de *L. plantarum*. Une différence de 11 dB a été notée en 25 kHz au jour 7-10, mais résolue au jour 28. Aucun animal recevant des probiotiques n'a développé un dysfonctionnement du nerf vestibulaire. Il n'y avait aucune preuve histologique d'endommagement des cellules ciliées auditives, tel que vérifié par microscopie.

Conclusion : Cette étude de thèse suggère que *L. plantarum* à 10^9 UFC/mL ne provoque pas d'ototoxicité dans un modèle animal chinchilla. Ces évaluations préliminaires de l'innocuité auditive et les effets inhibiteurs des agents pathogènes de *L. plantarum* démontrés par des études antérieures suggèrent que ce probiotique est un candidat pour une enquête plus approfondie pour le traitement de l'otite moyenne chronique suppurée.

Acknowledgements

I would like to express my deepest gratitude to Dr. Sam J. Daniel for his inspiration, guidance and mentorship throughout this project and my clinical training. Also, to Dr. Lily H.P. Nguyen for her leadership, constructive counsel and encouragement. To Dr. Bernard Segal for chairing my thesis committee meeting and his guidance, critical review of my work and encouragement throughout the pursuit of a Master's of Science degree and in the preparation of this thesis.

I would like extend my sincere thanks to Dr. Dalius Briedis for his constructive advice through the lens of a microbiology expert, and to Dr. Shyamali Saha, Dr. Satya Prakash, and Susan Westfall for their kind collaboration and sharing their expertise in probiotic medicine.

I would also like to thank my colleagues of the McGill Auditory Sciences Lab who have been helpful in every way throughout this endeavour. In particular Dr. Aren Bezdjian, Dr. Mario Mujica-Mota, Dr. Yan Lu, as well as the staff at the Animal Care Facility of the McGill University Research Institute.

I thank my fellow residents, my friends who have been supportive throughout.

Last but not least, I wish to thank my family: my parents for nurturing my passion for learning and discovery and believing that I could achieve anything I set my imagination to. My siblings Charles and Cynthia who are always there for me. Rafal, who never fails to support me when I need it most, while helping me stay grounded. My daughter Amelia who is the light of my life.

This work was supported by grants from The Department of Otolaryngology Head and Neck Surgery –McGill University and the Fonds de recherche Santé du Québec.

Preface

Contributions of Authors

Carol Nhan performed the literature review, growth and preparation of cell cultures, animal experiments, data collection, the analysis and the writing of the manuscript included in this thesis. Carol Nhan, Dr. Aren Bezdjian, Dr. Lily Nguyen, and Dr. Sam Daniel contributed to the designing of the experiments cooperatively. Dr. Shyamali Saha and Dr. Satya Prakash provided the expertise, training, and laboratory facilities for the culture growth and preparation. Dr. Susan Westfall assisted with the preparation and maintenance of cell cultures. All authors of the manuscript, Carol Nhan, Dr. Aren Bezdian, Dr. Shyamali Saha, Dr. Satya Prakash, Dr. Lily H.P. Nguyen, and Dr. Sam Daniel reviewed and edited the manuscript. The study was conducted in animal models at the McGill Auditory Sciences Laboratory located at the Research Institute of the Montreal Children's Hospital of the McGill University Health Centre and The Biomedical Technology and Cell Therapy Research Laboratory of McGill University. The study was funded by were funded by a grant from The Department of Otolaryngology Head and Neck Surgery –McGill University and the Fonds de recherche Santé du Québec. Result interpretation and final approval of the manuscript were done by Carol Nhan and Dr. Sam Daniel. Carol Nhan and Dr. Aren Bezdjian conducted the statistical analysis.

Claims of Originality

The present thesis yielded new knowledge by (1) Determining based on literature review a candidate probiotic active against bacterial pathogens most commonly associated with chronic suppurative otitis media. (2) Using a validated animal model to confirm that *Lactobacillus plantarum* is not ototoxic.

PART ONE: Introduction

Chapter 1.1. Rationale

Chronic suppurative otitis media (CSOM) is a chronic infection of the middle ear with a perforated tympanic membrane and chronic purulent discharge. CSOM can greatly impair a patient's quality of life[1] by causing hearing loss, otalgia, social isolation associated with having a chronically draining ear, limiting activities, and requiring frequent health care visits. In developing countries, CSOM is the leading cause of childhood hearing impairment[2 3 4.] This in turn has serious implications for a child's speech and language development[2 5 6], impacting their cognitive and education outcomes[7 8 9]. Furthermore, chronic drainage from the ear prevents the use of hearing aids that could otherwise help children with a hearing loss. CSOM may additionally lead to complications which may be severe – even life-threatening -- and include mastoiditis, meningitis, intracranial abscess, facial nerve palsy, and lateral sinus thrombosis[10 11 12 13].

Despite good antimicrobial therapy, infections may be resistant to treatment due to antibiotic resistance or biofilms that help these pathogens evade the host immune system as well as antibiotics. Additionally, recurrent courses of antibiotic treatment may also lead to otomycosis[14], an opportunistic fungal infection in the ear that occurs when antibiotics disturb the local microbiome. Furthermore, certain antibiotics are themselves ototoxic, causing hearing loss. These challenges speak to a need for novel therapeutic options.

Probiotics are beneficial bacteria that prevent the overgrowth of pathological bacteria that cause disease. A disruption of the natural nasopharyngeal flora is noted in children with otitis media. Normally, in healthy children non-typable *Haemophilus influenza* accounts for less than 2% of their normal nasopharyngeal flora[15], whereas levels up to 90% are seen in children known for otitis media[16]. Healthy children without a history of otitis media have a predominance of *Streptococcus viridians* in their nasopharynx, but also a minor level of the potentially pathogenic bacteria *Streptococcal pneumonia, Moraxella catarrhalis* and non-typable *Haemophilus influenza*[17 18] however a predominance of these pathogenic species is found in children known for otitis media[19]. It is postulated that probiotics help restore the native polymicrobial population in the nasopharynx, which typically shows reduced levels of nasopharyngeal commensals in cases of recurrent acute otitis media[20]. In a double-blinded study by Skovbjerg et al[21], children with chronic otitis media effusion scheduled for insertion of tympanostomy tubes were treated with nasal spray applications of probiotics or a placebo. A ten-day application of *Streptococcus sanguinis* was shown to significantly improve rates of resolution of the effusion as confirmed at the time of surgery.

Lactobacillus plantarum is a probiotic commonly used in foodstuff as well as normal intestinal microflora. It has been shown to prevent both *S. aureus* and *P. aeruginosa*, the main organisms found in CSOM[40], from establishing wound infections in mice and was the most effective of three species of *Lactobacilli* tested[22]. It also has antagonistic activity against *Peptostreptococcus*[23], an anaerobic organism also commonly found in CSOM[14]. In light of these findings, the topical application of the probiotic *Lactobacillus plantarum* may be promising for the prevention and treatment of CSOM; however, before considering further studies in the context of CSOM its ototoxicity potential must be established.

Chapter 1.2. Objectives and Organization

In view of the background previously outlined, the objective of this research is to test the otologic safety of the probiotic *Lactobacillus plantarum* in the middle ear using a validated Chinchilla animal model. This is a crucial first step before considering the evaluation of whether *L. plantarum* is effective in treating CSOM.

The thesis will be divided in four parts. Part two lays the background for an experimental study by discussing the problem of CSOM, a possible role for probiotics based on the existing literature, and the potential for microbacterial organisms to cause ototoxicity and thus further importance of testing the ototoxicity of any probiotic meant for otologic use. Part three presents an experimental study as published in the Journal of Otolaryngology – Head and Neck Surgery. Part four summarizes overall conclusions and proposed future research directions.

PART TWO: Background & LiteratureReview

Chapter 2.1. Chronic Suppurative Otitis Media (CSOM)

Microbiology

The propensity for otitis media is impacted by multiple risk factors including eustachian tube dysfunction which changes with age, genetics, environmental factors, the immune system, and viral or bacterial infections[24]. Acute otitis media is the precursor of chronic suppurative otitis media (CSOM) and its pathogenesis is usually initiated by a respiratory viral infection[25] and complicated by a bacterial infection that tracts from the nasopharynx[26], most commonly *Streptococcus pneumoniae*, *Haemophilus influenza* and *Moraxella catarrhalis*[27]. Despite widespread vaccination against some of the causative organisms such as the pneumococcus conjugate vaccines, the impact on reducing acute otitis media has been modest in healthy infants[28] and shifts in serotypes of *S. pneumoniae* and *H. influenza* have been reported[29]. Acute otitis media may cause perforation of the tympanic membrane, and with chronic inflammation and bacterial infection, persistent otorrhea (drainage of pus from the ear) may develop.

The majority of CSOM infections are polymicrobial[30 31] and involve chronic inflammation of the middle ear. Pathogens most commonly associated with CSOM are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Proteus* species, *Klebsiella pneumoniae, Streptococcus pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes*, *Peptostreptococcus*, *Fusobacterium nucleatum*, *Prevotella* species, *Bacteroides* species, *Clostridium* species and *Porphyromonas* species [32 33 34 35 36 37]. The most frequently isolated bacteria using culture techniques are *Pseudomonas aeruginosa* (up to 44%) and *Staphylococcus aureus* (up to 37%)[35 38,39,40 41 42 43]. Standard treatment involves aural toilet and topical antibiotics since the concentrations achieved locally are thousands of times greater than can be achieved using systemic antibiotics [44]. Treatment options however are limited to those without the potential to be ototoxic. Despite targeted therapy, treatment failure continues to be a problem in CSOM.

Antimicrobial Resistance and Otomycosis

CSOM is first treated empirically since the concentration of antibiotic achieved locally is thought to overcome even resistant bacterial strains in most cases. Culture-directed treatment is performed in cases of failure to respond to therapy[45]. However, with chronic antibiotic use, antimicrobial resistance can be problematic. The rate of resistant *pseudomonas* is reported as high as 54%[46] and appears to be increasing in some regions[47]. There is a significant prevalence of *S. aureus* that are methicillin resistant (MRSA) in patients with CSOM[48]. A study by Ahn et al found that patients with MRSA CSOM were three times as likely to continue having otorrhea even after mastoidectomy for recalcitrant disease[40].

Biofilms are formed when microbial cells form an extracellular polymeric matrix for protection and optimal growth conditions. They are sometimes found in chronic otitis media[49] and thought to contribute to resistance not only to antibiotics but also antimicrobial agents[50 51]. They tend to adhere to ulcerated middle ear mucosa as well as to foreign material such as tympanostomy tubes[52]. *Pseudomonas* in particular is known to employ biofilms[53], among other strategies such as antibiotic inactivation by enzymes[54] to evade the host's immune system. Biofilms contribute to treatment failure and recurrent infections[55 56].

In addition to antimicrobial resistance, chronic use of antibiotics in CSOM may lead to opportunistic fungal infections[57], most often by *aspergillus* and *candida* species[14]. These infections are also challenging to treat, and require frequent visits for debridement by a medical professional. They are also of particular concern when occurring in patients with immune suppression because they can become aggressive and cause significant morbidity and mortality.

Chapter 2.2. Therapeutic Use of Probiotics

The idea of treating pathogenic bacteria with healthy bacteria was first published in 1958 and reported the successful treatment of a patient with Clostridium difficile enterocolitis by way of fecal transplant[58]. Use of probiotics in the treatment of infections has been widely accepted in various applications such as infectious enterocolitis, Helicobacter pylori peptic ulcer disease, vaginal yeast infections, and wound infections. The use of Lactobacillus GG[59 60] and bididobacteria[61 62]are well documented at reducing the risk of viral diarrhea. Furthermore, probiotics are accepted prophylaxis against the development of necrotizing enterocolitis and overall mortality in preterm infants, as supported by a Cochrane Review[63].

Probiotics are thought to work in a multitude of ways. For example, certain probiotics appear to enhance the functions of the gut mucosal barrier[64] by inducing mucin secretion which may inhibit adherence of mucosal pathogens[65]. They are also found to interfere with adherence and proliferation of pathogens to epithelial cells, to make the microenvironment less habitable to pathogens (for example by competing for nutrients, lowering pH, and producing antagonistic agents)[32], as well as to suppress toxin production by pathogens[66]. Some probiotics stimulate innate immune responses once ingested, such as *lactobacilli*[67]. Probiotics such as *Lactobacillus* GGI[68] and *Bifidobacterium lactis* Bb-12[69] increase immunoglobulin A production and thus enhance humoral immunity.

Chapter 2.3. Probiotics and Otitis Media

Children with recurrent otitis media appear to have reduced levels of nasopharyngeal commensals. In studies comparing adenoid bacteriology of healthy children undergoing adenoidectomy for nasal obstruction to otitis-prone children undergoing adenoidectomy and tubes for recurrent otitis media, children with recurrent otitis media had reduced levels of *α-hemolytic streptococci*, along with an increase in *Haemophilus influenza* and *Streptococcus pneumoniae*[70 71 72].

Brook et al[71] designed a study that not only compared the pathogens isolated from the adenoids of otitis media prone to non-otitis media prone children, but also tested whether there were bacteria isolated from these children that could interfere in-vitro with the growth of known pathogens involved in otitis media. As expected, there was a significant increase in the rate of isolation of *S. pneumoniae, H. influenza, Moraxella catarrhalis* and/or *Streptococcus pyogenes* in otitis-prone children, defined as children having at least 6 episodes of acute otitis media in the 2 years prior to surgery. Twenty-seven of these pathogens were isolated from 21 of the 25 otitis media-prone children, compared to 10 of these pathogens isolated from 8 of the 20 control children ($P < 0.05$). They tested the ability of normal flora isolates from each of the children in these two groups to inhibit, or cause “interference” with, the growth of each of the four known pathogens above in-vitro. Interference refers to an antagonistic interaction between different bacterial strains in a way that negatively affects growth or activity. Bacterial interference against these known pathogens was found in 71 instances in the otitis media-prone group, but 193 times in the control group (*P* < 0.05), suggesting that normal children had more non-pathologic organisms in their nasopharynx with the ability to inhibit otitis-media associated pathogens in-vitro. Tano et al[73] found consistent results in a study comparing the ability of *α-hemolytic streptococci* isolated from the eustachian tubal orifice of healthy children, children with otitis media effusion, and recurrent otitis media. They specifically took specimens from the tubal orifice since prior studies suggest that the inhibitory activity of *α -hemolytic streptococci* varies depending on the anatomical site from which it is taken[74]. They found that the *α -hemolytic streptococci* from children with otitis media effusion and recurrent acute otitis media was significantly less effective at inhibiting *S. pneumoniae, H. influenza* (*P* < 0.001).

Probiotic nasal spray applications restoring the microbial flora of the nasopharynx appear to reduce rates of recurrent acute otitis media and improve rates of otitis media effusion resolution[75 21]. In Roos et al's[75] study, *α-hemolytic streptococci* from the eustachian tube openings of healthy children were tested for their ability to inhibit growth of *S. pneumoniae, H. influenza, M. catarrhalis* and *S. pyogenes*. Of the 800 strains tested, five strains were selected for their superior inhibitory activity and made into a nasal spray of 5x108 colony forming units (CFU) per millilitre by freeze drying in skimmed milk. The compound was reconstituted in normal saline immediately prior to treatment onset and kept refrigerated throughout the 10-day treatment course. The placebo spray consisted of skimmed milk powder with the same appearance as the probiotic spray. All patients received a 10 day course of antibiotics prior to beginning either the probiotic or placebo nasal spray. The children were treated with 10 days of nasal sprays, which was repeated after 55-60 days. In the three-month follow-up, there were significant differences in the rate free from recurrent otitis media (42% of the treatment group versus 22% of the placebo group) and rate of otitis media effusion without recurrence of acute otitis media (31% in the treatment group compared to 56% in the placebo group). Another double-blind randomised control study in children with chronic otitis media effusion found that nasal spray treatment with *Streptococcus sanguinis* resulted in significantly (*P*<0.05) improved recovery in the treatment group (7/19 patients) compared to the placebo group (1/17 patients)21.

A more recent double-blind randomized controlled study of children known for recurrent acute otitis media compared treatment with intranasal *Streptococcus salivarius* to placebo in reducing rates of acute otitis media, and also evaluated for uptake or colonization of the probiotic[76]. Otitis prone children received a course of antibiotics followed by twice per day sprays for 5 consecutive days each month. While simply comparing treatment to control group did not result in a significant reduction in rate of acute otitis media occurrence, when they specifically compared children who showed confirmation of *Streptococcus salivarius* colonization on nasopharyngeal swab to those who did not, the differences were significant. In those colonized, 12/28 (42.8%) did not experience acute otitis media following treatment versus 3/22 (13.6%) in the group that was not colonized ($P = 0.03$). The rate of nasopharyngeal colonization after a treatment with probiotics was 56% at the third month (immediately prior to third and final treatment course in this study) and 28% at 60 days after completion of therapy. This would suggest that the probiotic's impact on otitis media prevention was though local establishment and effects rather than through systemic immune modulation via probiotic that may have been swallowed. This is consistent with the literature, which does show mixed results for prevention of otitis media when children are treated with an oral supplementation of probiotics[82 83 77 78].

One such study, a randomized, double-blind, placebo-controlled study, found that feeding infants formula with probiotics significantly reduced their risk of acute otitis media and upper respiratory tract infections[79]. They recruited infants requiring formula as their sole source of food before 2 months of age from well-baby clinics and randomized them to receive 1 x 1010 colony-forming units of the probiotics *Lactobacillus rhamnosus GG* and *Bifidobacterium lactis Bb-12* supplemented to their formula daily versus a placebo over 12 months. The infants receiving this probiotic treatment had a significant reduction in acute otitis media, and in the need for antibiotic treatment in the first 7 months of life (22% versus 50%, $P = 0.014$). None of the infants treated with probiotics required tympanostomy tube placement in the first year of life, compared to four of the 40 (10%) of infants in the placebo group ($P = 0.066$). It is difficult to determine whether the effect on otitis media was independent, or secondary, to reduced respiratory infections since there have been other studies testing different probiotics that found no difference in rate of acute otitis media but a decreased rate of upper respiratory infections[77 78]. Also, this study was in infants bottle-fed milk containing probiotic and an infant's anatomy is such that the probiotic could have locally colonized the nasopharynx. Colonization of the nasopharynx was not confirmed in this study.

Chapter 2.4. Probiotics Against Organisms of Chronic Suppurative Otitis Media

If probiotics have the potential to reduce the risk of otitis media, it is conceivable to consider that they may help in recalcitrant chronic suppurative otitis media (CSOM) where antibiotic resistance and risk of complications from overuse of antibiotics such as otomycosis limit treatment options. While probiotics have not yet been studied in the context of CSOM, numerous studies have investigated the activity of probiotics against *Staphylococcus aureus and Pseudomonas aeruginosa,* the microorganisms most commonly associated with CSOM.

Lactobacillus fermentum inhibits growth, cytotoxicity, and biofilm formation in vitro by *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains in a dose dependent manner[80]. Even when tested against mature biofilms of *S. aureus* and *P. aeruginosa*, *Lactobacillus fermentum* cause dispersion of the biofilm in in-vitro conditions. Another in-vitro study screening lactobacilli samples isolated from yoghurt and fecal samples showed that *Lactobacillus fermentum* produced a bacteriocin that was effective against a clinical blood isolate of MRSA[81].

Bifidobacteria bifidum may be another promising probiotic. In a mouse model of staphylococcus aureus vaginosis, *Bifidobacteria bifidum* showed strong anti-staphylococcal activity. Treatment enhanced immune response in these splenectomized mice, and by day 3, mice that received the probiotic had returned to control-levels of CD3+ and CD4+82. In a clinical case report of multidrug-resistant *Pseudomona aeruginosa* ulcerative colitis infection refractory to steroid therapy and antibiotics, treatment with *Bifidobacteria* was reported to have been successful at effecting remission[83]. *Bifidobacteria* are reported to have an anti-inflammatory effect on a model of rat colitis[84], and in human studies have been shown to modulate inflammatory mediators[85 86].

Lactobacillus plantarum is a probiotic shown to prevent both *S. aureus* and *P. aeruginosa*, the main organisms found in CSOM, from establishing wound infections[22]. In an in-vitro comparison of the activity of three strains of lactobacilli applied to culture dishes of *S. aureus* and *P. aeruginosa, L. plantarum* was found to have the most inhibitory activity against both these pathogens[22] making it a good potential candidate for the treatment of CSOM. This study also investigated the effect of *L. plantarum* on wounds in a mouse model. They compared the treatment of *S. aureus* and *P. aeruginosa* inoculated wounds with *L. plantarum* to a control group treated with saline and found that *L. plantarum* was able to prevent *S. aureus* and *P. aeruginosa* from establishing wound infections. The wounds treated with the probiotic showed a resolution of inflammation and tissue histopathology improvement. Valdez et al[87] used a burned-mouse model to test the effect of *L. plantarum* on *P. aeruginosa* infected wounds as compared to a control. At a concentration of 10^5 CFU/mL, the probiotic significantly decreased necrosis and showed dermal regeneration and histologic evidence of capillary vessel formation, collagen matrix and fibroblasts marking active healing. The treated wounds also had significantly decreased tissue levels of *P. aeruginosa*, and increased levels of tissue phagocytes containing *P. aeruginosa* indicating an enhanced host response against the pathogen. This group also studied *L. plantarum in-vitro* and found that in addition to effectively inhibiting *P. aeruginosa,* it also significantly inhibited biofilm formation by the pathogen. In another *in-vitro* study, *L. plantarum* showed antagonistic activity against multiple bacteria including *Peptostreptococcus, Enterococci,* and *S. aureus*[23], an anaerobic organism sometimes found in CSOM[32]. All these findings suggest that *L. plantarum* may be an interesting candidate for the treatment of CSOM, however it must must first be determined whether it can be safely applied topically to the middle ear.

Chapter 2.5. Ototoxicity of Microbacteria

Not only does chronic suppurative otitis media (CSOM) cause conductive hearing loss up to 60 decibels (dB) due to perforation of the ear drum, fluid and disruption of the ossicular sound conducting mechanism[88], it can also cause sensorineural hearing loss[89 90 91] in up to 58% of patients[3 92 93]. There is evidence that bacterial infections are capable of causing damage to auditory structures. Studies testing *Streptococcus pneumoniae* in the middle ear of chinchilla and *Pseudomonas aeruginosa* exotoxin A found resulting sensorineural hearing loss[94 95]. Histologic studies comparing the CSOM ear to the contralateral normal ear in human temporal bones confirmed a loss of outer hair cells in the cochlear base of the CSOM affected ear[96].

The pathophysiology of sensorineural hearing loss in otitis media involves inflammatory mediators such as reactive oxygen species generated from the infectious process itself penetrating the inner ear through the round window and causing damage to the cochlear hair cells and the stria vascularis[97 98]. Mediators such as nitric oxide, cytokines and bacterial toxins can damage the auditory hair cells or the stria vascularis[97 99 100]. Arachidonic acid also alters blood flow to the cochlea, disrupting proper function[101]. Since the basal turn of the cochlea is closest to the round window, it is at greatest risk of injury, predisposing hearing loss at high frequencies[93 102]. Older patients are at higher risk of developing sensorineural hearing loss with CSOM, even when comparing the ear affected by CSOM to the other non-affected side and therefore controlling for the natural history of presbycusis (hearing loss in the high frequencies that occurs with age[)103 104]. These findings make it crucial to test any bacterial organism for ototoxicity before proposing its application to the middle ear.

An experimental study to be presented in Part Three, which follows, will describe such a study.

PART THREE: Safety of Transtympanic Application of Probiotics in a Chinchilla Animal Model

Manuscript published in

J Otolaryngol Head Neck Surg. 2017 Nov 22;46(1):63105

by

Carol Nhan[1], Aren Bezdjian[2], Shyamali Saha[3], Satya Prakash[3], Lily H P Nguyen[4], Sam J Daniel[5]

1. Department of Otolaryngology - Head & Neck Surgery and McGill Auditory Sciences Laboratory, The Montréal Children's Hospital, McGill University, 1001 Boulevard Décarie, Montréal, Québec, H4A 3J1, Canada.
2. Department of Experimental Surgery and McGill Auditory Sciences Laboratory, The Montréal Children's Hospital, McGill University, Montréal, Québec, Canada.
3. Departments of Biomedical Engineering, Physiology, and Artificial Cells and Organs Research Center, Biomedical Technology and Cell Therapy Research Laboratory, McGill University, Montréal, Québec, Canada.
4. Department of Otolaryngology - Head & Neck Surgery and Center for Medical Education, McGill University, Montreal, Québec, Canada.
5. Department of Otolaryngology - Head & Neck Surgery and McGill Auditory Sciences Laboratory, The Montréal Children's Hospital, McGill University, 1001 Boulevard Décarie, Montréal, Québec, H4A 3J1, Canada. sam.daniel@mcgill.ca.

Keywords: Chinchilla; Otitis; Ototoxicity; Probiotic; Transtympanic

---

Chapter 3.1 Abstract

Background: Chronic suppurative otitis media can be recalcitrant and difficult to treat, particularly with the increasing occurrence of antibiotic resistance. *Lactobacillus plantarum* is a probiotic that has been shown to decrease *S. aureus* and *P. aeruginosa* growth in wounds, making it a good candidate for the treatment of chronic suppurative otitis media. However, before it can be applied in the ear, its ototoxicity potential must be evaluated.

Methods: A prospective controlled trial was conducted in a chinchilla animal model at the Animal care research facilities of the Montreal Children's Hospital Research Institute to determine whether *Lactobacillus plantarum* is ototoxic when applied transtympanically. Ten chinchillas each had one ear randomly assigned to receive 10^9 CFU/mL of *Lactobacillus plantarum* solution, while the contralateral ear received saline. Auditory brainstem responses were measured bilaterally at 8, 20, 25 kHz before, at 7–10 days after application, and at 28 days after application of probiotic or saline. Facial nerve and vestibular function were assessed clinically.

Results: There were no statistically significant differences in hearing thresholds between control and experimental ears at 28 days after application. A difference of 11 dB was noted in the 25 kHz range at day 7–10, but resolved by day 28. No animals receiving probiotics developed vestibular nerve dysfunction based on behavioural observation. There was no histologic evidence of auditory hair cell damaged evidenced by scanning electron microscopy.

Conclusion: Our study suggests that a single application of *Lactobacillus plantarum* at 10^9 CFU/mL does not cause ototoxicity in a chinchilla animal model. These preliminary safety evaluations and the pathogen inhibitory effects of *L. plantarum* demonstrated by previous studies present this probiotic as a candidate of interest for further investigation.

Chapter 3.2 Background

Chronic suppurative otitis media (CSOM) can be challenging to treat, particularly when complicated by antibiotic resistance or secondary otomycosis. It is the leading cause of childhood hearing impairment in the developing countries[2] and has had serious implications for speech and language development in children[2 5 6], impacting cognitive and education outcomes[7]. Serious complications arising from these infections can cause meningitis, intracranial abscess, facial palsy, and lateral sinus thrombosis[10].

The majority of infections are polymicrobial and involve chronic inflammation of the middle ear. Pathogens most commonly associated with CSOM are *Pseudomonas*, *Staphylococcus*, *Peptostreptococcus*, *Fusobacterium*, *Prevotella*, and *Porphyromonas*[32].

Currently, the first-line treatment for uncomplicated CSOM involves antibiotics and anti-inflammatory agents applied topically to the ear. This treatment best achieves the highest dose delivery with the least secondary effects[106]. However, overuse of antibiotics has resulted in resistant pathogens. Moreover, prolonged use of antibiotics has been associated with development of otomycosis[14].

Probiotics are living microorganisms that can provide beneficial effects[107]. For over a decade, probiotic bacteria have successfully treated infections typically related to gastro-intestinal (GIT) diseases[108]. More recently, several non-GIT applications have been investigated[70]. In children with recurrent otitis media, nasal spray applications of probiotics have been shown to reduce their rates of both infection and middle ear effusion[21]. It is hypothesized that probiotics help restore the native polymicrobial population in the nasopharynx, which typically shows reduced levels of nasopharyngeal commensals in cases of recurrent acute otitis media[109]. These studies bring into question whether topical probiotic bacteria application could also be beneficial in the prevention and treatment of CSOM.

Lactobacillus plantarum is a probiotic shown to prevent both *S. aureus* and *P. aeruginosa*, the main organisms found in CSOM[32], from establishing wound infections[22]. It also has antagonistic activity against *Peptostreptococcus*[23], an anaerobic organism commonly found in COSM[32]. In a comparison of three strains of lactobacillus, *Lactobacillus plantarum* was found to have the best inhibitory activity against *S. aureus* and *P. aeruginosa*[22] making it a good potential candidate for the treatment of CSOM. Nonetheless, the ototoxicity potential of probiotics remains to be established prior to investigating otic applications. Thus, before studying whether Lactobacillus plantarum is effective in treating CSOM, its safety when applied topically to the middle ear must be determined.

Chapter 3.3 Materials and Methods

Study Overview

Chinchilla were used to verify the ototoxicity of *L. plantarum*. The chinchilla was chosen because it is a well-established animal model for hearing loss studies[110 111 112]. They also have large tympanic membranes and middle ears similar to humans and a cochlea that is readily dissectible. Each chinchilla had a solution of probiotic applied transtympanically to the randomly selected experimental ear and phosphate buffered saline (PBS) to the control ear. Hearing was assessed by auditory brainstem responses (ABR) prior to experimental application of probiotic, then at early and late intervals following application. The animals were euthanized and the cochlear structures were analyzed using scanning electron microscopy (SEM).

Animal Care and Ethics

The study received approval by the Animal Care Committee of the McGill University Health Centre Research Institute and was conducted at the McGill Auditory Sciences Laboratory in accordance with the guidelines of the Canadian Council for Animal Care. Ten female chinchillas (C. Laniger, Ryerson Chinchilla Ranch, OH) were included in the study. Throughout the study, chinchillas were kept in temperature and light controlled rooms with free access to water and commercial food by the animal care research facilities of the Montreal Children’s Hospital Research Institute.

Sample Size

The sample size of seven was calculated setting power at 80% and an alpha of 0.05 to show a difference of 20 dB with a standard deviation of 12.6 dB determined on a pilot study. Ten animals were used to account for the potential of animal loss during the study.

Hearing Evaluation

Hearing evaluations of the chinchilla were performed at three different times: at baseline prior to application of the probiotic bacteria, early (day 7-10) and late (day 28) after application of probiotic. Hearing was tested by ABR on chinchilla anesthetized by 5% Isoflurane and maintained with 3% Isoflurane. Acoustic stimuli of 8,000, 20,000, and 25,000 Hz pure tone bursts were presented to the chinchilla through insert earphones starting at 80 dB intensity and decreasing by 5 dB until a threshold was reached.

Probiotic Bacteria Preparation

L. plantarum ATCC 10241 was plated using MRS agar from an 80% (v/v) frozen MRS-glycerol stock. The plate was incubated for 24 hours at 37°C with 5% CO2 to ensure purity. A single colony from the MRS-agar plate was incubated for 24 hours at 37°C in 10mL of MRS broth. A standard curve was derived using the overnight culture of the bacteria to make a solution of 10^9 colony-forming-units (CFU)/mL in PBS for transtympanic application. The CFU count of the solution administered was determined again by standard colony counting to ensure accurate dosing. The pH and electrolyte content of the administered solution was verified in order to ensure no confounders in the ototoxicity study.

Transtympanic Application

Each of ten animals had one ear randomized to receive probiotic bacteria (experimental), while the contralateral ear received PBS (control). After anesthetizing, a radial incision in the antero-inferior quadrant of the tympanic membrane was made and 0.4-0.7 mL of probiotic solution (until the middle ear was filled) was administered into the middle ear via a soft sterile polyethylene tubing catheter. The same volume of PBS was instilled into the control ears following the same protocol.

Middle Ear Examination and Histology

Four weeks after application of the probiotic, all animals were euthanized. The middle ears were examined for bony or mucosal changes. The cochleae were dissected and fixed in 4% paraformaldehyde. Post-fixation staining with osmium tetroxide and graded dehydration with 30, 50, 70, 80, 90, and 100% alcohol was performed. Specimens were critical-point dried using Leica CPD 030, mounted, gold plated, and viewed using the Hitachi field emission electron microscopy (Hitachi S4700, Tokyo, Japan).

Statistical Analysis

Early (day 7-10) and late (day 28) shifts in ABR thresholds after application of the probiotics were compared using paired T-test between the experimental and control ears across all three frequencies tested (8, 20, 25 kHz). A p value < 0.05 was considered statistically significant.

Chapter 3.4 Results

Probiotic Preparation and Dose Selection

Previous studies testing the activity of *Lactobacillus plantarum* against *Staphyloccus aureus* or *Pseudomas aeruginosa* on wounds used concentrations of 10^5 to $1.5x10^8$ CFU/ml[87 113 114]. Standard colony counting of an aliquot of the probiotic solution used gave a count of $1.5x10^9$ CFU/mL. The solution had a neutral pH of 7.0. Na+ was 156 mmol/L, K+ 1.7 mmol/L, and Cl- 148 mmol/L.

Observations for Physical Signs of Toxicity

Three animals had to be euthanized before completion of the experiment due to unrelated illness and were therefore excluded from analysis. The remaining seven animals were in good health until the end of the experiment, maintaining steady weight gain and normal behaviors. Commonly accepted physical signs of ototoxicity are evidence of damage to cochleovestibular nerve, resulting signs of vestibular disturbance such as head tilt or disequilibrium.

Auditory Brainstem Response Threshold Shifts

To investigate ototoxicity, baseline hearing measured prior to application of solutions were compared to early post-application (day 7-10) and late post-application (day 28) using ABRs. On the early assessment (day 7-10 following transtympanic application of solution) a significant threshold shift was found at 25 kHz in the ear with the test probiotic doses (9.6 ± 2.3dB) when compared to the control ear receiving (-1.4 ± 3.5dB), P=0.02.

This threshold shift resolved by the day 28 ABR measurements. No significant long-term hearing loss was observed between experimental and control ears at all frequencies and time points tested (Fig.1).

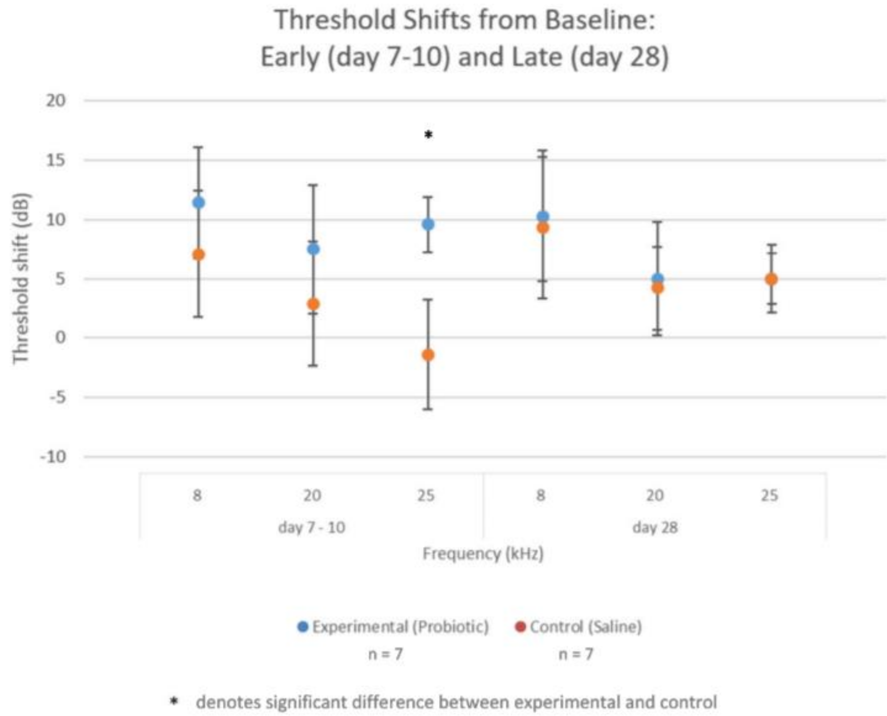

Fig. 1 Auditory brainstem response thresholds shifts from baseline. Error bars = 1 standard deviation. Abbreviations: dB, decibels; exp., experimental (probiotic); ctl, control (phosphate buffered saline)

Assessment of Structural Anatomy

Day 1 following application of solutions transtympanically, examination of the tympanic membranes under anesthesia confirmed that the middle ears were still fluid-filled. Prior to measuring early ABRs at day 7-10, ears were again examined otoscopically revealing small amounts of effusions remaining. After euthanasia and temporal bone dissection examination revealed no mucosal changes in the bulla of experimental and control ears.

Histology

Three randomly selected pairs of cochlea were examined under SEM, which revealed no observable changes to the cochlear hair cells between the experimental and control ears for each animal. The three rows of outer hair cells in the Organ of Corti were intact in both the control and experimental ears (Fig.2).

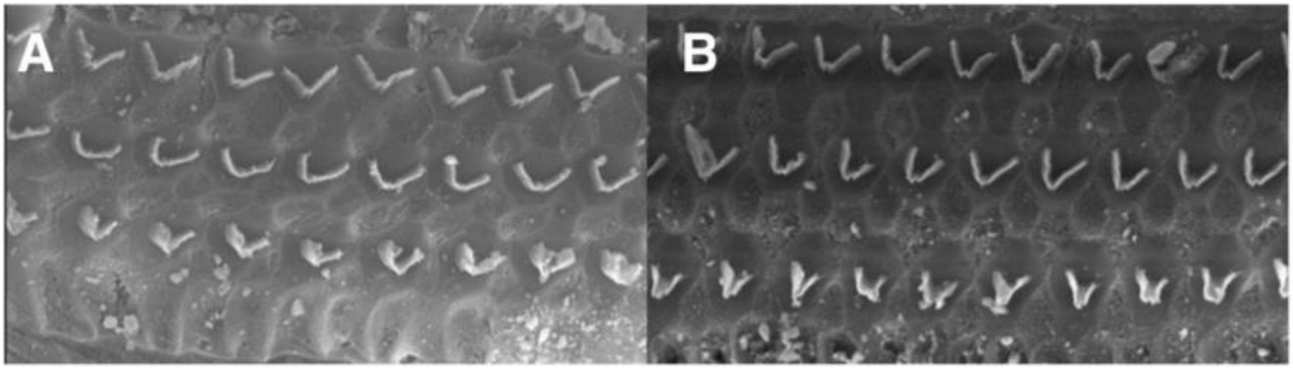

Fig. 2 Scanning electron microscopy showing comparison between control (**a**) and experimental (**b**) histological micrographs

Chapter 3.5 Discussion

Treatment of CSOM is problematic particularly when there is antibiotic resistance. Based on studies showing that probiotics can treat various infectious diseases[70 21 108], the question of whether probiotics could be effective in the treatment of CSOM is raised. Recolonization of the nasopharynx with commensal bacteria has been suggested as a strategy to treat recurrent otitis media[70 75 108]. Probiotic bacteria may be a safe and effective adjunct treatment for CSOM.

To date, topical application of probiotic directly to the ear has not been explored. An ideal probiotic would be effective against the pathogens common to CSOM, and have low potential of pathogenicity and ototoxicity. Studies show that *L. plantarum* is active *against P. aeruginosa* and *S. aureus* on wounds[22 87 114] and active against methicillin-resistant *S. aureus* in vitro[113], making it a good candidate for treating recalcitrant CSOM. However, the safety of its use in the ear precludes study of its efficacy particularly since some bacteria, such as *S. pneumoniae* and *H. influenzae*, are known to have the potential to cause sensorineural hearing loss through virulence factors[115], bacterial toxins[116], or inflammatory mediators[117]. This explains the association of otitis media with sensorineural hearing loss[90 118 94] above.

This study is the first to demonstrate that the probiotic *L. plantarum* at a concentration of 1.5 x 10^9 CFU/mL applied to the middle ear is not ototoxic as evidenced by ABR results 28 days post-application and by electron microscopy of the cochlea. Such a study is particularly relevant for an organism that is not pro-inflammatory, such as *L. plantarum*, since a non-inflammatory state tends to allow easier permeability through the round window membrane and greater risk of ototoxicity. Since *L. plantarum* has demonstrated ability to limit growth *of P. aeruginosa, S. aureus*[87] and *Peptrostreptococcus*[114], it may be a candidate for further studies investigating its safety and therapeutic use in recalcitrant CSOM.

Inhibition of *P. aeruginosa* growth, as well as inhibition of the production of biofilm and elastase by a 10^5 CFU/mL solution *L. plantarum* has been demonstrated both in vitro and in vivo[87]. In a burned-mouse model where burn wounds were infected with *P. aeruginosa*, 10^6 CFU/mL *L. plantarum* applied topically to wounds led to decreased pathogen growth and improved healing. A study of burn patients suggested that topical *L. plantarum* at a concentration of 10^5 CFU/mL was as effective as silver sulfadiazine (the gold standard in topical burn treatment) in decreasing bacterial load and promotion of wound healing[114].

A 1.5 x 10^9 CFU/mL solution of *L. plantarum,* a dose greater than that used in the studies on burn wounds, was administered into the middle ear cavity and confirmed that it did not cause any toxicity to Chinchilla cochlear structures. The transient statistically significant difference in threshold shift between experimental and control ears seen at 25 kHz during the early (day 7-10) ABR was likely due to the greater viscosity of the probiotic solution compared to PBS. It was noted on physical examination that the experimental ear demonstrated residual effusion at the time of early ABR. Even then this threshold shift was not considered clinically significant at only 11 dB, and most importantly was resolved by day 28.

Although this study serves as preliminary evidence that *L. plantarum* is safe for application directly to the middle ear, more complete testing must be done to confirm its safety and effectiveness in humans. Currently *L. plantarum* is widely used in fermented food products including yogurts, as well as probiotic supplements.

Limitations of this study include its small sample size, restricting its ability to detect hearing losses less than 20 dB, and the single application of probiotic rather than multiple applications at intervals, making its findings preliminary in nature. Moreover, euthanizing the animals at 28 days only gives us the short-term effect of the transtympanic Injection; any potential long-term toxicity cannot be ruled out with this study.

Further investigations evaluating *L. plantarum*'s otologic safety and clinical efficacy are needed. Chronic otitis media causes an increase in the thickness of the round window membrane by a factor of two, which could have a protective effect due to decreased permeability of the round window membrane[119 120 121]. The ability of substances to penetrate the round window membrane differs not only based on the properties of the substance itself but by complex and multifactorial mechanisms that affect permeability of the round window membrane. For example, methylprednisolone penetrates effectively while many high-molecular weight proteins do not penetrate at all[122]. Other local substances such as histamine increases the membrane's permeability due to its vasodilatory effect[123]. It would also be prudent to study the ototoxicity of *L. plantarum* when used in an animal model of CSOM since that may alter release of cytokines and other factors which may impact ototoxicity.

Chapter 3.6 Conclusion

This study demonstrates that a $1.5x10^9$ CFU/mL solution of *L. plantarum* applied intratympanically is not ototoxic in a chinchilla animal model. Given its activity against *P. aeruginosa* and *S. aureus*, this study's results present *L. plantarum* as a candidate for further investigation in the treatment of recalcitrant CSOM. Further pre-clinical and clinical investigations as well as efficacy studies will be invaluable for determining whether *L. plantarum* could be used therapeutically in COSM.

PART FOUR: Overall Conclusion

Chapter 4.1. Conclusion

The literature review described in Part two showed that chronic suppurative otitis media (CSOM) is a complex disease whose pathogenesis is multifactorial and involves multiple bacterial organisms, most commonly *Staphyloccoccus aureus* and *Pseudomonas aeruginosa*[35]. Probiotics appear to have beneficial effect in reducing acute otitis media and otitis media with effusion if the probiotics are able to colonize the nasopharynx, in particular near the eustachian tube orifice[71 75 76 81]. *Lactobacillus plantarum* is probiotic that has long been considered safe for ingestion and early evidence in in-vitro studies and wound care research suggest that it has antagonistic effect on *P. aeruginosa* and *S. aureus* wound infections and aids healing[22 23 87], making it an appropriate potential candidate for the treatment of CSOM.

The experimental study presented in Part three showed that at a concentration of $1.5x10^9$ CFU/mL, *L. plantarum* applied intratympanically was not ototoxic in a chinchilla animal model. Further pre-clinical and clinical investigations to understand the mechanism of actions responsible for such effects, its safety, and efficacy studies will be invaluable for determining whether *L. plantarum* could be used therapeutically in COSM.

Chapter 4.2. Future Studies

Recently a mouse model of CSOM has been described[124] whereby a eustachian tube obstruction is created unilaterally and the middle ear is inoculated with *P. aeruginosa* through an iatrogenic subtotal tympanic membrane perforation. This may be an appropriate animal model to test the effectiveness of *L. plantarum* in treating CSOM caused by *P. aeruginosa*, and perhaps the development of this model may be extended to include other pathogens or to create a multi-pathogen infection model to better represent what we see clinically. An important condition to consider in performing these investigations includes the use of pre-treatment antibiotics. Based on the studies outlined in this thesis by Roos et al[75] and Tano et al[73] it appears that pre-treatment with antibiotics prior to introduction of a probiotic is more effective, possibly by decreasing competition from other strains of bacteria and allowing more opportunity for the probiotic to adhere and establish itself.

If probiotics prove to be effective in treating CSOM, further study on the best way to deliver predictable viable doses locally would be pertinent. This may be a challenge outside of clinical studies. There is evidence that the supernatant of *L. plantarum* is also effective at inhibiting *P. aeruginosa* and *S. aureus,* albeit not as effectively as inoculation with the actual probiotic [22, 87]. It may be advantageous to characterize the elements in the supernatant responsible for this effect in order to produce therapeutic medicines based on these if colonization with beneficial probiotics proves to be impractical.

Lastly, this thesis evaluated only one probiotic, which based on the current literature, seemed to be the most appropriate. However, there are innumerable other probiotics that may also be effective in treating CSOM.

References

1 Baumann I, Gerendas B, Plinkert PK, Praetorius M. General and disease-specific quality of life in patients with chronic suppurative otitis media--a prospective study. Health Qual Life Outcomes. 2011 Jun 29;9:48. doi: 10.1186/1477-7525-9-48.

2 Madana J, Yolmo D, Kalaiarasi R, Gopalakrishnan S, Sujatha S. Microbiological profile with antibiotic sensitivity pattern of cholesteatomatous chronic suppurative otitis media among children. Int J Pediatr Otorhinolaryngol. 2011; 75(9):1104–1108.

3 Jensen RG, Koch A, Homoe P. The risk of hearing loss in a population with a high prevalence of chronic suppurative otitis media. Int J Pediatr Otorhinolaryngol. 2013; 77(9): 1530-5.

4 Aarhus, L., Tambs, K., Kvestad, E., Engdahl, B. Childhood otitis media: a cohort study with 30-year follow-up of hearing (The HUNT Study). Ear Hear 2015; 36: 302–308.

5 Daly KA, Hunter LL, Giebink GS. Chronic otitis media with effusion. Pediatrics in review/American Academy of Pediatrics. 1999; 20(3):85–93.

6 Bowd AD. Otitis media: health and social consequences for aboriginal youth in Canada’s north*. Int J Circumpolar Health.* 2005; 64(1):5–15.

7 Williams CJ, Jacobs AM. The impact of otitis media on cognitive and educational outcomes. *Med J Aust.* 2009; 191(9 Suppl):S69–S72.

8 Olatoke F, Ologe FE, Nwawolo CC, Saka, MJ. The prevalence of hearing loss among schoolchildren with chronic suppurative otitis media in Nigeria, and its effect on academic performance. Ear Nose Throat J 2008; 87:E19.

9 Khairi Md Daud M, Noor RM, Rahman NA, Sidek DS, Mohamad A. The effect of mild hearing loss on academic performance in primary school children. Int J Pediatr Otorhinolaryngol 2010; 74: 67–70.

10 Dubey SP, Larawin V. Complications of chronic suppurative otitis media and their management. Laryngoscope. 2007; 117(2):264-7.

11 Chew YK, Cheong JP, Khir A, Brito-Mutunayagam S, Prepageran N. Complications of chronic suppurative otitis media: a left otogenic brain abscess and a right mastoid fistula. Ear Nose Throat J 2012: 91, 428–430.

12 Sun J, Sun J. Intracranial complications of chronic otitis media. Eur Arch Otorhinolaryngol 2014: 271, 2923–2926.

13 O'Connor, T.E., Perry, C.F., Lannigan, F.J. Complications of otitis media in indigenous and non-Indigenous children. Medical Journal of Australia 2009: 191; S60-S64

14 Jackman A, Ward R, April M, Bent J. Topical antibiotic induced otomycosis. Int J Pediatr Otorhinolaryngol. 2005; 69(6):857-60.

15 Montgomery JM, Lehmann D, Smith T et al. Bacterial colonization of the upper respiratory tract and its association with acute lower respiratory tract infections in Highland children in Papua, New Guinea. Rev Infect Dis 1990; 12: S1006-1006.

16 Kuklinska D, Killian M. Relative proportions of Haemophilus species in the throat of healthy childrenand adults. Eur J Clin Microbiol 1984; 3: 249-51.

17 Berstein JM,Faden HF, Dryja DM, Wactawski-Wende J. Micro-ecology of the Nasopharyngeal Bacterial Flora in Otitis-prone and Non-otitis-prone Children. Acta Otolaryngol (Stockh) I 1993; I 13: 88- 92.

18 Faden HF, Stanievich J, Brodsky L, Bernstain JM. Changes in nasopharyngeal flora during otitis media inchildhood. Pediatr Infect Dis 1990; 9: 623-6.

19 Long SS, Henretig FM, Teter MJ, McGowan KL. Nasopharyngeal flora and acute otitis media. Infect Immun 1983; 41: 987-91.

20 Berstein JM,Faden HF, Dryja DM, Wactawski-Wende J. Micro-ecology of the Nasopharyngeal Bacterial Flora in Otitis-prone and Non-otitis-prone Children. Acta Otolaryngol (Stockh) I 1993; I 13: 88- 92.

21 Skovbjerg S , Roos K , Holm SE, Grahn Håkansson E, Nowrouzian F, Ivarsson M, Adlerberth I, Wold AE. Spray bacteriotherapy decreases middle ear fluid in children with secretory otitis media. Arch Dis Child 2009; 94(2):92–98.

22 Al-Mathkury HJF, Al-Aubeidi HJA-R. Probiotic effect of lactobacilli on mice in wound infections. J Al-Nahrain Univ 2008; 11:111–6.

23 Strus M, Malinowska M. The range of antagonistic effects of Lactobacillus bacterial strains on etiologic agents of bacterial vaginosis. Med Dosw Mikrobiol 1999; 51(1-2):47-57.

24 M.M. Rovers, A. Schilder, G. Zielhuis, R. Rosenfeld. Otitis Media. Lancet 200: 363; 465-473

25 Pettigrew MM, Gent JF, Pyles RB, Miller AL, Nokso-Koivisto J, Chonmaitree T. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. J Clin Microbiol 2011; 49(11): 3750–3755

26 Marom T, Nokso-Koivisto J, Chonmaitree T. Viralbacterial interactions in acute otitis media. Curr Allergy Asthma Rep 2012; 12(6):551–558

27 Kilpi T, Herva E, Kaijalainen T, Syrjänen R, Takala AK. Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life. Pediatr Infect Dis J 2001: 20(7):654–662.

28 Fortanier A, Venekamp R, Boonacker C, Hak E, Schilder A, Sanders E, Damoiseaux R. Document details - Pneumococcal conjugate vaccines for preventing otitis media. Cochrane Database Syst Rev 2014: 4; Article CD001480

29 Leach AJ, Morris PS, McCallum GB, Wilson CA, Stubbs L, Beissbarth J, Jacups S, Hare K, Smith-Vaughan HC. Emerging pneumococcal carriage serotypes in a high-risk population receiving universal 7-valent pneumococcal conjugate vaccine and 23-valent polysaccharide vaccine since 2001. BMC Infectious Diseases 2009: 9; 121

30 Bakaletz LO. Immunopathogenesis of polymicrobial otitis media. J Leukoc Biol 2010: 87, 213–222.

31 Vartiainen E, Vartiainen J. Effect of aerobic bacteriology on the clinical presentation and treatment results of chronic suppurative otitis media. J Laryngol Otol 1996: 110, 315–318.

32 Brook J. The role of anaerobic bacteria in chronic suppurative otitis media in children: implications for medical therapy. Anaerobe. 2008; 14(6):297-300.

33 Dayasena R, Dayasiri M, Jayasuriya C, Perera D. Aetiological agents in chronic suppurative otitis media in Sri Lanka. Australas Med J 2011; 4:101–104

34 Sharma K, Aggarwal A, Khurana PM. Comparison of bacteriology in bilaterally discharging ears in chronic suppurative otitis media. Indian J Otolaryngol Head Neck Surg 2010; 62:153–157

35 Prakash R, Juyal D, Negi V, Pal S, Adekhandi S, Sharma M, Sharma N. Microbiology of chronic suppurative otitis media in a tertiary care setup of Uttarakhand state, India. N Am J Med Sci 2013; 5:282–287

36 Orji FT, Dike BO. Observations on the current bacteriological profile of chronic suppurative otitis media in South eastern Nigeria. Ann Med Health Sci Res 2015; 5:124–128

37 Vishwanath S, Mukhopadhyay C, Prakash R, Pillai S, Pujary K, Pujary P. Chronic suppurative otitis media: optimizing initial antibiotic therapy in a tertiary care setup. Indian J Otolaryngol Head Neck Surg 2012; 64:285–289

38 Juyal D, Negi V, Pal S, Adekhandi S, Sharma M, Sharma N, Prakash R. Microbiology of chronic suppurative otitis media in a tertiary care setup of Uttarakhand state, India. North Am J Med Sci 2013; 5:282.

39 Shrestha BL, Amatya R, Shrestha I, Ghosh I. Microbiological profile of chronic suppurative otitis media. Nepalese J ENT Head Neck Surg 2012; 2:6 –7.

40 Ahn JH, Kim M-N, Suk YA, Moon BJ. 2012. Preoperative, intraoperative, and postoperative results of bacterial culture from patients with chronic suppurative otitis media. Otol Neurotol 2012;33:54 –59

41 Sattar A, Alamgir A, Hussain Z, Sarfraz S, Nasir J & Badar-e-Alam. Bacterial spectrum and their sensitivity pattern in patients of chronic suppurative otitis media. J Coll Physicians Surg Pak 2012: 22, 128–129.

42 Aduda DS, Macharia IM, Mugwe P, Oburra H, Farragher B, Brabin B & Mackenzie I. Bacteriology of chronic suppurative otitis media (CSOM) in children in Garissa district, Kenya: a point prevalence study. Int J Pediatr Otorhinolaryngol 2013: 77, 1107–1111.

43 Sharma S, Rehan HS, Goyal A, Jha AK, Upadhyaya S, Mishra SC. Bacteriological profile in chronic suppurative otitis media in Eastern Nepal. Trop Doct 2004; 34:102–104

44 Hannley MT, Denneny 3rd JC, Holzer SS. Use of ototopical antibiotics in treating 3 common ear diseases. Otolaryngol Head Neck Surg. 2000; 122(6):934–40.

45 Dohar JE, Kenna MA, Wadowsky RM. In vitro susceptibility of aural isolates of Pseudomonas aeruginosa to commonly used ototopical antibiotics. Am J Otol 1996; 17:207-9.

46 Lee SK, Park DC, Kim MG, Boo SH, Choi YJ, Byun JY, Park MS, Yeo SG. Rate of isolation and trends of antimicrobial resistance of multidrug resistant Pseudomonas aeruginosa from otorrhea in chronic suppurative otitis media. Clin Exp Otorhinolaryngol 2012;5:17

47 Kim SH, Kim MG, Kim SS, Cha SH, Yeo SG. Change in Detection Rate of Methicillin-Resistant Staphylococcus aureus and Pseudomonas aeruginosa and Their Antibiotic Sensitivities in Patients with Chronic Suppurative Otitis Media. J Int Adv Otol. 2015; 11(2):151-6.

48 Yang JA, Kim JY, Yoon YK, Kim S, Park DW, Sohn JW, Sim HS, Kim MJ. Epidemiological and genetic characterization of methicillin resistant Staphylococcus aureus isolates from the ear discharge of outpatientswith chronic otitis media. J Korean Med Sci 2008; 23:762.

49 Lampikoski H, Aarnisalo, AA, Jero, J, Kinnari, TJ. Mastoid biofilm in chronic otitis media. Otol Neurotol 2012; 33: 785–788.

50 Hall-Stoodley, L, Stoodley, P. Evolving concepts in biofilm infections. Cell Microbiol 2009; 11: 1034–1043.

51 Mah TF. Biofilm-specific antibiotic resistance. Future Microbiol 2012; 7: 1061–1072.

52 Wang, JC, Hamood, AN, Saadeh C, Cunningham, MJ, Yim, MT, Cordero, J. Strategies to prevent biofilm-based tympanostomy tube infections. Int J Pediatr Otorhinolaryngol 2014; 78: 1433–1438.

53 Alhede M, Bjarnsholt, T, Givskov M, Alhede M. Pseudomonas aeruginosa biofilms: mechanisms of immune evasion. Adv Appl Microbiol 2014; 86: 1–40.

54 Gellatly SL, Hancock RE. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. Pathog Dis 2013; 67: 159–173.

55 Garcıa-Cobos S, Moscoso M, Pumarola F, Arroyo M, Lara N, Perez-Vazquez, M, Aracil B, Oteo J, Garcıa E, Campos J. Frequent carriage of resistance mechanisms to b-lactams and biofilm formation in Haemophilus influenzae causing treatment failure and recurrent otitis media in young children. J Antimicrob Chemother 2014: 69, 2394–2399.

56 Saunders J, Murray M, Alleman A. Biofilms in chronic suppurative otitis media and cholesteatoma: scanning electron microscopy findings. Am J Otolaryngol 2011; 32:32–37

57 Schrader N, Isaacson G. Fungal otitis externa – its association with fluoroquinolone eardrops. Pediatrics 2003; 111: 1123.

58 Eiseman B, Silen W, Bascom GS, Kauvar AJ. Fecal enema as an adjunct in the treatment of pseudomembranous. Surgery 1958: 44; 854-859

59 Oberhelman RA, Gilman RH, Sheen P, et al. A placebo-controlled trial of Lactobacillus GG to prevent diarrhea in undernourished Peruvian children. J Pediatr 1999: 134, 15–20.

60 Szajewska H, Kotowska M, Mrukowicz JZ, et al. Efficacy of Lactobacillus GG in prevention of nosocomial diarrhea in infants. J Pediatr 2001: 138, 361–365.

61 Chouraqui JP, Van Egroo LD & Fichot MC (2004) Acidified milk formula supplemented with Bifidobacterium lactis: impact on infant diarrhea in residential care settings. J Pediatr Gastroenterol Nutr 2004: 38, 288–292.

62 Saavedra JM, Bauman NA, Oung I, et al. Feeding of Bifidobacterium bifidum and Streptococcus thermophilus to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet 1994: 344, 1046–1049.

63 AlFaleh K, Anabrees J. Probiotics for prevention of necrotizing enterocolitis in preterm infants. Cochrane Database of Systematic Reviews 2014: 4, Article CD005496

64 Isolauri E, Majamaa H, Arvola T, et al. Lactobacillus casei strain GG reverses increased intestinal permeability induced by cow milk in suckling rats. Gastroenterology 1993: 105, 1643 –1650.

65 Mack DR, Michail S, Wei S, et al. Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. Am J Physiol 1999: 276, G941 – G950.

66 Cebra JJ. Influences of microbiota on intestinal immune system development. Am J Clin Nutr 1999; 69:1046S–1051S

67 Perdigo´n G, de Macias ME, Alvarez S, et al. Effect of perorally administered lactobacilli on macrophage activation in mice. Infect Immun 1986 : 53, 404 – 410.

68 Fukushima Y, Kawata Y, Hara H, et al. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. Int J Food Microbiol 1998: 42, 39– 44.

69 Rautava S, Arvilommi H & Isoaluri E. Specific probiotics in enhancing maturation of IgA responses in formula-fed infants. Pediatr Res 2006: 60, 221 – 224.

70 Bernstein JM, Sagahtaheri-Altaie S, Dryja DM, Wactawski-Wende J. Bacterial interference in nasopharyngeal bacterial flora of otitis-prone and non-otitis-prone children. J. Acta Otorhinolaryngol Belg. 1994; 48(1):1-9.

71 Brook I, Yocum P. Bacterial interference in the adenoids of otitis media-prone children. Pediatr Infect Dis J 1999;18:835-7.

72 Fujimori I, Hisamatsu K, Kikushima K, Goto R, Murakami Y, Yamada T. The nasopharyngeal flora in children with otitis media with effusion. Eur Arch Otorhinolaryngol 1996;253:260-3.

73 Tano K, Grahn-Håkansson E, Holm SE, Hellström E. Inhibition of OM pathogens by alpha-hemolytic streptococci from healthy children, children with SOM and children with rAOM. Int J Pediatr Otorhinolaryngol 2000 Dec 22;56(3):185-90.

74 Tano K, Olofsson C, Grahn-Hakansson E, Holm SE. In vitro inhibition of S. pneumoniae, nontypable H. influenzae and M. catarrhalis by alpha-hemolytic streptococci from healthy children, Int. J. Pediatr. Otorhinolaryngol 1999; (47): 49–56.

75 Roos K, Håkansson EG, Holm S. Effect of recolonisation with "interfering" alpha streptococci on recurrences of acute and secretory otitis media in children: randomised placebo controlled trial. Brit Med J 2001; 322(7280):210–212.

76 Marchisio P, Santagati M, Scillato M, Baggi E, Fattizzo M, Rosazza C, Stefani S, Esposito S, Principi N. Streptococcus salivarius 24SMB administered by nasal spray for the prevention of acute otitis media in otitis-prone children. Eur. J. Clin. Microbiol. Infect. Dis 2015: 34: 2377-2383

77 R. Cohen, E. Martin, F. de La Rocque, F. Thollot, S. Pecquet, A. Werner, M. Boucherat, E. Varon, E. Bingen, C. Levy. Probiotics and prebiotics in preventing episodes of acute otitis media in high-risk children: A randomized, double-blind, placebo-controlled study. Pediatr. Infect. Dis. J. 2013; 32: 810-814

78 F. Di Pierro, M. Colombo, M. Giuliani, M. Danza, I. Basile, T. Bollani, A. Conti, A. Zanvit, A. Tottoli. Effect of administration of Streptococcus salivarius K12 on the

occurrence of streptococcal pharyngo-tonsillitis, scarlet fever and acute otitis media in 3 years old children. Eur. Rev. Med. Pharmacol. Sci. 2016: 20; 4601-4606

79 Rautava S, Salminen S, Isolauri E. Specific probiotics in reducing the risk of acute infections in infancy--a randomised, double-blind, placebo-controlled study. Br J Nutr. 2009; 101(11):1722-6.

80Varma P, Nisha N, Dinesh K, Kumar, Biswas R. Anti-infective Properties of Lactobacillus fermentum against Staphylococcus aureusand Pseudomonas aeruginosa. J Mol Microbiol Biotechnol 2011; 20:137-143.

81 Nawaz SK, Riaz S, Riaz S, Hasnain S. Screening for anti-methicillin resistant Staphylococcus aureus (MRSA) bacteriocin producing bacteria. Afr J Biotechnol 2009: 8, 365-368

82 Sikorska H, Smoragiewicz W. Role of probiotics in the prevention and treatment of meticillin-resistant Staphylococcus aureus infections. International Journal of Antimicrobial Agents 2013; 42: 475–481.

83 Nagasaki A, et al. Ulcerative Colitis with Multidrug-Resistant Pseudomonas aeruginosa Infection SuccessfullyTreated with Bifidobacterium. Digestion 2010; 81:204–205.

84 Peran L, Camuesco D, Comalada M, et al: A comparative study of the preventative effects exerted by three probiotics, Bifidobacterium lactis , Lactobacillus casei and Lactobacillus acidophilus , in the TNBS model of rat colitis. J Appl Microbiol 2007; 103: 836–844.

85 Imaoka A, Shima T, Kato K, et al: Anti-inflammatory activity of probiotic Bifidobacterium: enhancement of IL-10 production in peripheral blood mononuclear cells from ulcerative colitis patients and inhibition of IL-8 secretion in HT-29 cells. World J Gastroenterol 2008; 14: 2511–2516.

86 Bai AP, Ouyang Q, Xiao XR, et al: Probiotics modulate inflammatory cytokine secretion from inflamed mucosa in active ulcerative colitis. Int J Clin Pract 2006; 60: 284–288.

87 Valdéz JC, Peral MC, Rachid M, Santana M, Perdigón G. Interference of Lactobacillus plantarum with Pseudomonas aeruginosa in vitro and in infected burns: the potential use of probiotics in wound treatment. Clin Microbiol Infect 2005;11(6):472-9.

88 Varshney S., Nangia A., Bist S. S., Singh R. K., Gupta N., Bhagat S. Ossicular chain status in chronic suppurative otitis media in adults. Indian J Otolaryngol Head Neck Surg 2010; 62:421–426

89 Mittal R, Lisi CV, Gerring R, Mittal J, Mathee K, Narasimhan G, Azad RK, Yao Q, Grati M, Yan D, Eshraghi AA, Angeli SI, Telischi FF, Liu XZ. Current concepts in the pathogenesis and treatment of chronic suppurative otitis media. J Med Microbiol. 2015; 64(10):1103-16.

90 Papp Z, Rezes S, Jo´ kay I, Sziklai I. Sensorineural hearing loss in chronic otitis media. Otol Neurotol 2003; 24: 141–144.

91 da Costa SS, Rosito LP, Dornelles C. Sensorineural hearing loss in patients with chronic otitis media. Eur Arch Otorhinolaryngol 2009; 266, 221–224.

92 de Azevedo AF, Pinto DC, de Souza NJ, Greco DB, Goncalves DU. Sensorineural hearing loss in chronic suppurative otitis media with and without cholesteatoma. Braz J Otorhinolaryngol. 2007; 73(5): 671-4.

93 Joglekar S, Morita N, Cureoglu S, Schachern PA, Deroee AF, Tsuprun V, Paparella MM, Juhn SK. Cochlear pathology in human temporal bones with otitis media. Acta Otolaryngol 2010; 130: 472–476.

94 Morizono T, Giebink GS, Paparella MM, Sikora MA, Shea D. Sensorineural

hearing loss in experimental purulent otitis media due to Streptococcus pneumoniae. Arch Otolaryngol 1985; 111:794Y8.

95 Stenqvist M, Anniko M, Pettersson A. Effect of Pseudomonas aeruginosa exotoxin A on inner ear function. Acta Otolaryngol 1997; 117(1):73-9.

96 Cureoglu S, Schachern P, Paparella MM, Lindgren BR. Cochlear changes in chronic otitis media. Laryngoscope 2004; 114:622Y6.

97 Clerici WJ, DiMartino DL, Prasad MR. Direct effects of reactive oxygen species on cochlear outer hair cell shape in vitro. Hear Res 1995; 84:30–40

98 Jung TT, Llaurado RJ, Nam BHHH, Park SK, Kim PD, John EO. Effects of nitric oxide on morphology of isolated cochlear outer hair cells: possible involvement in sensorineural hearing loss. Otol Neurotol 2003; 24:682–685

99 Juhn SK, Jung MK, Hoffman MD, Drew BR, Preciado DA, Sausen NJ, Jung TT, Kim BH, Park SY. The role of inflammatory mediators in the pathogenesis of otitis media and sequelae. Clin Exp Otorhinolaryngol 2008; 1:117–138.

100 Guo Y, Wu Y, Chen W, Lin J. Endotoxic damage to the stria vascularis: the pathogenesis of sensorineural hearing loss secondary to otitis media? J Laryngol Otol 1994; 108: 310–313.

101 Jung TT, Park YM, Miller SK, Rozehnal S, Woo HY, Baer W. Effect of exogenous arachidonic acid metabolites applied on round window membrane on hearing and their levels in the perilymph. Acta Otolaryngol Suppl 1992; 493: 171–176.

102 Kolo ES, Salisu AD, Yaro AM, Nwaorgu OG. Sensorineural hearing loss in patients with chronic suppurative otitis media. Indian J Otolaryngol Head Neck Surg 2012; 64: 59–62.

103 Yoshida, H., Miyamoto, I. & Takahashi, H. (2009). Is sensorineural hearing loss with chronic otitis media due to infection or aging in older patients? Auris Nasus Larynx 2009; 36: 269–273.

104 Amali A, Hosseinzadeh N, Samadi S, Nasiri S, Zebardast J. Sensorineural hearing loss in patients with chronic suppurative otitis media: Is there a significant correlation? Electron Physician. 2017; 25:9(2):3823-3827.

105 Nhan C, Bezdjian A, Saha S, Prakash S, Nguyen LHP, Daniel SJ. Safety of transtympanic application of probiotics in a chinchilla animal model. J Otolaryngol Head Neck Surg. 2017 Nov 22;46(1):63.

106 Daniel S. Topical Treatment of Chronic Suppurative Otitis Media. Curr Infect Dis Rep. 2012; 14:121-127.

107 Sanders ME. Probiotics: Definition, Sources, Selection, and Uses. Clin Infect Dis. 2008; 46 Suppl 2:S58-S61; discussion S144-S151.

108 Jones ML, Martoni CJ, Ganopolsky JG, Sulemankhil I, Ghali P, Prakash S. Improvement of gastrointestinal health status in subjects consuming lactobacillus reuteri NCIMB 30242 capsules: a post-hoc analysis of a randomized controlled trial. Expert Opin Biol Ther. 2013;13(12):1643–51.

109 Berstein JM, Faden HF, Dryja DM, Wactawski-Wende J. Micro-ecology of the nasopharyngeal bacterial Flora in Otitis-prone and non-otitis-prone children. Acta Otolaryngol. 1993;13:88–92.

110 Nhan C, Bezdjian A, Alarfaj A, Daniel SJ. Can floseal™ be applied safely during otologic surgery? Assessment of ototoxicity in a chinchilla animal model. J Otolaryngol Head Neck Surg. 2017; 29; 46(1):24.

111 Nader ME, Kourelis M, Daniel SJ. Hydrogen peroxide ototoxicity in unblocking ventilation tubes: a chinchilla pilot study. Otolaryngol Head Neck Surg. 2007; 136(2):216-20.

112 Aron M, Victoria Akinpelu O, Dorion D, Daniel S. Otologic safety of manuka honey. J Otolaryngol Head Neck Surg. 2012 Apr;41 Suppl 1:S21-30.17

113 Brachkova MI, Marques P, Rocha J, Sepodes B, Duarte MA, Pinto JF. Alginate films containing lactobacillus plantarum as wound dressing for prevention of burn infection. J Hosp Infect. 2011;79(4):375–57.

114 Peral MC, Martinez MA, Valdez JC. Bacteriotherapy with lactobacillus plantarum in burns. Int Wound J. 2009;6(1):73–81.

115 Tsuprun V, Cureoglu S, Schachern PA, Ferrieri P, Briles DE, Paparella MM, Juhn SK. Role of pneumococcal proteins in sensorineural hearing loss due to otitis media. Otol Neurotol. 2008; 29(8):1056-60.

116 Demaria TF. Localization of nontypeable Haemophilus influenza endotoxin in the middle and inner ear during experimental otitis media. Acta Otolaryngol. 1999;119(5):583-7.

117 Juhn SK, Jung TTK, Lin J, Rhee CK. Effect of inflammatory mediators on middle ear pathology and inner ear function. Ann NY Acad Sci 1997; 830:130-142.

118 Margolis RH, Hunter LL, Saupe JR, Giebink GS. Effect of otitis media on extended high frequency hearing in children. Ann Otol Rhinol Laryngol 1993; 102:1-5.

119 Sahni RS, Paparella MM, Schachern PA, Goycoolea MV, Le CT. Thickness of the human round window membrane in different forms of otitis media. Arch Otolaryngol Head Neck Surg 1987; 113:630-634.

120 Schachern PA, Paparella MM, Goycoolea MV, Duvall AJ III, Choo YB. The permeability of the round window membrane during otitis media. Arch Otolaryngol Head Neck Surg 1987; 113:625-629.

121 Schachern PA, Paparella MM, Goycoolea MV. Experimental grafting of the round window membrane: Part I. Laryngoscope 1987; 97:790-796.

122 Kashio, A., Sakamoto, T., Kakigi, A. et al. Topical application of the antiapoptotic TAT-FNK protein prevents aminoglycoside-induced ototoxicity. Gene Ther 2012; 19, 1141–1149.

123 F. Wessel, M. Winderlich, M. Holm, M. Frye, R. Rivera-Galdos, M. Vockel, et al. Leukocyte extravasation and vascular permeability are each controlled in vivo by different tyrosine residues of VE-cadherin. Nat. Immunol., 15 (3) (2014), pp. 223-230.

124 Santa Maria PL, Kim S, Varsak Yk, Yang YP. Heparin binding-epidermal growth factor-like growth factor for the regeneration of chronic tympanic membrane perforations in mice. Tissue Eng 2015; Part A 21:1483–1494.