UNIVERSITÀ DEGLI STUDI DI CATANIA
DOTTORATO IN BIOLOGIA UMANA E BIOINFORMATICA:
BASI CELLULARI E MOLECOLARI DEL FENOTIPO
XXVI CICLO
DIPARTIMENTO DI SCIENZE BIOMEDICHE E BIOTECNOLOGICHE

Clinical Application of *S.salivarius* 24SMBc in the Prevention of Recurrent Otitis Media in Paediatric Age

Applicazione clinica di *S.salivarius* 24SMBc nella prevenzione dell'otite media ricorrente in età pediatrica

TESI DI DOTTORATO
Dott.ssa Marina Scillato

Coordinatore: Chiar.mo Prof. MICHELE PURRELLO

Tutor: Prof.ssa MARIA SANTAGATI

ANNO ACCADEMICO 2013 / 2014

TABLE OF CONTENTS

ABSTRACT (English)

Introduction. Much attention has recently been devoted to the analysis of the oral microbiota to develop a bacteriotherapy focused on prevention and/or treatment of upper respiratory tract infections. The oral cavity harbours some beneficial bacterial species such as *Streptococcus salivarius* which is considered the predominant 'safe' colonizer, capable of fostering more balanced, health-associated oral microflora, interfering with potential pathogens. This antagonist activity is often mediated by competition for nutriments, better adhesion to target cells and release of bioactive agents such as bacteriocins. In our laboratory, we characterized one strain, *S.salivarius* 24SMBc, isolated from healthy children which showed excellent inhibitory activity against *S.pneumoniae* and *S.pyogenes* and potent capacity of adhesion to HEp-2 cells.

These properties encouraged us to evaluate a possible application of *S.salivarius* 24SMBc as an oral probiotic for children with recurrent otitis media.

Material and methods. We sequenced the *S.salivarius* 24SMBc genome by pyrosequencing to verify the presence of virulent factors and to look for genes encoding bacteriocins that inhibit the growth of the pathogens previously described.

Then, we included *S.salivarius* 24SMBc in a clinical trial protocol conducted on 17 healthy adult volunteers to evaluate its safety for the human host and its ability to colonize and persist in the upper respiratory tract. The presence of *S.salivarius* 24SMBc in rhinopharynx tissue was determined after different time intervals from nasal administration by molecular identification, antagonism test to evaluate BLIS production and RAPD-PCR to distinguish *S.salivarius* 24SMBc's genotype from other *S.salivarius* strains. The following phase for the assessment of the colonization of *S.salivarius* 24SMBc in the upper respiratory tract of children and its efficacy to reduce the number of episodes of otitis media (OM), was realized through a paediatric randomized, placebo-controlled, double-blind trial. This study enrolled 120 "otitis prone" children and included phenotypic and molecular identification of *S.salivarius* 24SMBc and pathogenic strains of OM from the biological samples. Moreover, the level of colonization of our strain was determined by qPCR using a specific genomic target to identify *S.salivarius* 24SMBc.

Results and conclusion. Genome annotation showed that *S.salivarius* 24SMBc is free of streptococcal virulent factors. The results of clinical trials demonstrated the absence of adverse effects for the human host and a good capability of *S.salivarius* 24SMBc to colonize the human rhinopharynx tissue. Prophylactic administration of *S.salivarius* 24SMB to children with a history of recurrent OM reduces episodes of this disease as well the incidence of infection by some causative pathogens such as *S.pneumoniae* and *S.pyogenes*. Therefore, *S.salivarius* 24SMBc appears a competitive nasopharyngeal - localized strain with a good potential for use as an oral probiotic to prevent OM in paediatric subjects.

ABSTRACT (Italian)

Introduzione. Recentemente, lo studio della microflora orale ha suscitato molto interesse al fine di sviluppare una batterioterapia mirata alla prevenzione e/o trattamento delle infezioni delle alte vie respiratorie. La cavità orale ospita alcune specie di batteri benefici come *Streptococcus salivarius* che è considerato un microrganismo colonizzatore 'sicuro' e predominante in questo microhabitat, in grado di promuovere una microflora orale più equilibrata e associata allo stato di benessere, interferendo con i potenziali patogeni. Questa attività antagonista è spesso mediata dalla competizione per i nutrienti, una migliore adesione alle cellule target e il rilascio di agenti bioattivi quali le batteriocine. Nel nostro laboratorio, abbiamo caratterizzato un ceppo, *S.salivarius* 24SMBc, isolato da bambini sani, che ha mostrato un'eccellente attività inibitoria nei confronti di *S.pneumoniae* e *S.pyogenes* e una buona capacità di adesione alle cellule HEp-2. Queste proprietà ci hanno indotto a valutare una possibile applicazione di *S.salivarius* 24 SMBC come probiotico orale nei bambini con otite media ricorrente.

Materiali e metodi. Abbiamo sequenziato il genoma di *S.salivarius* 24SMBc mediante pyrosequencing per verificare la presenza di fattori virulenza e determinare la presenza di geni codificanti le batteriocine che inibiscono la crescita dei patogeni descritti in precedenza.

Successivamente, abbiamo incluso *S.salivarius* 24SMBc in un protocollo di sperimentazione clinica condotto su 17 volontari adulti sani per valutare la sicurezza di questo microrganismo nei confronti dell'ospite umano e la sua capacità di colonizzare e persistere nelle alte vie respiratorie.

La presenza di *S.salivarius* 24SMBc nel tessuto rinofaringeo è stata determinata dopo diversi intervalli di tempo in seguito alla somministrazione nasale del ceppo in studio, mediante identificazione molecolare, test dell'antagonismo per valutare la produzione di batteriocine e RAPD-PCR per distinguere il genotipo di *S.salivarius* 24SMBc da quello di altri ceppi appartenenti alla specie *S.salivarius*.

Lo step seguente, finalizzato alla valutazione della colonizzazione di *S.salivarius* 24SMBc nelle alte vie respiratorie dei bambini e della sua efficacia nel ridurre il numero di episodi di otite media, è stato realizzato attraverso uno studio pediatrico randomizzato, controllato con placebo, in doppio cieco. Questo studio ha arruolato 120 bambini con otite ricorrente ed ha previsto la ricerca di *S.salivarius* 24SMBc e

dei ceppi responsabili di otite media dai campioni biologici, mediante identificazione fenotipica e molecolare.

Inoltre, è stato determinato il livello di colonizzazione del nostro ceppo mediante qPCR, utilizzando una specifica sequenza genomica come target molecolare per identificare *S.salivarius* 24SMBc.

Risultati e conclusioni. La sequenza del genoma ha dimostrato che *S.salivarius* 24SMBc è privo dei fattori di virulenza che possono ritrovarsi nel genere *Streptococcus*.

I risultati ottenuti dagli studi clinici hanno dimostrato che il nostro ceppo batterico è sicuro per l'uomo ed ha una buona capacità di colonizzare il tessuto rinofaringeo umano. La somministrazione profilattica di *S.salivarius* 24SMB nei bambini con una storia di otite media ricorrente, riduce gli episodi di questa malattia e anche l'incidenza di infezione da parte di alcuni agenti patogeni responsabili come *S.pneumoniae* e *S.pyogenes*.

Pertanto, *S.salivarius* 24SMBc appare essere un microrganismo dalla localizzazione nasofaringea, competitivo e con un buon potenziale per un suo uso come probiotico orale nella prevenzione dell'otite media nei soggetti pediatrici.

1. INTRODUCTION

1.1 Probiotics

Probiotics are microorganisms, principally bacteria, which confer health benefits maintaining or restoring a host's natural microbial flora. The use of microorganisms to promote health is very ancient and can even be traced back to classical Roman literature, where food fermented with microorganisms was used therapeutically **[1]**. The modern history of probiotics dates back to 1877, when Pasteur and his associate Joubert, noting suppression of anthrax bacillus growth in co-cultures with 'common bacilli' (probably *Escherichia coli*), commented that "these facts perhaps justify the highest hopes for therapeutics" **[2]**.

The concept of probiotics evolved at the turn of the 20th century from a hypothesis first proposed by the Ukrainian bacteriologist and Nobel Laureate Elie Metchnikoff. In 1908, working at the Pasteur Institute, Dr. Metchnikoff observed that a surprising number of people in Bulgaria lived more than 100 years. This longevity could not be attributed to the impact of modern medicine because Bulgaria, one of the poorest countries in Europe at the time, had not yet benefited from such life-extending medical advances. Dr. Metchnikoff further observed that Bulgarian peasants consumed large quantities of yogurt that contains bacteria which conferred the observed health-promoting benefits. He suggested that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" **[3]**.

The term 'probiotics', the antonym of the term antibiotics, was introduced in 1965 by Lilly & Stillwell as Substances produced by microorganisms which promote the growth of other microorganisms **[4]**. After several definitions, the final one, officially adopted by the World Health Organization, outlining the breadth and scope of probiotics as they are known today, was: "Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" **[5]**.

In contrast, prebiotics are generally defined as not digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, and thus, in fact, improve host health. These prebiotics include inuline, fructo-oligosaccharides, galacto-oligosaccharides and lactulose. The concept of prebiotics essentially has the same aim as probiotics, which is to improve host health via

modulation of the intestinal flora, although by a different mechanism. However, there are some cases in which prebiotics may be beneficial for the probiotic, especially with regard to bifidobacteria. This is known as the symbiotic concept. Synbiotics are defined as mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract of the host **[6]**.

Before the advent of antibiotics, there were a series of brave attempts by physicians to give protection against diseases such as tuberculosis, anthrax and diphtheria by dosing patients with putatively innocuous commensal bacteria **[7]**. However, except for the treatment of minor ailments or as supplemental therapy, the application of so-called 'bacteriotherapy' or 'bacterio-prophylaxis' was largely discontinued on the spectacular discovery of antibiotics.

Indeed, in the middle of the twentieth century, antibiotics offered the promise of efficient and cheap treatment of bacterial infections and even the possibility to eliminate infectious diseases. However, within the span of a single human generation many bacterial species adapted to their antibiotic-laced ecosystems and variants flourished that are capable of resisting our most potent designer antimicrobials **[8]**.

Thus, alternative antimicrobial approaches are being developed and probiotics have gained special interest in the last few years.

Usually, the use of antibiotics implies that the infection is already in progress otherwise probiotics, living microbes part of the natural microflora, can be used as means of prevention of diseases and maintaining of the human health **[9]**.

Probiotic therapy or "bacteriotherapy" is based on the implantation and persistence within the normal microflora of relatively innocuous 'effector' bacteria, whose action can be directed at the host, the pathogens or both. Probiotics may modulate the host's innate or acquired immune response by products like metabolites, cell wall components and DNA. The concept of "bacteriotherapy" seems to be related to bacterial interference. Mechanisms contributing to microbial interference might typically include either the greater ability of the effector bacterium to adhere to epithelial surface, blocking contact between pathogens and host cells, and to compete with others for limited space, essential for the multiplication of all microoganisms, and for nutrients from environment. Another desirable mechanical property for probiotics is their capacity to aggregate among themselves (auto-aggregation), with

other probiotics or with pathogens (co-aggregation). Aggregation also enables the formation of a barrier that protects the host's epithelium from colonization by pathogens. Moreover, the ability to co-aggregate with a pathogen allows the probiotics to entrap it **[10]**. At last, one of the most important attributes of a 'good' probiotic strain is thought to be the strain's ability to produce and be resistant to a variety of anti-competitor molecules, some relatively non-specific in their targeting (e.g. acids, hydrogen peroxide) and others (e.g. bacteriocins, and bacteriocin-like inhibitory substances (BLIS) and bacteriophages) apparently principally targeted against relatively similar bacteria **[8]**.

1.2 Bacteriocins

Allelopathy is the production of chemical compounds which are toxic to other organisms but not to the producers. Microbes produce a remarkable array of substances which help them to compete in their local environments for the limited niche space and nutritional resources available, such as bacteriocins **[11]**.

Bacteriocins were first identified in 1925, when Grazia demonstrated that *E.coli* V produced in a liquid media a dialyzable and heat-stable substance (later referred to colicin V) that inhibited in high dilution the growth of *E.coli* S. There followed a period in which a whole series of colicins produced by *E.coli* and closely related members of the *Enterobacteriaceae* were discovered **[12]**. In 1946, Fredericq demonstrated that colicins were proteins and that they had a limited range of activity due to the presence or absence of specific receptors on the surface of sensitive cells **[13]**. The study of bacteriocins of Gram-positive bacteria got off to a relatively faltering start, largely focusing on staphylococci, and with various attempts to apply similar principles of classification to those that had been established for colicins **[14]**. In recognition of the discovery that antibiotic substances of the colicin type may also be produced by non-coliform bacteria, the more general term "bacteriocin" was coined by Jacob et al. in 1953. Bacteriocins were specifically defined as protein antibiotics of the colicin type, i.e., molecules characterized by lethal biosynthesis, predominant intraspecies killing activity and adsorption to specific receptors on the surface of bacteriocin-sensitive cells **[15]**.

Bacteriocins have been found in all major lineages of bacteria and, more recently, have been described as universally produced by some members of the Archaea. Indeed, the Archaea have their own distinct family of bacteriocin-like antimicrobials, known as archaeocins. According to Klaenhammer, 99% of all bacteria may make at least one bacteriocin, and the only reason we have not isolated more is that few researchers have looked for them **[16]**.

The frequency and diversity of bacteriocin production varies greatly among bacterial populations and the dynamic interactions occurring among bacteriocin-producing, sensitive and resistant cells are likely responsible for much of this variation. However, the frequency of bacteriocinogeny and the diversity of bacteriocins produced are also determined by the habitat in which the population lives and by the genomic background of the producing strains **[11]**.

Two main features distinguish the majority of bacteriocins from classical antibiotics: bacteriocins are ribosomally synthesized and have a relatively narrow killing spectrum **[17]**. The bacteriocin family includes a diversity of proteins in terms of size, microbial target, mode of action, release, and immunity mechanisms and can be divided into two main groups: those produced by Gram-negative and those produced by Gram-positive bacteria **[11, 14]**.

1.2.1 Bacteriocins of Gram Negative bacteria

Gram-negative bacteria produce a wide variety of bacteriocins, which can be divided into three groups based on size: (1) large colicin-like (25–80 kDa) bacteriocins, (2) the much smaller microcins (<10 kDa) and (3) phage tail-like bacteriocins.

- **Colicins and Colicin-like Bacteriocins**

Since their discovery, the colicins of *E.coli* have been the most extensively studied Gram-negative bacteriocins, and utilized as a model system for investigating the mechanisms of bacteriocin structure/function, genetic organization, ecology, and evolution **[18]**.

Other members of the *Enterobacteriaceae* family also exhibit a high frequency (30–50%) of bacteriocin production **[19]**. Many of these bacteriocins are similar to colicins in structure and function, and share many molecular, evolutionary and ecological features as well. They are often referred to as colicin-like bacteriocins (CLBs) and similar to colicins have narrow killing spectra which are generally restricted to closely related species. The colicin and many CLB toxin proteins are organized into three functional domains: the N-terminal translocation, the central receptor binding, and the C-terminal killing domains **(Figure 1)**.

Figure 1: Colicin toxin.

The interaction of a colicin molecule with the target cell is initiated by the binding of the receptor-binding domain to a specific cell surface receptor located on the outer cell surface. The colicin protein is subsequently imported into the cell via the

translocation domain utilizing either the translocation system to move across the cell's outer membrane to reach the inner membrane (in the case of ionophore colicins) or the cytoplasm (in the case of the nuclease colicins). The killing domain then mediates the killing of a target cell by pore formation in the cell membrane or nuclease activity. Nuclease colicins have DNase or RNase activities which degrade 16S rRNA or tRNAs. Additionally, a muraminidase function has been described for colicin M that degrades murein in the bacterial cell wall and thereby affects the cell's structural integrity, resulting in cell lysis.

Colicin operons consist of three tightly linked genes encoding the toxin, immunity and lysis proteins, and are usually found on plasmids. The first gene encodes the toxin whose activity kills the target cells. The immunity gene encodes a protein that binds adjacent to the active site of the colicin protein and inhibits its activity by steric hindrance and electrostatic repulsion mechanisms, protecting the producer cell. In the case of ionophore colicins, the immunity gene is orientated opposite to the toxin gene while it is co-linear with toxin gene in nuclease colicins. The last gene encodes a protein (also called the bacteriocin release protein) which lyses the host cell to release the expressed bacteriocin proteins outside the cell **(Figure 2)**.

Colicin expression is regulated by the SOS induction system and when it is triggered in cells at times of stress, colicin genes are rapidly induced to express high levels of protein. In the case of nuclease colicins, the co-linear arrangement of the immunity and colicin genes within the gene cluster results in increased co-expression of the immunity protein which will bind to newly synthesized colicins and protect the cells from its nuclease activity. In the case of pore-forming colicins, induction does not result in increased levels of immunity protein, as the immunity gene is transcribed from the other strand. Pore-forming colicins, unlike the nuclease colicins, can kill the cells only from the exterior by punching holes in the cell membrane. Therefore, it may not be necessary for the cells to increase the levels of immunity protein during a phase of rapid colicin expression.

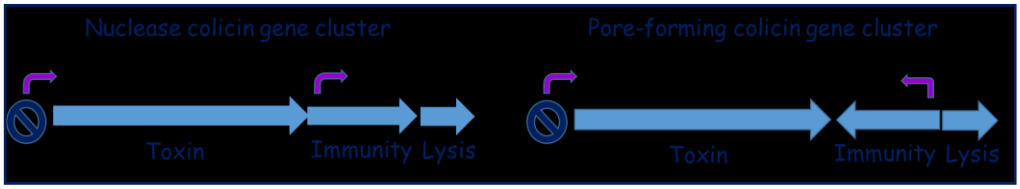


Figure 2: Genetic organization of nuclease and pore-forming colicins.
The LexA-binding region is indicated by ⊘.

Colicin expression results in lysis of the producer cell, due to co-expression of a lysis protein which mediates the release of colicin into the extra-cellular environment **[20]**. However, experimental evidence suggests that the expression of colicin is induced in only a small fraction of the population **[21]**. These colicin-expressing cells eventually die but produce enough colicin to kill related, but competing, cells. Thus, a fraction of colicin-harbouring cells display altruistic behaviour by "sacrificing themselves" for the larger benefit of their clonal kin. Indeed, colicins have been implicated as a defence mechanism in competition between cells with more similar nutritional and niche requirements.

Although colicins are representative of Gram-negative bacteriocins, there are intriguing differences found within this subgroup of the bacteriocin family. *E.coli* encodes its colicins exclusively on plasmid replicons. The nuclease pyocins of *Pseudomonas aeruginosa*, which show sequence similarity to colicins, and other, as yet uncharacterized, are found exclusively on the chromosome. Other close relatives to the colicin family, the bacteriocins of *Serratia marcescens*, are found on both plasmids and chromosomes **[20]**.

- **Microcins**

Microcins are non-SOS-inducible low molecular weight peptides similar to the bacteriocins of Gram-positive bacteria **[22]**. All the microcins characterized to date are secreted from the cell, rather than being released as a consequence of cell lysis. It has also been suggested that as much as 90% of the microcins produced by a cell may be retained within the cell **[23]**.

- **Phage tail-like bacteriocins**

Phage tail-like bacteriocins are nuclease- and protease-resistant rod-like particles resembling a bacteriophage tail, which kill sensitive cells by depolarization of the

cell membrane **[24]**. These are multi-meric peptide assemblies, proposed to be defective phages or to have originated from phages which evolved to function as bacteriocins. For example, pyocin R2 (produced by *Pseudomonas* spp.) appears to be a remnant of phage P2 whereas pyocin F2 is similar to phage lambda **[25]**.

1.2.2 Bacteriocins of Gram Positive bacteria

Bacteriocins of Gram-positive bacteria are as abundant as and even more diverse than those found in Gram-negative bacteria.

Bacteriocins produced by lactic acid bacteria (LAB), which have a long history of use in fermentation and meat and milk preservation, are the best characterized of this group **[20]**.

In 1993, Klaenhammer attempted to put some order into the classification of the bacteriocins of LAB, by proposing four major classes **[26]**:

1. Class I - post-translationally modified bacteriocins, i.e., the lantibiotics,
2. Class II - small (<10 kDa) heat-stable membrane-active bacteriocins,
3. Class III - larger (>30 kDa) heat-labile bacteriocins,
4. Class IV - complex bacteriocins composed of essential lipid or carbohydrate moieties in addition to protein.

This provisional scheme was adopted by most investigators in the field but it has been reviewed by several authors such as Cotter et al. **[27]**. They have proposed a more radical modification to the Klaenhammer classification scheme for LAB bacteriocins, in which there are essentially only two principal categories: lantibiotics (class I) and non- lanthionine-containing bacteriocins (class II). The former class III (large heat-labile murein hydrolases) and class IV (the lipid- or carbohydrate-containing bacteriocins) are withdrawn.

Heng et al., proposed classification schema based on that of Cotter, but modified so as to be applicable to most, if not all, known bacteriocins of Gram-positive bacteria **(Figure 3) [14]**.

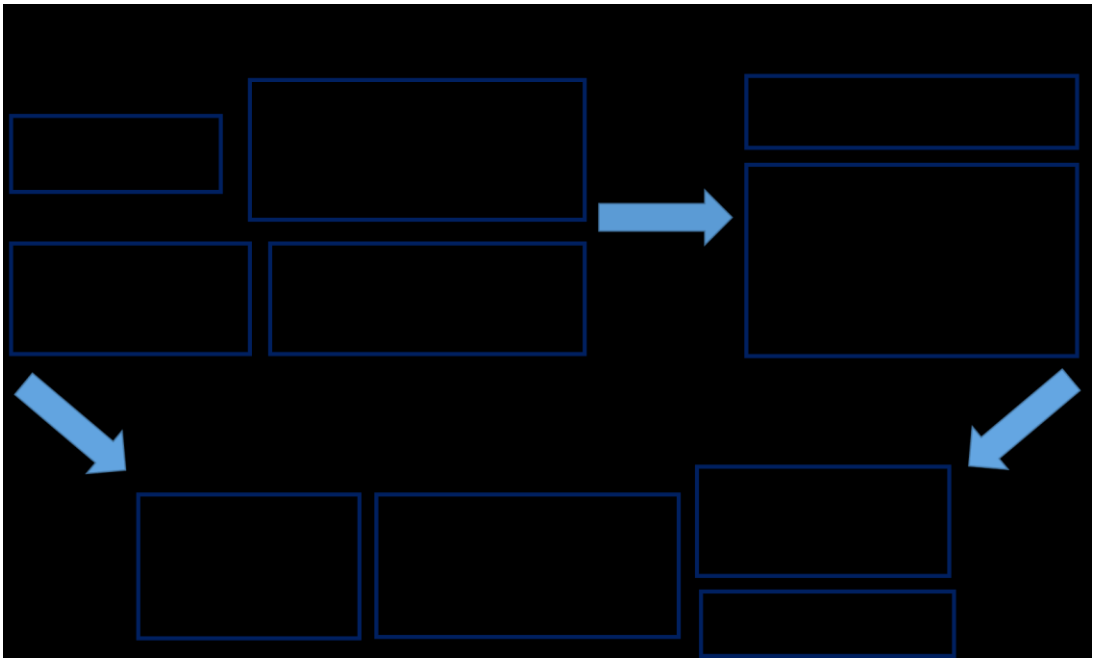


Figure 3: Classifications of bacteriocins of Gram-positive bacteria.

Class I: The lantibiotic

The term "lantibiotic" was coined to refer to the diverse array of Gram-positive bacterial antibiotic peptides that contain the non-genetically encoded amino acids lanthionine (Lan) and/or 3-methyllanthionine (MeLan), as well as various other highly modified amino acids, commonly including the 2,3-unsaturated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb). The lantibiotics described to date, all of which are produced exclusively by Gram-positive bacteria, are initially produced as ribosomally synthesized precursor peptides, which then undergo a series of post-translational modifications to produce the unusual amino acids that are intrinsic components of the biologically active peptides. As the family of lantibiotic molecules grew, the individual members were initially classified according to the topology of their ring structures and their biological activities **[28]**, as either type A (elongated amphipathic structures) or type B (globular and more compact structures). In order to encompass the more recently described two-component varieties, type C lantibiotics have been proposed. Type A lantibiotics are further divided into subtypes AI and AII based on the size, charge and sequence of their leader peptides **[29]**.

Class II: The Unmodified Peptide Bacteriocins

Class II represents the largest collection of bacteriocins as it essentially encompasses all of the currently described small (<10 kDa) unmodified peptide bacteriocins of Gram-positive bacteria. This class comprises over 50 members with diverse origins, ranging from genera inhabiting the oral cavity and gastrointestinal tract (of humans and other animals) to species best known for their involvement in the dairy and food industries. Class II peptides are divided into three types.

- **Type IIa: The Pediocin-like Peptides**

The largest single collection of class II bacteriocins, consisting of over 20 members, sharing strong activity against *Listeria monocytogenes* **[14]**.

- **Type IIb: Multi-Component Bacteriocins**

Class IIb includes some bacteriocins that can require two or more peptides to effect optimal inhibitory activity. Garneau et al. proposed that two-component bacteriocins are subdivided into synergistic (S) and enhancing (E) type inhibitory agents. S-type two-component bacteriocin activities are dependent on the concerted action of both peptides, and neither component appears inhibitory on its own **[30]**. Conversely, for an E-type two-component bacteriocin, either each component peptide or only one peptide of the duet possesses inhibitory activity, but combination of the components results in greatly enhanced killing action toward the target species.

- **Type IIc: Miscellaneous Unmodified Bacteriocins**

All single-peptide non-modified bacteriocins that do not fulfill the criteria of type IIa or type IIb are automatically members of type IIc.

Class III: The Large (>10 kDa) Bacteriocins

This class consists of several large antimicrobial proteins that can generally be subdivided into two distinct groups: (1) the bacteriolytic enzymes (or bacteriolysins), which facilitate the killing of sensitive strains by cell lysis, and (2) the non-lytic antimicrobial proteins.

Class IV: The Cyclic Bacteriocins

These inhibitory agents are ribosomally synthesized peptides, but possess a circular structure as they are post-translationally processed such that the first and last amino acids of the mature peptide are covalently bonded, corresponding to the so-called head-to-tail ligation **[14]**.

Bacteriocins of Gram-positive bacteria differ from Gram-negative bacteriocins in two fundamental ways. First, bacteriocin production is not necessarily lethal to the producing cell. This critical difference is due to the transport mechanisms Gram-positive bacteria encode to release bacteriocin toxin. Typically, their biosynthesis is self-regulated with specifically dedicated transport mechanisms facilitating release, although some employ the sec-dependent export pathway. Second, Gram-positive bacteria have evolved bacteriocin-specific regulation, whereas bacteriocins of Gram-negative bacteria rely solely on host regulatory networks. A mechanism of auto-regulation can be observed for the production of nisin by *Lactobacillus lactis*. Nisin was the first bacteriocin to be isolated and approved by FDA in 1988 as a bio-preservative for a narrow range of foods, specifically to prevent the outgrowth of *Clostridium botulinum* spores **[20]**.

The nisin gene cluster contains genes encoding the nisin precursor (nisA), and proteins involved in post-translational modification of the pre-nisin (nisB and nisC), secretion of the modified precursor (nisT) and immunity of the producing *L.lactis* (nisIFE). Other genes constitute the nisin gene cluster, such as *nisP* gene that encodes for an extracellular protease involved in removal of the pre-nisin leader peptide to generate the mature nisin molecule. Moreover, in the nisin gene cluster there are genes that encode a two-component regulation system, composed of a sensor kinase (nisK) and a response regulator (nisR) **(Figure 4)**.

The production of nisin is cell-density dependent and was revealed to be regulated at the transcriptional level. Nisin acts as AMP (antimicrobial peptide) and as a secreted peptide pheromone that induces its own biosynthesis by triggering the corresponding signal transduction system in a quorum sensing-like manner.

The ribosomally synthesized precursor (nisA) is modified and transported by a membrane-anchored multi-meric complex composed of the factors B, C and T. Modified pre-nisin processing is performed by the protease nisP. Then, the mature

nisin molecule, primarily, acts as AMP and producing cells are protected by the immunity system composed of the factors I, and FGE. The second role of nisin is as a peptide pheromone that is sensed by the input domain of its corresponding sensor kinase domain (K_I). Subsequent phosphotransfer from the sensor kinase transmitter domain (K_T) to the receiver domain of the response regulator (R_R) leads to its activation. The output domain (R_O) of the active response regulator will bind to a specific target nis-box within the promoter present in the biosynthetic gene cluster, leading to transcriptional activation and increased production **[31, 32, 33]**.

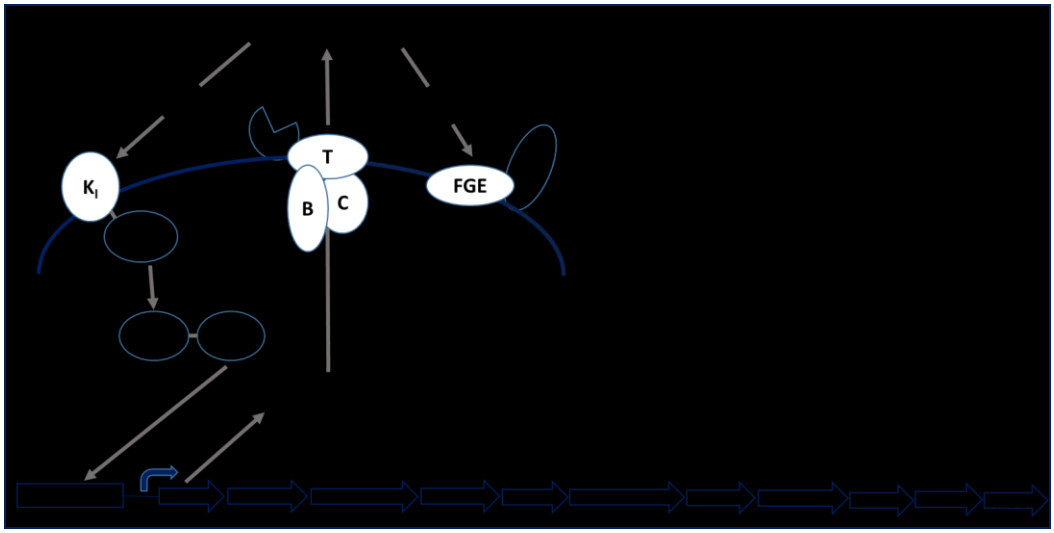


Figure 4: Nisin biosynthesis and regulation.

Table 1 shows bacteriocins produced by Gram negative and Gram positive bacteria known in literature **[34]**.

Bacteriocin	Producing organism	Source	Reference
Bacteriocins produced by gram-negative bacteria			
Colicin E1-E9	Escherichia coli	Human feces	Gordon and O'Brienn (2006) Smajs et al. (2010)
Colicin K	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Colicin N	*Escherichia coli*	Human feces	Smajs et al. (2010)
Colicin S4	*Escherichia coli*	Human feces	Smajs et al. (2010)
Colicin U	*Escherichia coli*	Human feces	Smajs et al. (2010)
Colicin Y	*Escherichia coli*	Human feces	Smajs et al. (2010)
Colicin B	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Colicin Ia	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Colicin Ib	*Escherichia coli*	Human feces	Smajs et al. (2010)
Colicin M	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Colicin 5	*Escherichia coli*	Human feces	Smajs et al. (2010)
Colicin Js	*Escherichia coli*	Human feces	Smajs et al. (2010)
Microcin B17	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Microcin C7/C51	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Microcin J25	*Escherichia coli*	Human feces	Salomon and Farias (1992)
Microcin 15m	*Escherichia coli*	Human feces	Aguilar et al. (1982)
Microcin 15n	*Escherichia coli*	Human feces	Aguilar et al. (1982)
Microcin V	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Microcin L	*Escherichia coli*	Human feces	Gordon and O'Brienn (2006)
Microcin E492	*Klebsiella pneumoniae*	Human feces	De Lorenzo (1984)
Microcin H47	*Escherichia coli*	Human feces	Lavina et al. (1990)
Microcin 147	*Escherichia coli*	Human feces	Poey et al.(2006)
Microcin M	*Escherichia coli*	Human feces	Patzer et al. (2003)
Pyocin S2	*Pseudomonas aeruginosa*	Clinical isolate	Abdi-Ali et al. (2004)

Table 1

Bacteriocin	Producing organism	Source	Reference
Bacteriocins produced by Gram-positive bacteria			
BHT A BHT B	*Streptococcus rattus and mutans*	Oral cavity	Hyink et al. (2005)
Cytolysin	*Enterococcus faecalis*	Human feces	Haas and Gilmore (1999)
Mutacin I	*Streptococcus mutans UA140*	Oral cavity	Qi et al. (2001)
Mutacin II	*Streptococcus mutans T8*	Oral cavity	Chikindas et al. (1995)
Mutacin III	*Streptococcus mutans UA787*	Oral cavity	Qi et al. (1999)
Mutacin K8	*Streptococcus mutans K8*	Oral cavity	Robson et al. (2007)
Mutacin 1140	*Streptococcus mutans JH1000*	Oral cavity	Hillman et al. (1998)
Mutacin B-Ny226	*Streptococcus mutans Ny226*	Oral cavity	Mota-Meira et al. (1997)
Nisin Z	*Lactococcus lactis MM19*	Human feces	Millette et al. (2008)
Ruminococcin A	*Ruminococcus gnavus, Ruminococcus hansenii*	Human feces	Dabard et al. (2001) Marcille et al. (2002)
Salivaricin A	*Streptococcus salivarius 20P3*	Oral cavity	Ross et al. (1993)
Salivaricin A1	*Streptococcus pyogenes*	Oral cavity	Upton et al. (2001)
Salivaricin A2-5	*Streptococcus salivarius*	Oral cavity	Wescombe et al. (2006)
Salivaricin B	*Streptococcus salivarius K12*	Oral cavity	Hyink et al. (2007)
Streptococcin A-FF22	*Streptococcus pyogenes FF22*	Throat	Hynes et al. (1993)
Streptin	*Streptococcus pyogenes, Bifidobacterium longum*	Oral cavity Health adult feces	Wescombe and Tagg (2003) Lee et al. (2008)
Thuracin CD	*Bacillus thuringiensis*	Health adult feces	Rea et al. (2010)
Avicin A	*Enterococcus avium*	Infant feces	Birri et al. (2010)
Bacteriocin RC714	*Enterococcus faecium*	Human feces	del Campo et al. (2001)
Bacteriocin 32	*Enterococcus faecium*	Clinical isolate	Inoue et al. (2006)
Bacteriocin GM-1	*Enterococcus faecium*	Infant feces	Kang and Lee (2005)
Bifidocin B	*Bifidobacterium bifidum*	Nursling stools	Yiildirim et al. (1999)
Enterocin A	*Enterococcus faecium*	Infant feces	O'Shea et al. (2009)
Pediocin PA-1	*Pediococcus acidiilactici UVA1*	Breast fed feces	Mathys et al. (2007)

Table 1

Bacteriocin	Producing organism	Source	Reference
Bacteriocins produced by gram-positive bacteria			
Abp118	*Lactobacillus salivarius*	Human feces ileal -cecal region	Flynn et al. (2002)
Blp	*Streptococcus pneumoniae*	Clinical isolate	David et al. (2007)
Lactacin F	*Lactobacillus jojnsonii*	Human feces	Pridmore et al. (2004)
Mutacin IV	*Streptococcus mutans*	Oral cavity	Qi et al. (2001)
Plantaricin EF and JK Plantaricin A	*Lactobacillus plantarum*	Oral cavity	Kleerebezem et al. (2003)
Salivaricin T	*Lactobacillus salivarius*	Infant feces	O'Shea et al. (unpublished)
Gassericin Reutericin 6	*Lactobacillus gasseri LA39*	Breast fed feces	Toba et al. (1991)
ESL5	*Enterococcus faecalis*	Human feces	Kang et al. (2009)
Mutacin N	*Streptococcus mutans*	Oral cavity	Hale et al. (2004)
Gassericin T	*Lactobacillus gasseri*	Human feces	Kawai et al. (2000)
Gassericin KT7	*Lactobacillus gasseri*	Breast fed feces	Zhu et al. (2000)
Acidocin LF221A/B	*Lactobacillus gasseri*	Child fecal isolate	Majhenic et al. (2004)
Acidophilucin A	*Lactobacillus acidophilus*	Breast fed feces	Toba et al. (1991)

Table 1

1.3 Guidelines for the Evaluation of Probiotics

Over the past 20 years, there has been an increase in research on probiotic bacteria and a rapidly growing commercial interest in the use of them in food and medicine **[35]**. Indeed, scientific evidence continue to accumulate on the properties, functionality, and benefits of probiotics for the promotion of human health, with suggestions that they can play an important role in immunological, digestive, and respiratory functions and could have a significant effect in alleviating infectious diseases in children **[36]**.

Therefore, the Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food, held in Cordoba, Argentina 1-4 October, 2001 recognized the need for guidelines to set out a systematic approach for the evaluation of probiotics in food leading to the substantiation of health claims. Consequently, a Working Group was convened by the FAO/WHO in London, Ontario, April 30 and May 1, 2002 to generate guidelines and recommend criteria and methodology for assessment of probiotics and to identify and define what data need to be available to substantiate health claims accurately. A scheme outlining these guidelines is shown in **figure 5 [5]**. While the recommended guidelines focus particularly on intestinal probiotics, can be considered generally applicable to all probiotics **[37]**.

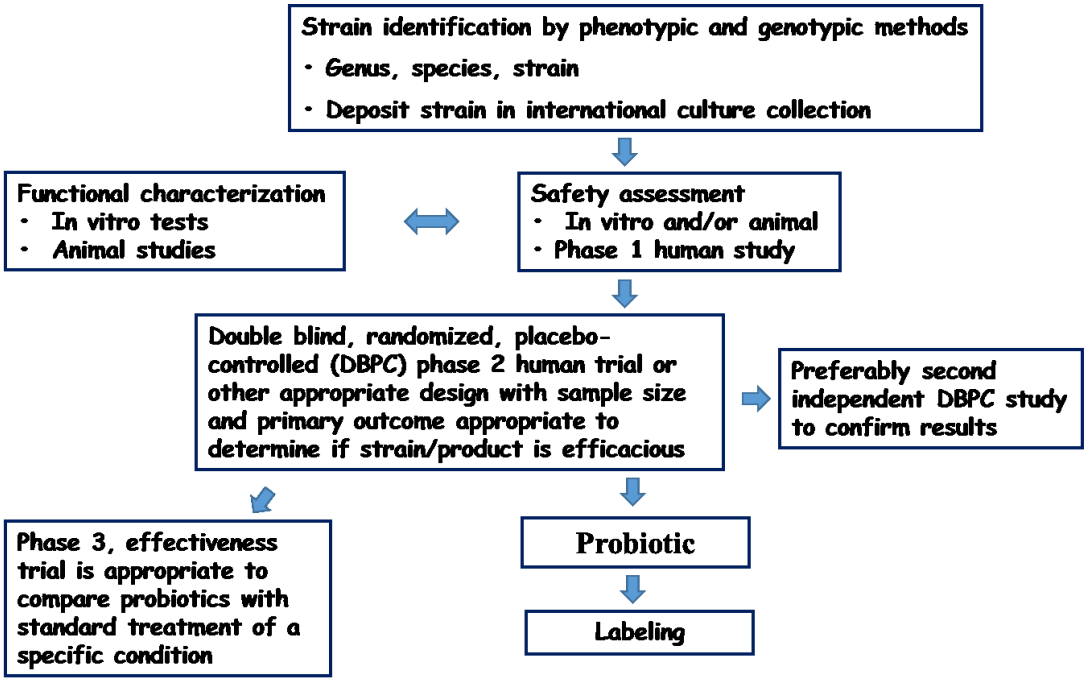


Figure 5: Guidelines for the evaluation of probiotics.

- **Strain identification**

It was recognized that it is necessary to know the genus and species of the probiotic strain. The current state of evidence suggests that strain identity is important to link a particular microorganism to a specific health effect as well as to enable accurate surveillance and epidemiological studies.

Speciation of the bacteria must be established using the most current, valid methodology. It is recommended that a combination of phenotypic and genetic tests be used.

Following the predevelopmental screening phase, probiotic candidate should be deposited in an internationally recognized culture collection, such as the American Type Culture Collection (ATCC) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

Nomenclature of the bacteria must conform to the current, scientifically recognized names. Current nomenclature can be retrieved as follows:

· Approved Lists of Bacterial Names (Int. J. Syst. Bacteriol, 1980, 30:225-420) also available at http://www.bacterio.cict.fr/

· Validation Lists, published in the International Journal of Systematic and Evolutionary Microbiology (or International Journal of Systematic Bacteriology, prior to 2000) **[5, 37]**.

- **Functional characterization**

In vitro test are useful for functional characterization and to gain knowledge of the mechanism of the probiotic effect. However, it was noted that currently available *in vitro* tests are not fully adequate to predict functionality of probiotic microorganisms in the human body and that probiotics for human use require substantiation of efficacy with human trials. Thus, appropriate target-specific *in vitro* tests that require validation with *in vivo* performance are recommended, for example: adherence to mucus and/or human epithelial cells and cell lines, ability to reduce pathogen adhesion to surfaces, resistance to specific environments (i.e. gastric acidity) **[5].**

- **Safety**

An important first step in safety evaluation is a thorough search of the literature. Identification of the history of use and reports of infection resulting from the chosen species/strain should be noted. Safety is verified prior to commercial release but, in practice, it is an ongoing process and requires continual *in vitro* and *in vivo* analysis. *In vitro* safety checks include: metabolic profiling to assess the production of deleterious byproducts (i.e. D-lactate); antibiogram determination to accepted standards such as those established by the Clinical and Laboratory Standard Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST), to indicate antibiotic resistance; toxicity to cell lines and blood (hemolysis), and the presence of virulence factors. Many genetic techniques are available to evaluate the safety of potential probiotics, such as PCR and PFGE, but full genome sequencing allows for rapid strain identification and determination of known virulence and antibiotic resistance genes, colonization factors and genetic transfer mechanisms. Following *in vitro* testing, trials in animals allow for an *in situ* safety assessment of the probiotic and help predict potential toxicity for the human host. Typically, researchers study the effect of the probiotic analyzing changes to total body weight, individual organ weight, key biochemistry markers, urine, and blood. Human trials should be carried out using the foreseen commercial formulation and dosage levels to ensure its safety is evaluated in a "real world situation" **[37, 38, 39, 40, 41]**.

- **Efficacy**

The principal outcome of efficacy studies on probiotics should be proven benefits in human trials, such as statistically and biologically significant improvement in condition, symptoms, signs, well-being or quality of life; reduced risk of disease or longer time to next occurrence; or faster recovery from illness. Each should have a proven correlation with the probiotic tested.

The double blind, randomized, placebo-controlled studies measure efficacy compared with placebo (where the placebo is the food carrier devoid of the test probiotic). Sample size needs to be calculated for specific endpoints, and statistically significant differences must apply to biologically relevant outcomes.

It is recommended that human trials be repeated by more than one Center for confirmation of results that have to be published in peer-reviewed scientific or medical journals. Furthermore, publication of negative results is encouraged as these contribute to the evidence to support probiotic efficacy.

- **Effectiveness**

Probiotics can be studied by comparison with standard therapy.

- **Labeling**

The following information must be given on the label:

- ✓ Genus, species and strain designation. Strain designation should not mislead consumers about the functionality of the strain
- ✓ Minimum viable numbers of each probiotic strain at the end of the shelf-life
- ✓ The suggested serving size must deliver the effective dose of probiotics related to the health claim
- ✓ Health claim(s)
- ✓ Proper storage conditions
- ✓ Corporate contact details for consumer information **[5]**

1.4 Oral Probiotics

A variety of probiotic bacteria, such as LAB, have been targeted as potential therapeutic agents, differing in terms of their bioavailability, metabolic activity, and mode of action. Until recently, conventional probiotics have typically comprised selected bacteria obtained from intestinal sources (especially lactobacilli and bifidobacteria) and their application has almost exclusively focused on the gastrointestinal benefits **[37]**. However, with the more widespread acceptance of the potential for probiotic intervention to also effect health benefits for non-intestinal body sites, there has come the increased application of effector strains of species that are indigenous to alternative target tissue in order to obtain more specific and enduring benefits **[41]**. Moreover, the realization that much human illness can be linked either directly or indirectly to the development of oral microbiota disequilibria has diverted much contemporary probiotic research to products that are capable of fostering a healthy oral microbiota **[42]**. Although there have been some attempts to use conventional approved intestinal bacteria such as lactobacilli for oral cavity probiotics, it appears more likely that bacteria isolated directly from the natural oral microbiota in healthy humans will be efficacious for such purposes **[43]**.

The microorganisms that inhabit the human oral cavity have been designated as the human oral microbiome. The term microbiome was coined by Joshua Lederberg "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and diseases" **[44]**. The human being and its microbiome together make up a "supraorganism" and the number of microbial cells within a human body exceeds the total number of human cells in the body by nearly 10 times **[45]**. Remarkably, these potentially overwhelming populations coexist with the host, with harmful effects occurring only if the immune status is altered or if there is a loss of control of epithelial cell sensing and discriminatory systems. It is now generally accepted that some resident commensal bacteria have been shown to provide significant benefit to the host by blocking pathogen colonization and by influencing the normal development of cell structure and the immune system **[46, 47]**.

There are various microhabitats throughout the body that contribute to the overall microbiome, such as mouth, skin, gut, etc **(Figure 6)**. Each microhabitat maintains a unique ecosystem with distinct atmospheric and nutritional compositions that provide

a setting for symbiotic interactions among the various microbes within that ecosystem and the host **[42]**. The Human Microbiome Project (HMP) that explores the role of the human microbiome in physiology, health, and disease through metagenomic research, states that an understanding of human health and disease is impossible without understanding the human microbiome **[48]**.

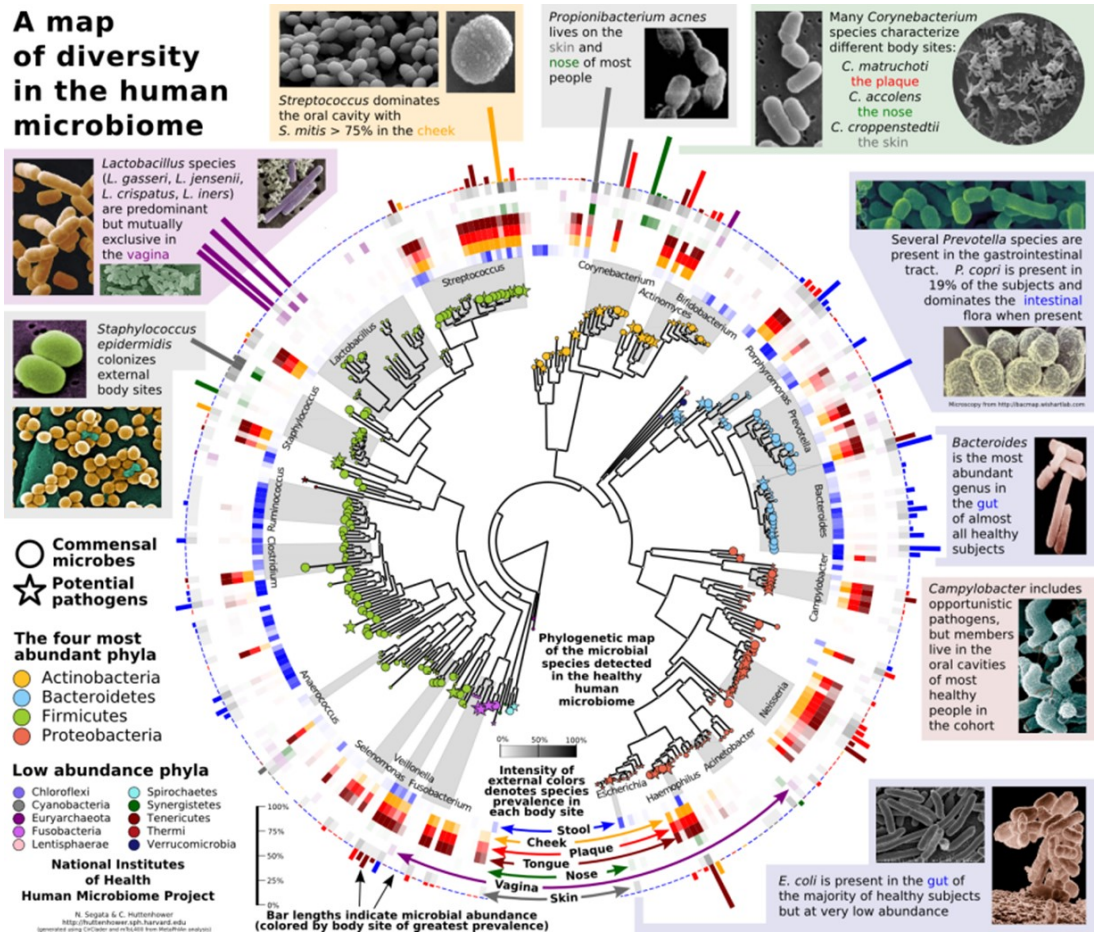


Figure 6: Human microbiome.

Specifically, studies have shown the oral cavity's microbiome to be a key source in the etiology of many oral and systemic diseases **[49]**. Indeed, the oral cavity is the primary gateway to the body and when severe cases of oral disease result in the spread of infection to other body sites, may produce systemic diseases such as cardiovascular disease or others **[42]**. Because the oral microbiome is vital to a body's overall health, it is crucial to unravel its complexities to learn the mechanisms by which it maintains health or causes disease. To understand the role of the oral

microbiome within the oral cavity, it is important to analyze its fundamental characteristics and dynamics.

The oral cavity is a complex and heterogeneous microbial habitat. Food particles and cell debris provide some nutrients, thus contributing to the establishment of favourable conditions for microbial growth. This environment is consolidated by a constant humidity and an atmosphere that is mainly composed of expired air. Saliva contains enzymes such as lysozyme, lactoperoxidase and amylase, which may have an antibacterial action **[50]**.

More than 700 bacterial species are present in the oral cavity and, maintaining the bacterial communities unaltered, has a significant impact on general health by either preventing or causing infections. The major genera with the largest representation in healthy oral cavities include the following: *Streptococcus*, *Veillonella*, *Granulicatella*, *Gaemella*, *Actinomyces*, *Corynebacterium*, *Rothia*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Capnocytophaga*, *Neisseria*, *Haemophilus*, *Treponema*, *Lactobacterium*, *Eikenella*, *Leptotrichia*, *Peptostreptococcus*, *Staphylococcus*, *Eubacteria*, and *Propionibacterium* **[51, 52, 53]**.

The need for biodiversity in health may suggest that every species carries out a specific function that is required to maintain equilibrium and homeostasis within the oral cavity.

The key to oral health is an ecologically balanced and diverse microbiome that practices commensalism within itself and mutualism with its host. Commensal relationships among microbes allow them to flourish at no expense to their co-habitants and, in turn, maintain biodiversity within the oral cavity.

However, certain pathological changes within the microbial ecosystem may occur and cause a oncebeneficial microorganism to initiate disease within the oral cavity. Ecological shifts that cause pathological changes are: (1) a change in the relationships between the microbes and the host; (2) an increase in relative abundance; and (3) acquisition of virulence factors. In disease, microbes alter their relationship with their host from mutualistic to parasitic and with other microbes from commensal to opportunistic. Pathogens will grow with disregard of their co-habiting bacteria, and any beneficial bacteria will not be able to inhibit the disease manifestation **[42, 52, 54]**.

The members of the genus *Streptococcus*, in particular nonpathogenic streptococci, are the most abundant bacterial species at the oropharyngeal level, and they have been proposed to exert an important role in the protection against pathogenic agents, which cause inflammation and infections **[8]**. It is well known that the first studies of oral probiotics can be traced to the use of oral alpha-haemolytic streptococci, isolated from the human pharynx, with inhibitory activity against potential pathogens of the upper airways. Roos at al. investigated the effect of alpha-haemolytic streptococci administered as a nasal spray containing two *Streptococcus sanguinis*, two *Streptococcus mitis* and one *Streptococcus oralis*, on the incidence of OM in otitis-prone children. The results of this double-blind randomized, placebo-controlled trial showed that the nasal spray was effective in reducing the incidence of OM. Based on these findings, Tano et al. tested a nasal spray containing alpha-haemolytic streptococci but there was no significant change in the nasopharyngeal microbiota or the number of OM episodes compared to the control group when the spray was used without a prior appropriate antibiotic treatment **[55, 56, 57]**. However, the three species utilized in the formulation are recognized as potential pathogens, for example *Streptococcus mitis* has been associated with lung infection and abscess formation, and both *Streptococcus oralis* and *Streptococcus sanguinis* have been implicated in bacterial endocarditis **[37]**.

Other strains of streptococci from the human oral cavity and belonging to commensal species known to have extremely low pathogenic potential are now being developed. In this regard, a key species is *Streptococcus salivarius*, which has been investigated for its role in the prevention of upper respiratory tract diseases.

S.salivarius is a lactic acid bacterium that is mainly encountered in the mouths of human beings. It is the first commensal bacterium that appears in the oral cavity of newborns where it colonizes the upper respiratory tract **[51]** and persists there as a predominant member of the native microbiota throughout the life of its human host **[58]**. *S.salivarius* has an exclusive and intimate association with humans that are its sole natural host. Since it has no other known reservoir in nature and its survival time elsewhere is short, the inevitable source of *S.salivarius* for the newborn baby is the saliva of its closest early contacts. Predictably, the mother will have the major contributing role towards her baby's pioneer oral microbiota, and indeed, it seems

that over the first few days of life the baby's *S.salivarius* population progressively changes to more closely resemble that of the mother.

According to several studies, large populations of *S.salivarius* efficiently adhere to the oral epithelial cells, especially the papillary surface of the tongue that is a strategically location to carry out a population surveillance and modulation role within the oral microbiota **[59, 60]**. The presence of an adhesion system such as pili, fibrils, saliva-binding proteins and host-cell-binding proteins, together with its high competition rate, helps this species to stay in the human mouth **(Figure 7) [61]**.

Figure 7: Scanning electron micrograph showing the attachment of *S.salivarius* K12 to microspikes on Hep-2 cells through pilus-like appendages **[60]**.

S.salivarius is an oral streptococcal species that is not known to have any disease associations in healthy humans **[62]**. Indeed, there have been occasional reports of infections involving *S.salivarius*, though their occurrence (even in adverse medical conditions) is extremely low **[63, 64]**.

S.salivarius, is still generally classified as a risk group 2 organism in Europe **[37]** and has not been granted a positive QPS status by European Food Safety Authority (EFSA). 'Qualified Presumption of Safety' is a safety assessment system based on four parameters: establishing the identity, body of knowledge, possible pathogenicity and end use of the microorganism. However, a microorganism might not be approved by EFSA but may have gained a GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (FDA) in the USA that indicates the use of the product without any demonstrable harm to consumers. This is the case for *Streptococcus salivarius* K12 which has been approved as a food ingredient and has been commercialized in both Australia and New Zealand for several years as an oral probiotic (BLISK12TM Throat Guard). Its safety has recently been assessed in a

clinical trial which shows that the intake of this bacterium is well tolerated by humans. Moreover, *Streptococcus salivarius* is closely related to *Streptococcus thermophilus*, a benign organism used in the manufacture of yogurt, which has both the QPS and the GRAS status **[10, 65, 66, 41, 67]**. Many comparative genomic studies regarding taxonomy and phylogeny among dairy streptococci have demonstrated that *Streptococcus spp*. are clustered in two main groups, one comprising *S.macedonicus*, and *S.bovis* species and the other *S.thermophilus* and *S.salivarius*: the species in each group show strong similarities in the DNA sequence of the ribosomal locus **[67, 68]**.

Many strains of *S.salivarius* are producers of bacteriocin-like inhibitory substances (BLIS) that are diverse in their activity spectra and are thought to play an important role in both stabilizing the oral microbiota and preventing overgrowth (or infection) by potential pathogens **[60]**.

Table 2 shows *S.salivarius* bacteriocins **[60]**.

***S. salivarius* bacteriocins**	**Reference**
Salivaricin A	**[69]**
Salivaricin A1	**[69]**
Salivaricin A2	**[69]**
Salivaricin A3	**[69]**
Salivaricin A4	**[69]**
Salivaricin A5	**[69]**
Salivaricin B	**[70]**
Salivaricin G32	**[71]**
Salivaricin 9	Genbank accession number DQ889747
Streptin	Heng NCK et al. (unpublished)
Salivaricin MPS	**[60]**

Table 2: BLIS produced by strains of *S. salivarius*.

Salivaricin A (SalA) was the first completely characterized *S.salivarius* BLlS. It is a type AII lantibiotic, produced by members of the species *S.salivarius*, *S.pyogenes*, *S.dysgalactiae and S.agalactiae*. Six subtypes of SalA (A-A5) have been described,

each of these peptides is capable of inducing production of any one of the salivaricin A subtypes **[69]**.

In *S.salivarius* the genetic locus comprises eight open reading frames designated *salABCTXYKR*. The first gene is *salA* encoding the precursor lantibiotic peptide, downstream of it, the *salB* and *salC* genes are predicted to encode peptides involved in post-translational modification. The next two genes, *salT* and *salX*, encode for the SalTX protein complex located within the cell membrane that carries out cleavage and export of modified SalA. Then, there is *salY* encoding a protein associated with self-protection against SalA while *salKR* genes, situated at the distal end of the locus, encode products forming a two-component sensor kinase-response regulator system. Production of this lantibiotic is auto regulated, like the production of nisin, previously described. An interesting finding was that SalA production by one streptococcal species may be induced by sensing of the homologous peptide from another streptococcal species. Indeed, the SalA peptide sensing system apparently does not discriminate between SalA and SalA1 produced by *S.salivarius* and *S. pyogenes,* respectively. The ability of SalA1-producing *S.pyogenes* strains to respond to SalA from *S.salivarius* (and vice versa) could provide a selective mechanism for co-colonization of the mucosal epithelium by pathogen and commensal cell populations. For example, SalA1 produced by rapidly multiplying *S.pyogenes* cells might stimulate production of SalA by *S.salivarius* strains, leading to modulation of the number of *S.pyogenes* cells **[72]**. It represents a model of regulated coexistence of streptococcal populations in the oral microbial community structure.

Many advantageous and different characteristics make *S.salivarius* strains promising candidates for the development of oral probiotics and several of these microorganisms have been studied for their bacterial interference against human infections regarding the oral cavity and/or upper respiratory tract **(Figure 8)**. Moreover, different studies on *S.salivarius* show that strains of this genus are potential probiotics for all ages as they may alleviate many diseases that generally tend to manifest during human life **(Figure 9)**.

The pioneering studies on *S.salivarius* strains affected *S.salivarius* TOVE-R (R for rough colony morphology) which demonstrated to colonize rat dental plaque

reducing populations of *S.mutans* and *S.sobrinus* that are commonly implicated in the etiology of dental caries **[73, 74]**.

The first *S.salivarius* specifically selected for its potential to interfere with the colonization of the upper respiratory tract by *S.pyogenes* was strain *S.salivarius* K58. Its antagonist activity was due to the production of the bacteriocin enocin whose action appeared to involve interference with pantothenate utilization, inhibiting the growth of organisms requiring exogenous pantothenate such as *S.pyogenes* **[55]**.

Another *S.salivarius* strain, well-known in scientific literature, that was selected initially for its antagonist activity against *S.pyogenes* was *S.salivarius* K12. It harbours two lantibiotics, salivaricin A and salivaricin B that are responsible for the inhibitory growth of the principal causative agent of streptococcal pharyngitis. Further studies showed additional features of *S.salivarius* K12, such as anti-inflammatory effect, good adhesion to epithelial cells, inhibitory spectrum encompassing some of the key Gram-negative anaerobes that have been implicated in halitosis, and a protective effect against *Candida albicans*. Since 2001, strain K12 has been used as an oral probiotic and marketed internationally by the New Zealand company BLIS Technologies Ltd. **[70, 75, 76, 77]**.

Similar to *S.salivarius* TOVE-R, *S.salivarius* M18 is a strain that inhibits *S.mutans* and *S.sobrinus*, moreover, its antagonist activity affects other bacterial species implicated in diseases of the upper respiratory tract, through production of four salivaricins: A2, 9, MPS, and M. Furthermore, *S.salivarius* M18 inhibits the expression of pro-inflammatory cytokines IL-6 and -8 **[62, 78]**.

Another interesting strain of *S.salivarius* is *S.salivarius* ST3 which has the ability to inhibit *S.pyogenes*, to reduce the levels of pro-inflammatory cytokines IL-6 and -8 and has a good adhesion to epithelial cells **[75]**.

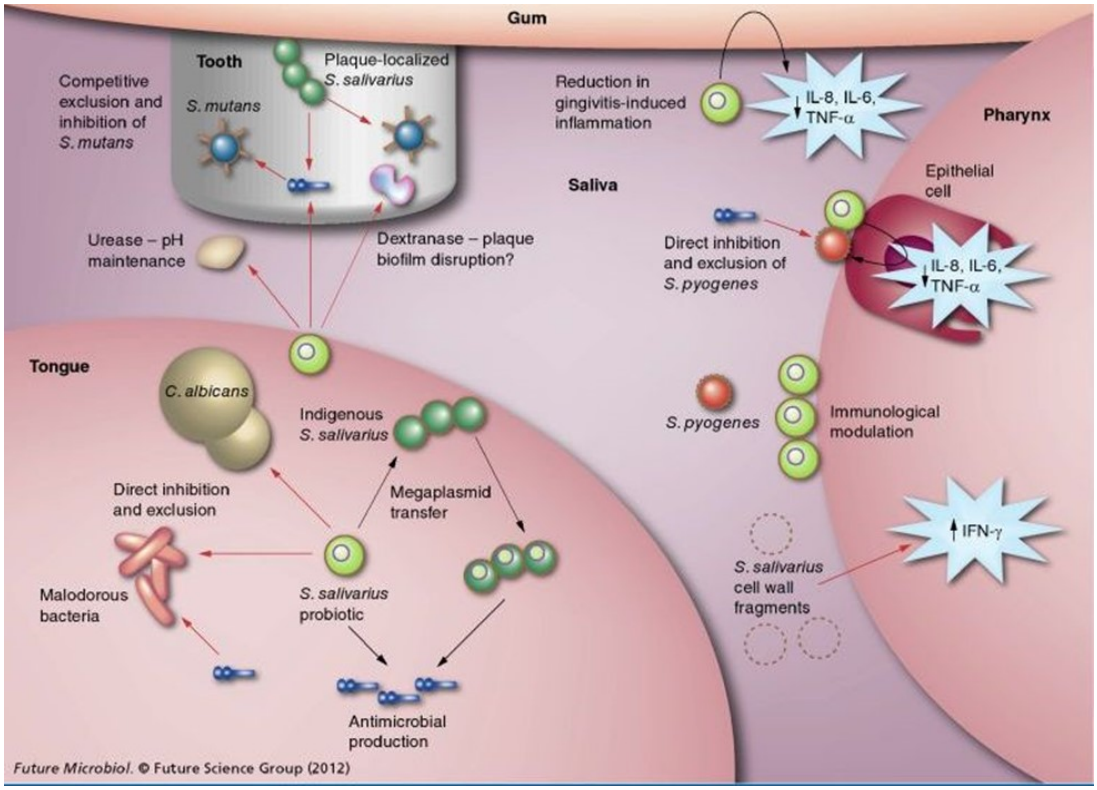


Figure 8: Influence of *S.salivarius* strains in the oral cavity.

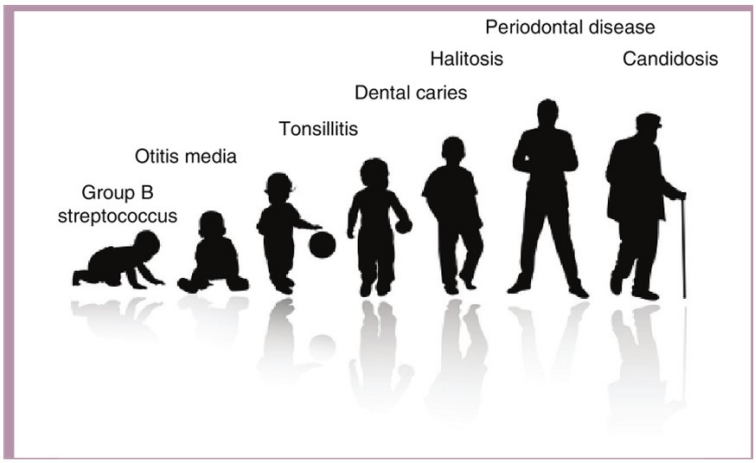


Figure 9: Diseases that may be alleviated by *S.salivarius* probiotics and the ages at which they generally tend to manifest.

I characterized with my research group a strain of *S.salivarius*, specifically selected for its potential to interfere with the colonization of upper respiratory tract (URT) pathogens: *S.salivarius* 24SMBc.

S.salivarius 24SMBc was selected by screening of 81α-hemolytic streptococci isolated from 62 nasal and/orpharyngeal swabs of healthy children and identified by currently available phenotypical and molecular techniques. This strain is susceptible to a variety of commonly utilized antibiotics for URT infections treatment and has no hemolytic or harmful enzymatic activity. In addition, it also lacks the main streptococcal virulence genes encoding for streptolysin S, mitogenic exotoxin Z, pyrogenic toxin B, fibronectin-binding protein, serum opacity factor, and exotoxin type C, and G, that can be found in the *Streptococcus* genus.

S.salivarius 24SMBc was assessed through deferred antagonism test for antagonist activity against representative strains of URT infections including OM pathogens. It showed an inhibitory spectrum for *S.pneumoniae* when tested on Columbia agar base supplemented with 5% horse blood and 0.1% $CaCo_3$, and *S.pyogenes* when tested on TSYCa.

In particular, *S.salivarius* 24SMBc inhibits *S.pneumoniae* strains that include different serotypes, such as 19A, responsible for cases of pediatric meningitis in Sicily, and one *S.pyogenes* group that is represented by some serotypes such as M1 that is very virulent and very diffused in Italy, and involved in severe infections in children and adults.

Furthermore, it does not interfere with other *S.salivarius* strains, thus it can coexist with other "friendly bacteria" that colonize the host oral cavity.

This strain demonstrated a good capability to adhere to epithelial cells, indeed, the cells of *S. salivarius* 24SMBc remained attached to the *in vitro* HEp-2 monolayer test.

These characteristics led my research group to patent (Pat. num: WO 2011/125086) and register as DSM 23307 our *S.salivarius* 24SMBc strain **[38, 79]**.

2. AIM OF STUDY

During my PhD carried out at the Microbial Molecular Antibiotic Resistance (MMAR) laboratory my research line focused on further analysis of *S.salivarius* 24SMBc strain, finalized to its application as an oral probiotic for children with recurrent OM. Indeed, this microorganism showed a good *in vitro* antagonist activity against some pathogens of OM, in particular *S.pneumoniae* and *S.pyogenes*.

The first phase was the analysis of the *S.salivarius* 24SMBc genome in order to verify the presence of virulent factors and look for genes encoding bacteriocins that determine the capability of our strain to interfere with the growth of the pathogens previously described.

To realize our aim, focused on the use of *S.salivarius* 24SMBc strain as an oral probiotic, we followed the standard guidelines for the evaluation of probiotics, recommended by the FAO and WHO **[5]**.
Thus, we assessed the safety and human tolerance of *S.salivarius* 24SMBc including it in a clinical trial protocol conducted on healthy adult volunteers to verify the lack of adverse events. Moreover, in this study we determined the adherence to human epithelial cells *in vivo* analysing its colonization and persistence in the human upper respiratory tract.

The following phase focused on efficacy of *S.salivarius* 24SMBc and was realized through a paediatric randomized, placebo-controlled, double-blind trial that involved children with recurrent OM. This clinical study was finalized for the assessment of persistence and the level of colonization of *S.salivarius* 24SMBc in the upper respiratory tract of these children and its efficacy to prevent or reduce the presence of causative bacteria of OM.

3. ANALYSIS OF S. SALIVARIUS 24SMBc STRAIN'S GENOME SEQUENCE

The genome of *S.salivarius* 24SMBc strain was sequenced by Pyrosequencing 454. The chromosome reads were assembled into 376 contigs. The estimated length of the chromosome is 1,893,903 bp with a GC content of 40%.

Genome annotation was performed using RAST **[80]**. *S.salivarius* 24SMBc was found to be free of streptococcal virulent factors (**Figure 10**) and this assessment suggested that this microorganism is safe for use as a probiotic.

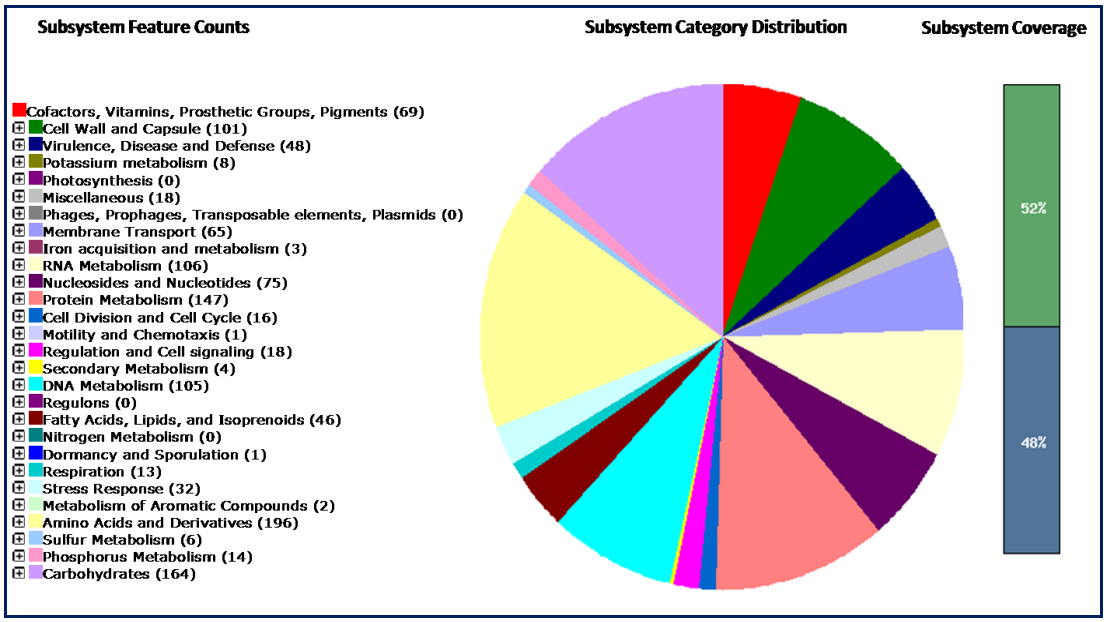


Figure 10: Organism Overview for *Streptococcus salivarius* 24SMBc

Sequencing the genome of *S.salivarius* 24SMBc, we detected a *blp* (bacteriocin-like protein) locus that resembles the *blp* locus that has been described in *S.pneumoniae* and *S. thermophilus* [**81, 82**].

The *blp* locus of *S.thermophilus*, when fully functional, is organized in independent transcription units coding specific functions related to bacteriocin production: *blpABC*, encoding an ABC-transporter (blpA), a transport accessory protein (blpB), and a peptide pheromone (blpC); *blpRH*, encoding a histidine kinase (blpH) and a response regulator (blpR). The other operons are: *blp*D-*orf*2, *blp*U-*orf*3 and *blp*E-F, encoding bacteriocin precursors and proteins involved in immunity, and *blpG-X*, whose function is unknown **(Figure 11)**.

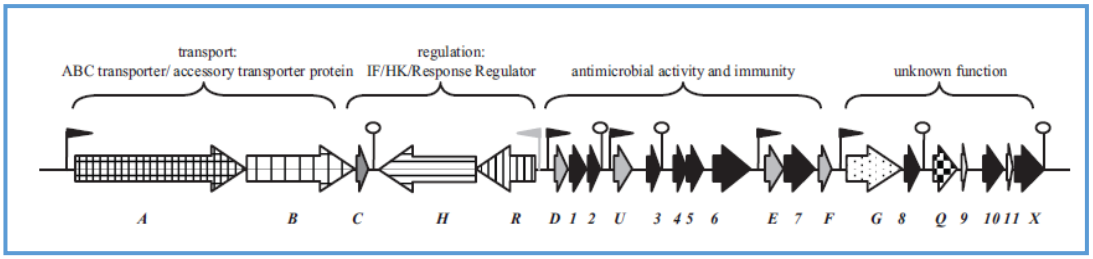


Figure 11: The *blp* locus of *S.thermophilus*

This locus contains all the genetic information required for the production of bacteriocin and is regulated at the transcriptional level by a Quorum Sensing mechanism in which the mature form(s) of the induction factor blpC trigger(s) the expression of the bacteriocin and immunity genes through the blpR-blpH TCS (Two Component System). The mechanism of regulation by cell density implies that there is a basal level of secretion of IF (induction factor) and that a critical concentration of IF triggers its auto-induction, resulting in the amplification of the response.

The ABC transporter recognizes the N-termini of both the pheromone and the bacteriocins and transports these peptides across the cytoplasmatic membrane, concurrent with cleavage at a conserved double-glycine motif. Cleaved extracellular blpC can then bind the sensor kinase, blpH. This interaction results in the activation of blpR and upregulation of the entire gene cluster via binding to consensus sequences within each promoter **[81, 82]**.

Sequence analysis of *S.salivarius* 24SMBc *blp* locus of 4.2 kb in size showed the presence of a specific sequence of 258 bp missing in other strains of *S.pneumoniae*, *S.thermophilus* and *S.salivarius* previously described **(Figure 12)**. Therefore, this sequence was used to discriminate our microorganism from other strains of *S.salivarius*.

TAAGCCAAAGTCAGATGACATTGCCTTATTA
GTCTTTATCAAAAAAATCCCATAGGATGATGAGTCCTATGGGAATTTTTTATTGTGTGGAAAC
AAGCAAGATTTAGGCTTCAATTTTTACGATTCATTTTAATGTTATTTAAACAAATGATGTAGCC
AAGACAGTGGAACAAGACAAAGATATGCCAGTAAAAATAAAGCATAAAGCTATCAGATTTGG
TTATCGTATAAAAAATAAAAATTGCAACAAATAAAATG

Figure 12: Specific sequence of 258 bp in size in *S.salivarius* 24SMBc strain.

4. CLINICAL TRIAL PROTOCOL OF A NASAL SPRAY FORMULATION OF S. SALIVARIUS 24SMBc

S.salivarius 24SMBc, thanks to its significant probiotic characteristics, was included in a clinical trial protocol conducted on healthy adult volunteers to evaluate its safety and ability to colonize and persist in the human upper respiratory tract.

The study enrolled 17 subjects that were treated with the nasal spray formulation of *S.salivarius* 24SMBc following a 6-days course of cefixoral. This antibiotic treatment was necessary to effect a temporary reduction in the levels of native oral bacterial populations in order to facilitate subsequent colonization by *S.salivarius* 24SMBc.

The presence of *S.salivarius* 24SMBc was determined after 2h, 4h, 24h and 7 days from nasal spray administration, collecting rhino-pharyngeal swabs and plating them for each time determination onto Columbia Agar Base and Mitis Salivarius agar.

Furthermore, rhino-pharyngeal swabs were obtained just prior to the antibiotic treatment to evaluate the pre-existent microbiota in the upper respiratory tract of volunteers and after the antibiotic treatment to verify the permanence of antibiotic-resistant bacteria.

Each α-haemolitic streptococcal colony isolated on Mitis Salivarius agar that showed a typical aspect of a *S.salivarius* colony was analyzed by antagonism test to evaluate BLIS production and RAPD-PCR to distinguish *S.salivarius* 24SMBc from other *S.salivarius* strains through genotype profiling.

The levels of colonization by *S.salivarius* 24SMBc were estimated by calculating the proportion of samples containing *S.salivarius* colonies with the same characteristics of the strain under study, and all the samples collected from volunteers enrolled in this study.

4.1 Materials and methods

4.1.1 Preparation of test material

The *S.salivarius* 24SMBc strain was formulated for a nasal spray device containing not less than $1x10^9$ CFU/ml. The cell counts were obtained just prior to commencement and at completion of the study. The product is manufactured by DMG, Rome, Italy.

4.1.2 Clinical trial

The clinical trial involved 17 health subjects, males and females (aged 18-54 years), enrolled in the area of Catania, Italy. This research was carried out during routine clinical practice, following international guidelines and in line with the principles outlined in the Declaration of Helsinki, such that approval from local ethics boards was not required. Exclusion criteria considered were: pregnancy and breast feeding, morpho-functional disorders of the nasal passages and nasal airflow, inflammatory hypertrophic vasomotor diseases. Moreover, the clinical trial excluded patients with diabetes, cystic fibrosis, gastroesophageal reflux, chronic renal failure, recurrent or relapsing inflammation of the upper respiratory tract, mucosal atrophy and impaired mucociliary clearance deficit, hypersensitivity to cephalosporins and subjects treated with immunosuppressants and antibiotics.

The nasal spray was administered 3 times daily for 3 days after an antibiotic treatment with cefixoral (400 mg daily) for 6 days. Rhino-pharyngeal swabs were collected from the volunteers before and later the antibiotic treatment and after 2h, 4h, 24h and 7 days following the nasal spray administration. Then, the biological samples were sent to our MMAR laboratory to be tested for their content of *S.salivarius* 24SMBc.

4.1.3 Isolation of bacteria and culture conditions

The rhino-pharyngeal swabs were plated directly onto Columbia Agar Base (Oxoid, Basingstoke, UK), plus 5% horse blood to determine a total microflora population and Mitis Salivarius agar (Difco Laboratories), a selective medium for streptococci to isolate viridans strains. Cultures were incubated overnight at 37 °C in 5% CO2 in air atmosphere.

4.1.4 Test for BLIS production

Each morphologically distinct colony grown on Mitis Salivarius agar was tested for BLIS production using a deferred antagonism test on Columbia Agar Base (Oxoid, Basingstoke, UK) supplemented with 5% horse blood and 0.1% CaCO3. The test strain was inoculated diametrically across the test agar plate as a 1-cm wide streak. The visible growth of the test strain was removed using a glass slide, and the surface of the plate was sterilized by exposure to chloroform vapours for 30 min.

The agar surface was then aired to remove residual chloroform for 15 min. Then, Todd Hewitt broth cultures of the indicator strains, grown for 18 h at 37 °C, were streaked across the growth line of the original producer strain for BLIS production. The plates were incubated for 18h at 37°C and examined for interference zones with the indicator. The isolates that inhibited the growth of an indicator strain were considered to be inhibitory for that species. The indicator strains were representative strains of URT infections including OM pathogens: *S.pyogenes* group (*S.pyogenes* 2812A serotype M18, *S.pyogenes* Spy35370 serotype M1 and F222 serotype M2), *H.influenzae* 3ATF, *S.aureus* 10F, *E.coli* 12I, *P.aeruginosa* 115, *S.salivarius* ATCC13419, and *M.catarrhalis* 120. Regarding *S.pneumoniae*, this group included three not-typed clinical isolates of *S.pneumoniae* (11ATN, 22ATN and 148) and three *S.pneumoniae* serotype 19A (BT *S.pneumoniae*; CR *S.pneumoniae*; GC *S.pneumoniae*), which are responsible for cases of paediatric meningitis in Sicily, Italy. All *S.pneumoniae* strains used were resistance to erythromycin and clindamycin, and susceptibility to penicillin and ampicillin. All strains used as indicator strains in the deferred antagonism test were clinical strains except *S.salivarius* ATCC13419. The BLIS production was also tested using a deferred antagonism test on Trypticase Soy Yeast Extract Calcium agar (Trypticase Soy Broth; Oxoid, Basingstoke, UK) + 2% Yeast extract (Oxoid, Basingstoke, UK) + 1.5 agar (Oxoid, Basingstoke, UK) + 0.1% CaCO3 **[38]**.

4.1.5 DNA extraction

Bacterial colonies grown on Mitis Salivarius agar and positive for deferred antagonism test were plated onto Columbia Agar Base (Oxoid, Basingstoke, UK),

plus 5% horse blood and collected in order to carry out DNA extraction as previously described **[84]**.

4.1.6 RAPD-PCR

Genotyping by RAPD analysis was carried out using the primers OPA3 (5'-AGT CAG CCA C-3') and OPA18 (5'- AGG TGA CCG T-3') **[85]**. The RAPD reaction was performed in a total volume of 25 µl containing: 2.5 Mm $MgCl_2$, 1x DyNAzime buffer, 2 Mm dNTPs mixture, 0.04 U/µl of DyNAzime II DNA Polymerase (Thermo SCIENTIFIC), 1.2 Mm of primers and 50 ng/µl of genomic DNA. The amplification was run in the thermocycler as follows: initially 5 min at 94°C, followed by 30 cycles of 1 min at 32°C (annealing), 2 min at 72°C (extension), 1 min at 94°C (denaturation) and a final extension step of 72°C for 10 min.

The PCR products were resolved by electrophoresis on 1.5% (w/v) agarose-TBE gel containing 1X SYBER Safe DNA gel stain, applying a mixture of 7 µl of PCR product and 3 µl of BFB (0.25% bromophenol blue, 0.25% Xylene Cyanole, 30% glycerol) and run in 0.5X TBE buffer for 75 min at 100 V. A 1Kb DNA ladder and a 100 bp DNA ladder (NEW ENGLAND BioLabs) were used as molecular weight markers. The RAPD fragment patterns were visualized with blue light emission.

4.2 Results

From the clinical point of view, no adverse events were manifested by the volunteers enrolled in the trial. The averages of the total microflora population determined after 2h, 4h, 24h and 7 days from nasal spray administration were approximately from 10^4 CFU/ml to $>10^6$ CFU/ml. At the same time intervals, α-haemolitic streptococcal colonies grown on Mitis Salivarius agar with the typical aspect of a *S.salivarius* colony (large, soft and pale blue) **(Figure 13)** were isolated from 16 samples until the 7^{th} day and from one sample until the 4^{th} hour with a range from 10 CFU/ml to $>10^6$ CFU/ml **(Table 3)**.

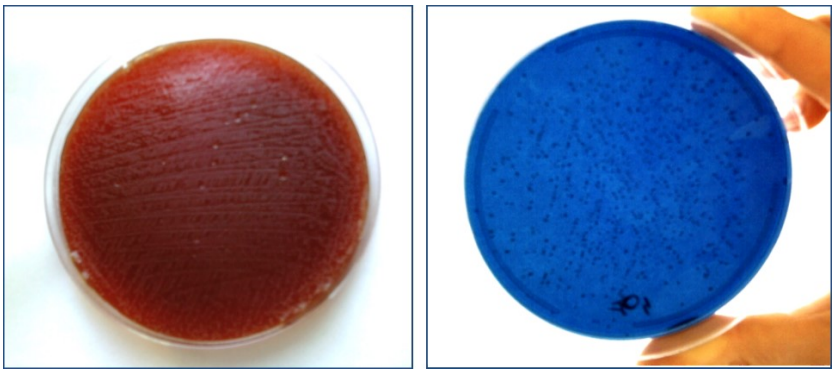


Figure 13: *S.salivarius* 24SMBc growth on Columbia Agar Base and Mitis Salivarius agar.

All streptococcal colonies grown on Mitis Salivarius agar with typical aspect of *S.salivarius* were analyzed by deferred antagonism test and several of them showed the same inhibitory activity of *S.salivarius* 24SMBc, in particular, against *S.pneumoniae* serotype 19A and *S.pyogenes* serotype M1 **(Figure 14)**.

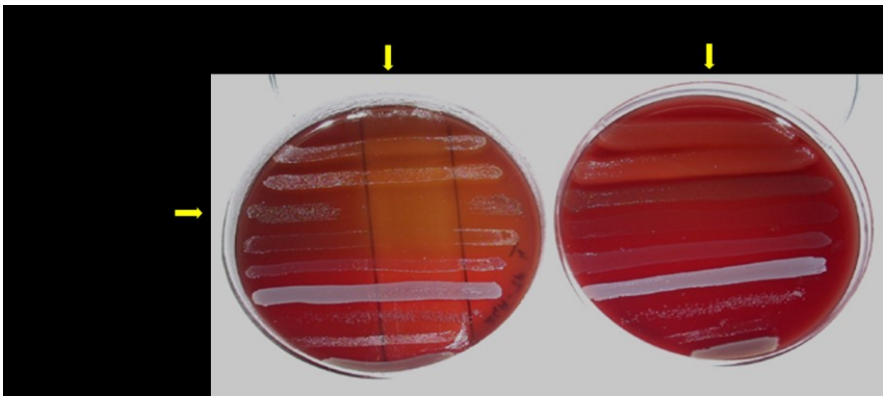


Figure 14: Deferred antagonism test.

All streptococcal colonies that were positive with the deferred antagonism test, were evaluated by RAPD analysis using primers OPA3 and OPA18 that provide distinct fragment patterns. This analysis showed that all colonies tested had an identical profile to *S.salivarius* 24SMBc with the exception of one colony that demonstrated that one volunteer (patient 005) was colonized both by our strain and another *S.salivarius* **(Figure 15)**.

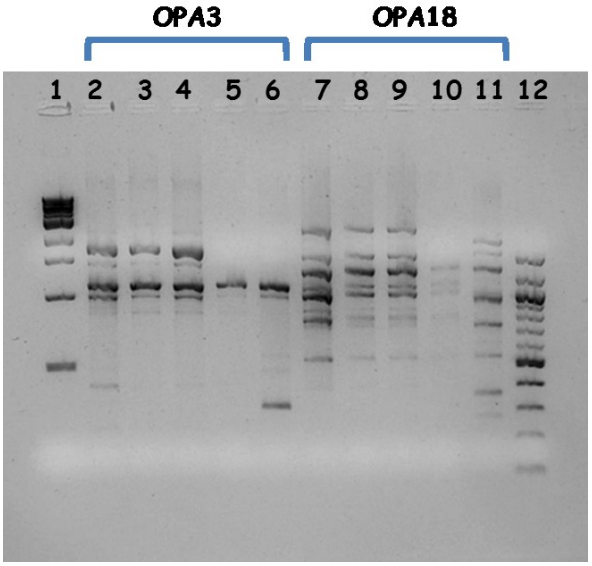


Figure 15: RAPD fingerprinting with primers OPA3 and OPA18
1: 1Kb DNA ladder; 2-7: *S. salivarius* 24SMBc; 3-8: patient 3; 4-9: patient 4;
5-10: patient 5; 6-11: patient 5; 12: 100 bp DNA ladder.

Our results allowed us to determine the level of colonization by *S.salivarius* 24SMBc in the upper respiratory tract of adults. In this clinical trial protocol, 8 volunteers were colonized by *S.salivarius* 24SMBc until the 7th day and 1 volunteer (patient 001) was colonized until the 4th hour from nasal spray administration. In this last case, the sample presented colonies of cefixoral-resistent *S.aureus* (>10^6 CFU/ml) just prior to the initiation of the antibiotic treatment and after the nasal spray administration. All in all, samples of the remaining 8 volunteers were negative for *S.salivarius* 24SMBc **(Table 3)**. In addition, the nasal spray was well tolerated in all patients.

Patient	Age	Bacterial count (CFU/mL)	T_1 2h	T_2 4h	T_3 24h	T_4 7thdays	S.salivarius 24SMBc
001	45	Total count	$>10^6$	$>10^5$	$>10^6$	$>10^6$	
		a-haemolitic streptococci count	6,5x10	10	10^4	10^4	T_1, T_2
002	20	Total count	$>10^6$	10^2	$3x10^2$	10^5	
		a-haemolitic streptococci count	10^3	2x10	-	-	-
003	18	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	
		a-haemolitic streptococci count	5x10	3x10	10^2	10^2	T_1,T_2,T_3,T_4
004	28	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	
		a-haemolitic streptococci count	3x10	10	10	2x10	T_1,T_2,T_3,T_4
005	19	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	
		a-haemolitic streptococci count	10^2	4x10	5x10	3x10	T_1,T_2,T_3,T_4
006	26	Total count	$2x10^2$	$>10^6$	2x10	5x10	
		a-haemolitic streptococci count	5x10	$4x10^2$	10	10	-
007	24	Total count	$>10^6$	$>10^6$	10^4	$>10^6$	
		a-haemolitic streptococci count	10^4	10^3	10^2	10^3	T_1,T_2,T_3,T_4
008	54	Total count	$>10^6$	10^4	$>10^6$	$>10^6$	
		a-haemolitic streptococci count	10^6	10^3	10^3	10^2	T_1,T_2,T_3,T_4
009	31	Total count	$>10^6$	10^5	10^5	10^5	
		a-haemolitic streptococci count	10	30	-	10^2	-
010	38	Total count	$>10^6$	10^5	10^5	$>10^6$	
		a-haemolitic streptococci count	10^3	10^2	10	10^2	T_1,T_2,T_3,T_4
011	30	Total count	10^6	10^5	10^6	10^6	
		a-haemolitic streptococci count	10^2	50	10^2	70	T_1,T_2,T_3,T_4
012	27	Total count	10^5	10^5	10^4	10^5	
		a-haemolitic streptococci count	20	50	10	80	-
013	23	Total count	$>10^6$	10^5	10^5	10^4	
		a-haemolitic streptococci count	10^2	5x10	10^2	8x10	-
014	32	Total count	$>10^6$	10^5	10^5	10^5	
		a-haemolitic streptococci count	9x10	5x10	7x10	10	-
015	43	Total count	$>10^6$	$>10^6$	10^5	10^6	
		a-haemolitic streptococci count	-	10	8x10	5x10	-
016	42	Total count	$>10^6$	$>10^6$	$>10^6$	$>10^6$	
		a-haemolitic streptococci count	10^2	5x10	5x10	3x10	T_1,T_2,T_3,T_4
017	52	Total count	10^6	10^6	10^5	10^4	
		a-haemolitic streptococci count	-	20	10	20	-

Table 3: Colonization by *S.salivarius* 24SMBc.

5. CLINICAL EVALUATION OF S. SALIVARIUS 24SMBc IN A PAEDIATRIC RANDOMIZED, PLACEBO-CONTROLLED, DOUBLE-BLIND TRIAL

On the basis of its (i) good oral cavity colonization capability, (ìì) demonstrated tolerability in the human host, and (iii) antagonist activity against oral pathogens, such as *S.pneumoniae* and *S.pyogenes* strains, we evaluated the efficacy of *S.salivarius* 24SMBc when administered to children having a history of recurrent OM.

OM is the second most common childhood infection. Children less than 3 years of age have a high prevalence of OM, with the peak incidence occurring around 1 year of age. Children under 2 years of age with six or more episodes of acute otitis media (AOM) are termed "otitis-prone".

The interaction between bacteria, viruses and the host immune response plays a role in the pathogenesis of OM which is usually initiated by respiratory viruses and can be complicated by bacterial infection, which worsens the clinical outcome. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and less often *Streptococcus pyogenes* are the main bacteria involved in OM that, typically, translocate from the nasopharynx to the middle ear via the eustachian tube.

Children may be asymptomatic carriers of such pathogens, or may develop infections such as suppurative OM. Under normal conditions, the low density of bacterial colonisation in the nasopharynx may initiate a host response that regulates the inflammatory process and eradicates these bacteria without causing mucosal damage. In contrast, high bacterial loads in the nasopharynx can cause repeated inflammation, mucosal damage and persistence of infection **[86, 87, 88, 89, 90, 91, 92]**.

There have been some pilot studies aimed at protecting the human host against development of OM using commensal inhabitants of the healthy nasopharynx. In particular, alpha-haemolytic streptococci have been investigated in previously described clinical trials **[56, 57]**.

In our study, 120 subjects with a diagnosis of not less than 4 episodes of recurrent otitis media in the previous year, were enrolled in a paediatric randomized, placebo-controlled, double-blind trial.

23 children represented a control group for eventual adverse events after administration of the nasal spray containing *S.salivarius* 24SMBc, 50 children were

selected for the rinogermina group while the other 47 were selected for placebo group. Rinogermina group was made up of children that received the nasal spray containing *S.salivarius* 24SMBc.

The main aims of this trial were to assess: i) the persistence and the level of colonization of *S. Salivarius* 24SMBc in the upper respiratory tract of children with recurrent OM; ii) the efficacy to prevent or reduce the presence of OM pathogens by phenotypical and molecular approaches. The presence of *S.pneumoniae*, *S.pyogenes*, *H.influenzae*, *M.catarrhalis* and *S.salivarius* were determined by isolation of bacterial colonies and PCR.

In addition, we determined the presence of *S.salivarius* 24SMBc in the rhino-pharyngeal samples by PCR using the *blp*C gene sequence as the molecular target. Moreover, the persistence of *S.salivarius* 24SMBc and its total count for each sample at the different times were determined by qPCR using a TaqMan probe designated into a specific sequence of the *blpC* gene. This DNA region is able to discriminate our strain from other *S.salivarius*.

5.1 Materials and methods

5.1.1 Clinical trial

The paediatric randomized, placebo-controlled, double-blind trial was conducted on 120 children followed-up by an Italian day care centre located in Milan: Clinic Pediatrics of IRCCS Ca' Granda Ospedale Maggiore Policlinico. This research was carried out during routine clinical practice, following international guidelines and in line with the principles outlined in the Declaration of Helsinki, such that approval from local ethics boards was not required. Inclusion criteria were: informed signed consent from parents, age 12-72 months and previous episodes of OM. Exclusion criteria were: age above 72 months, positive history for serious allergic diseases, acute febrile illness, immunodeficiency, presence of cancer, kidney and liver diseases, and use of transtympanic drainage tubes. The population consisted of males and females, was subdivided into three groups: the first, made up of 23 children, was a control for adverse events; the second, made up of 50 children, was treated with *S.salivarius* 24SMBc; the third, made up of 47 children, received the placebo.

According to the treatment protocol, standardised in the previous clinical trial with adult volunteers, the nasal spray containing $1x10^9$ CFU/ml of *S.salivarius* 24SMBc was administered 3 times daily for three days. Furthermore, before administration of the nasal spray, Augmentin (80 mg/Kg) was given for 7 days to decrease the endogenous population inhabiting the oral cavity in order to enhance the colonization process of the probiotic strain.

These children were followed-up for a period of 160 days with medical examinations and collection of rhino-pharyngeal swabs at specific time intervals: T_6 (six days), T_{61} (sixty-one days), T_{120} (four mouths) and T_{150} (five mouths) after administration of *S.salivarius* 24SMBc or placebo.

Moreover, a rhino-pharyngeal swab (sample T_0) was collected from each child after antibiotic treatment in order to analyse the bacterial population that was present before the commencement of the colonization protocol.

All specimens from the paediatric patients were kept in 5 ml of Brain-heart infusion (BHI) broth plus 20% of glycerol and frozen at -80°C. Then, these rhino-pharyngeal swabs were sent to our MMAR laboratory to be tested for their *S.salivarius* 24SMBc

content and OM pathogens: *S.pneumoniae*, *H.influenzae*, *M.catarrhalis* and *S.pyogenes*.

5.1.2 Culture conditions

The rhino-pharyngeal swabs were plated directly onto Columbia Agar Base (Oxoid, Basingstoke, UK), plus 5% horse blood to determine a total microflora population, Mitis Salivarius agar (Difco Laboratories), a selective medium for streptococci, used for isolation of the viridans strains. Moreover, the swab samples were plated onto chocolate agar with the addition of bacitracin and chocolate agar that are selective media for *Haemophilus spp.* and *Moraxella spp.,* respectively.
Cultures were incubated overnight at 37 °C in 5% CO2 in air atmosphere.

5.1.3 DNA extraction

For each sample, all colonies grown on Columbia Agar Base (Oxoid, Basingstoke, UK), plus 5% horse blood, were collected and washed with 1 ml of solution of 0.9 % w/v NaCl. Then microbial DNA was extracted and purified through a QIAcube Extractor using QIAamp DNA Mini kit (Qiagen).
The DNA concentration (absorbance at 260 nm; A_{260}) and the purity (A_{260}/ A_{280}) were calculated using a BioPhotometer D30 (Eppendorf).

5.1.4 Molecular identification of S.salivarius and pathogenic strains

The presence of *S.pneumoniae*, *S.pyogenes* and *S.salivarius* strains was determined by PCR using primers that amplify species-specific sequences, respectively; *lytA*, *M-type* and *sodA* genes, described in **table 4**. We used primers to amplify the *16S rRNA* gene as a positive control for DNA-templates. *S.pneumoniae* 22A-TN, *S.pyogenes* 5005 and *S.salivarius* 24SMBc were used as positive controls for *lytA*, M-type and sodA genes, respectively.
For detection of *S.salivarius* 24SMBc, we used specific primers (MS442 and MS443) designed to amplify the *blp*C gene, described in **table 4**. *S.salivarius* K12, *S.salivarius* 3C30 were used as negative controls.

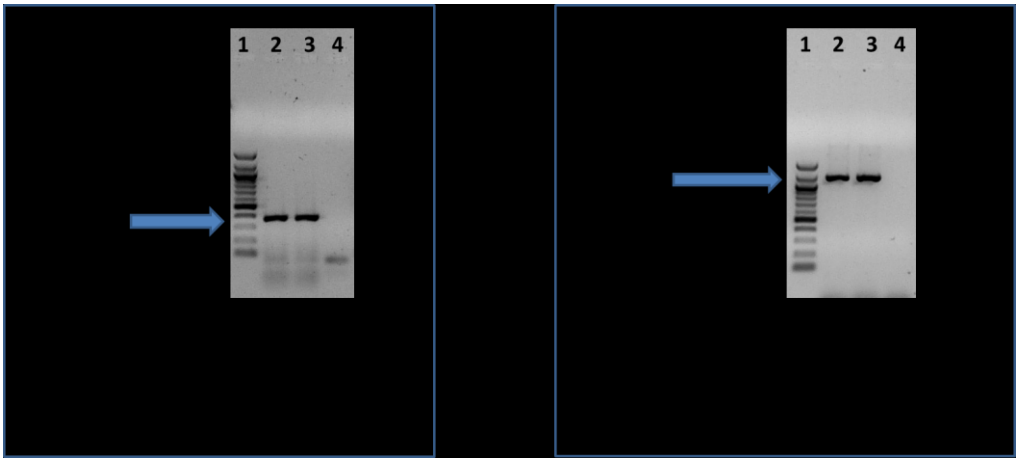
Gene	Primer name	Primer sequence (5'-3')	Amplicon size (bp)
lytA	lytA up	5'-AGTTGCCGTTGTGCTTCCT-3'	400
	lytA reverse	5'-ACGGACTACCGCCTTTATATCGAAC-3'	
M-type	*M-type-up1*	5'-TATT(C/G)GCTTAGAAAATTAA-3'	1200
	M-type-reverse2	5'-GCAAGTTCTTCAGCTTGTTT-3'	
sodA	sodA up	5'-ATGCTGAAACAATGACTCTTCACC-3'	400
	sodA reverse	5'-ACCGTCTGAGATAGGAGTGTCTTG-3'	
16S rRNA	16S	5'-TGGGGTGAATCGTAACAAG-3'	350
	23S	5'-CCAAGGCATGCCACCGTGT-3'	
blpC	MS 442	5'-GCCCTAAGCCAAAGTCAGATGA-3'	280
	MS443	5'-GGTATGGCTCACCCTTTTATGTG-3'	

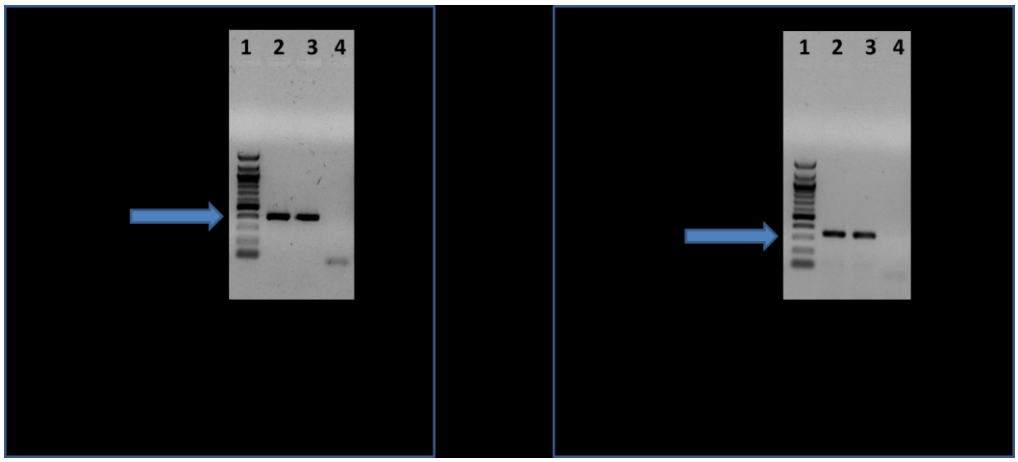
Table 4: Primers used for PCR.

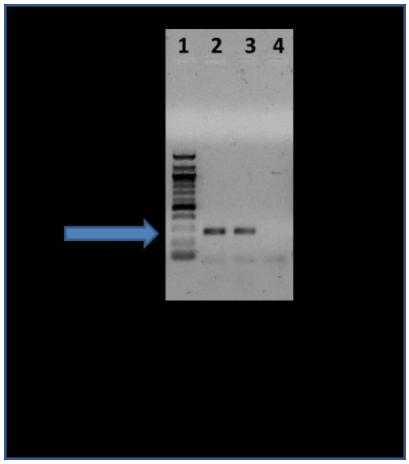
The PCR reaction mixture for amplification of the *16S rRNA* gene, *lytA* gene, *sodA* gene and *blpC* gene was performed in a total volume of 25 µl containing: 1x polymerase chain reaction buffer, 0.5 Mm dNTPs mixture, 0.024 U/µl of DyNAzime II DNA Polymerase (Thermo SCIENTIFIC), 0.4 Mm of each primer and 50 ng/µl of genomic DNA. The amplification was run in the thermocycler as follows: initially 5 min at 94°C, followed by 30 cycles of 30s at 58°C (annealing), 1 min at 68°C (extension), 30s at 93°C (denaturation) and a final extension step of 68°C for 10 min.

For M-type PCR, the reaction was performed in a total volume of 25 µl containing: 1x polymerase chain reaction buffer, 0.8 Mm dNTPs mixture, 0.024 U/µl of DyNAzime II DNA Polymerase (Thermo SCIENTIFIC), 1.4 Mm of each primer and 50 ng/µl of genomic DNA. The amplification was run in the thermocycler as follows: initially 15s at 94°C, 30s at 46.5°C, 1 min 15s at 72°C, followed by 20 cycles of 15s at 94°C, 30s at 46,5°C, 1 min 15s at 72°C with a 10 sec increment for each of the subsequent 19 cycles. Then a final extension step of 72°C for 10min.

The PCR products were resolved by electrophoresis on 1% (w/v) agarose-TBE gel containing 1X SYBER Safe DNA gel stain, applying a mixture of 5 µl of PCR product, 2µl of BFB (0.25% bromophenol blue, 0.25% Xylene Cyanole, 30% glycerol), 3µl of water and run in 0.5X TBE buffer for 30'at 120 V. A 100 bp DNA ladder (NEW ENGLAND BioLabs) was used as molecular weight markers. The PCR products were visualized with blue light emission **(Figures 15, 16, 17, 18, 19, 20)**.


1 2 3 4
1 2 3 4


1 2 3 4
1 2 3 4


1 2 3 4

5.1.5 Real-time quantitative-polymerase chain reaction

The detection and quantification of *S.salivarius* 24SMBc at various time intervals, was determined by qPCR using a TaqMan probe designed to amplify *blp*C: blp-Tq (5'- FAM-TCCACTGTCTTGGCTACATCATT-3'-BHQ1) and a couple of primers: Blp-upTq (5'-GGATGATGAGTCCTATGG-3') and Blp-revTq (CTGGCATATCTTTGTCTTG). The analytical sensitivity of the real-time assay was determined by serial dilutions of target DNA. Standard curves of C_t (threshold cycle) versus genome copies were then constructed. Genome copy numbers were calculated on the basis of a genome size of 2.2 Mb. The blp assay was able to detect bacterial DNA over a linear range between 10^2 and 10^8 genome copies.

qPCR was performed with the aid of the Mx3000P Instrument (STRATAGENE) using optical grade 96-well plates. All samples were analysed using the QuantiNova Probe PCR Kit. Final reactions contained: 1x QuantiNova Probe PCR Master Mix, 0.25 pmol of each primer, 0.1 pmol of probe, 1x QN ROX Reference Dye and 1 µl template DNA (approximately 50 ng/µl).

The temperature profile was as follows: initial denaturation of 95°C for 4 min, followed by 40 cycles of 95°C for 5s, 60°C for 30s, and a final cycle at 95°C for 1 min, 58°C for 1 min and 95°C for 30s.

Data acquisition and subsequent analysis were performed using the MxPro-Mx3000P software. The amount of initial template DNA was calculated by determining C_t, which is the number of polymerase chain reaction cycles required for the fluorescence to exceed a threshold value significantly higher than the background fluorescence. All samples were run in triplicate, and the mean value was used for analysis **(Figure 21)**.

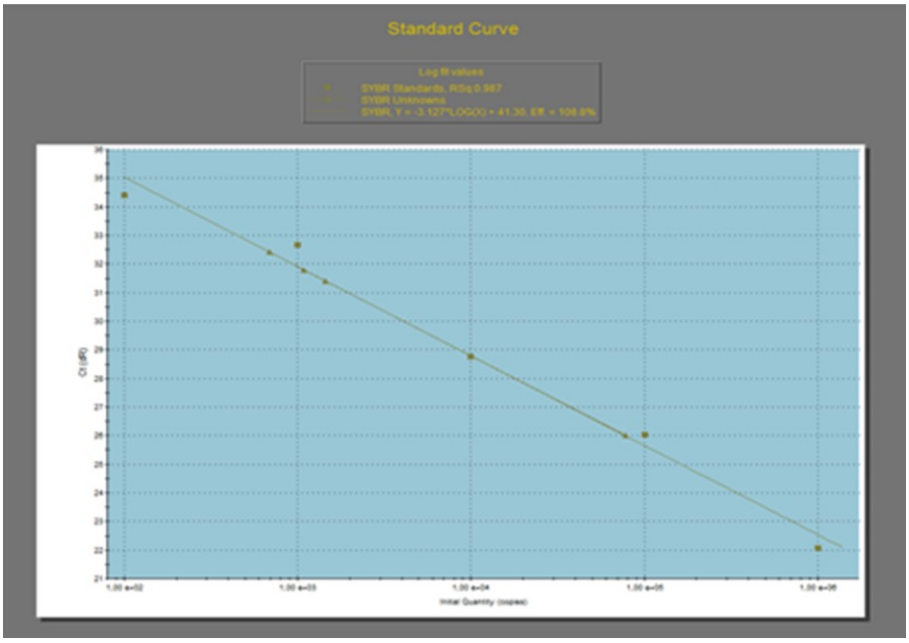


Figure 21: Standard Curve of blpC target

5.2 Results

In this study, we attempted to establish the preventive role played by *S.salivarius* 24SMBc when administered to children with a history of recurrent acute otitis media. Results obtained by qPCR demonstrated that *S.salivarius* 24SMBc was present in 21 of the 50 treated children, showing different percentages of colonization and ranges of bacterial counts, for specific time intervals: T_6 (six days), T_{61} (sixty-one days), T_{120} (four mouths) and T_{150} (five mouths) after administration of our strain. It is interesting that among 21 children who were positive for the presence of *S.salivarius* 24SMBc, the level of colonization reached a value of 66.7 % with a range of bacterial count from 10 to 10^4 CFU/ml at T_{150} compared to lower values in T_6, which is the first sample after nasal spray administration (**Table 5)**.

	Time intervals	Level of colonization	Bacterial count
21 positive patients colonized by S.salivarius 24SMBc	T_6	19%	10^2-10^3 CFU/ml
	T_{61}	9.5%	10-10^3 CFU/ml
	T_{120}	4.8%	10^2 CFU/ml
	T_{150}	66.7%	10-10^4 CFU/ml

Table 5

Statistically significant results were seen in terms of episodes of OM that were more in the group of children treated with placebo than in the group of children treated with *S.salivarius* 24SMBc, as shown in **table 6**.

Number episodes of OM	Rinogermina group (50 patients treated with S.salivarius 24SMBc)	Placebo group (47 patients)
0	15	7
1	11	17
2	9	13
3	10	6
4	2	3
5	3	0
6	0	0
7	0	1

Table 6

No episode of otitis media was manifested in the 15 patients belonging to the rinogermina group and in 7 patients belonging to the placebo group. Moreover, among these 15 children treated with rinogermina, 8 were colonized by our strain. In particular, the presence of *S.salivarius* 24SMBc was determined after 150 days from the spray administration in 6 children belonging to this group (**Table 7**).

Sample	T_6	T_{61}	T_{120}	T_{150}
024	+	+	+	+
029	+	+	+	+
030	+	+	+	+
047	+	-	-	-
067	+	+	-	-
089	+	+	+	+
102	+	+	+	+
117	+	+	+	+

Table 7

In this study, we evaluated the efficacy of *S.salivarius* 24SMBc to prevent or reduce the presence of *S.pneumoniae*, *H.influenzae*, *M.catarrhalis* and *S.pyogenes* responsible for OM. The presence of *S.pneumoniae*, the principal pathogen of OM, was determined in the samples at T_0, T_6, T_{61}, T_{120} with similar percentages in the groups of children (rinogermina and placebo groups) enrolled in the study. However, the group of children treated with *S.salivarius* 24SMBc, showed a reduction of colonization by *S.pneumoniae* in the samples at T_{150} **(Table 8)**.

	S.pneumoniae				
	T_0	T_6	T_{61}	T_{120}	T_{150}
Rinogermina group	44%	10%	24%	12%	28%
Placebo group	46.8%	10.6%	12.8%	6.4%	36.1%

Table 8

Regarding *S.pyogenes*, its presence was lower in almost all samples belonging to the rinogermina group compared with the placebo group **(Table 9)**.

	S.pyogenes				
	T_0	T_6	T_{61}	T_{120}	T_{150}
Rinogermina group	2%	6%	10%	6%	8%
Placebo group	4.3%	8.5%	8.5%	19.1%	10.6%

Table 9

In addition, in all samples, the presence of *H.influenzae* and *M.catarrhalis*, other causative bacterial strains of OM, was not demonstrated with significant differences in the two groups of children (rinogermina and placebo groups) enrolled in the study **(Tables 10, 11)**. This result is in agreement with the antagonist ability of *S.salivarius* 24SMBc which is not able to inhibit the growth of *H.influenzae* and *M.catarrhalis*.

	H.influenzae				
	T_0	T_6	T_{61}	T_{120}	T_{150}
Rinogermina group	4%	4%	6%	4%	0%
Placebo group	8.5%	0%	4.3%	14.9%	2.1%

Table 10

	M.catarrhalis				
	T_0	T_6	T_{61}	T_{120}	T_{150}
Rinogermina group	2%	0%	0%	0%	0%
Placebo group	0%	0%	0%	2.1%	0%

Table 11

DISCUSSION

The beneficial role of bacterial interference in infections of the URT has been largely attributed to the presence of normal flora in the nasopharynx, mainly α-haemolytic and non-haemolytic streptococci **[56, 57]**. The use of oral probiotics has become a realistic prophylactic strategy for many inflammatory diseases and infections. To date, several clinical trials with humans have demonstrated the probiotic properties of commensal bacteria in the oral cavity in the reduction of URT infections.

The probiotic potential of the *S.salivarius* species stemmed from (i) its numerical predominance in the oropharynx, (ii) the capability to produce diverse anti-competitor molecules and (iii) demonstrations of its beneficial application for the relief or control of various upper respiratory tract ailments, led our research group to study an interesting strain: *S.salivarius* 24SMBc. This bacterial strain showed antagonist activity against some representative pathogens of OM, *S.pneumoniae* and *S.pyogenes*, and a good capability to adhere to human epithelial cells **[38]**.

The sequencing of its genome showed that *S.salivarius* 24SMBc is free of streptococcal virulent factors and allowed us to detect a *blp* (bacteriocin-like protein) locus with a specific nucleotide sequence that discriminates our strain from other strains of *S.salivarius*.

The clinical trial protocol conducted on healthy adult volunteers (in elaboration) showed that *S.salivarius* 24SMBc is tolerated by the human host since no adverse events were observed. Definitively, the proportion of volunteers colonized by *S.salivarius* 24SMBc until the 7^{th} day, after nasal spray administration, was 8 out of 17 (~ 47%) and this level of colonization may be increased with a better antibiotic pre-treatment to reduce the pre-existent bacteria of the oral microbiota. Indeed, the presence of a cefixoral-resistent *S.aureus* strain in one sample (patient 001) is associated with a brief persistence of *S.salivarius* 24SMBc demonstrating that probably contaminant strains can interfere with colonization by probiotics. It is well known that an appropriate antibiotic pre-treatment is important when oral probiotics are assessed for their efficacy in adhering to target tissue and competing with other bacteria **[57]**. However, our strain showed a good capability to adhere to host tissues if we compare its level of colonization with that of *S.salivarius* K12. This strain of *S.salivarius* is a model of an oral probiotic which in a preliminary study to assay its

binding efficacy to human epithelial cells, showed a level of colonization of 33% **[93]**.

The main aim of this study was to evaluate the clinical evidence of a probiotic application of *S.salivarius* 24SMBc for the prevention or reduction of recurrent OM in children.

In the paediatric randomized, placebo-controlled, double-blind trial that involved 120 children, no episodes of OM were seen in 15 out of the 50 children treated with the probiotic spray (rinogermina group) and in 7 out of the 47 children treated with placebo. This result underlined a protection role of our strain against OM with about 50% efficacy compared with the placebo.

Moreover, 8 out of the 15 children (rinogermina group) that didn't manifest any episodes of OM were colonized by *S.salivarius* 24SMBc, 1 patient until T_6, 1 patient until T_{61} and the other 6 until T_{150}. Overall, 21 out of the 50 children treated with rinogermina showed the presence of our probiotic strain, in particular, at time interval T_{150} when we could see a decreased presence of *S.pneumoniae*. Regarding the other pathogens of OM, only *S.pyogenes* was prevalent in the placebo group while the presence of *H.influenzae* and *M.catarrhalis* was similar in the two groups of children (rinogermina and placebo groups).

This clinical study highlights that *S.salivarius* 24SMBc has the key characteristics of an oral probiotic that can be administrated to children for prevention and treatment of OM: (i) absence of virulence determinants, (ii) colonization capability, and (iii) the ability to compete with pathogens such as *S.pneumoniae* and *S.pyogenes*.

Moreover, *S.salivarius* 24SMB was able to reduce episodes of OM without permanently colonizing the site. Probably, it is a consequence of its modulation of the host's immune system and the next endpoint of our research will be to examine this aspect.

In this setting, bacteriotherapy has the potential of treating diseases in a natural way, aiming at infection prevention using beneficial bacteria that can restore or increase the microfloral biodiversity as well as interfering with potential pathogens.

For this reason, the use of bacteriotherapy and its application is in continuous development encompassing different areas of human health. Nowadays the use of oral cavity probiotics is still a relatively undeveloped area, but it is steadily becoming an interesting approach for prevention and therapy, especially for pediatric patients.

7. REFERENCES

1. Pradeep K, Kuttappa MA, Prasana KR (2014) Probiotics and oral health: an update. SADJ **69**:20-24
2. Pasteur L, Joubert JF (1877) Charbon et septicèmie. C. R. Soc. Biol. Paris **85**: 101-115
3. Metchnikoff E (1908) Prolongation of life: optimistic studies. Putnam,New York (translate by Mitchell PC)
4. Lilly DM, Stillwell RH (1965) Probiotics: growth-promoting factors produced by microoganisms. Science **147**:747-748
5. FAO/WHO (2002) Report of a joint FAO/WHO expert consultation on guidelines for the evaluation of probiotics in food. World Health Organization and Food and Agriculture Organization of the United Nations, London Ontario, Canada.
6. Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr. **125**:1401-1412.
7. Florey H.W. (1946) The use of micro-organisms for therapeutic purposes. Yale J. Biol. Med **19**:101-117
8. Tagg John R, Dierksen Karen P (2003) Bacterial replacement therapy: adapting 'germ warfare' to infection prevention. ELSEVIER. **21**:217-223
9. Rajendhran J, Gunasekaran P (2010) Human microbiomics. Indian J Microbiol **50**:109-112
10. Popova M, Molimard P, Courau S, Crociani J, Dufour C, Le Vacon F. and Carton T (2012) Beneficial effects of probiotics in upper respiratory tract infections and their mechanical actions to antagonize pathogens. Journal of Applied Microbiology **113**:1305-1318
11. Gordon DM, Oliver E and Littlefield-Wyer J: The Diversity of Bacteriocins in Gram-Negative Bacteria. In: Bacteriocins-*Ecology and Evolution*. Riley MA, Chavan MA (2007) (Eds). Springer-Verlag, Berlin, Germany, 5-17
12. Grazia A (1925) Sur un remarquable exemple d'antagonisme entre deux souches de coilbacille. Comp Rend Soc Bio **93**:1040-1041
13. Fredericq P (1946) Sur la pluralitè des recepteurs d'antibiose de E.coli. CR Soc Bio (Paris) **140**:1189-1194

14. Heng NCK, Wescombe PA, Burton JP, Jack RW and Tagg J R. (2007) The Diversity of Bacteriocins in Gram-Positive Bacteria. In: Bacteriocins-Ecology and Evolution. Riley MA, Chavan MA (Eds). Springer-Verlag, Berlin, Germany, 45-92
15. Jacob F, Lwoff A, Siminovitch A, Wollman E (1953) Dèfinition de quelques termes relatifs à la lysogènie. Ann. Inst. Pasteur (Paris) **84**:222-224
16. Klaenhammer TR (1988) Bacteriocins of lactic acid bacteria. Biochimie **70**: 337-349
17. Riley MA, Wertz JE (2002) Bacteriocins: evolution, ecology and application. Annu Rew Microbiol **56**:117-137
18. Cascales E, Buchanam SK, Duche D, Kleanthous C, Lloubles R, Postle K, Riley M, Slatin S, Cavard D (2007) Colicin biology. Microbiol Mol Biol Rev **71**:158-229
19. Pugsley AP (1984) The ins and outs of colicins. Part I. Production, and translocation across membranes. Microbiol Sci **1**:168-175
20. Riley MA (2009) Bacteriocins, Biology, Ecology, and Evolution. Encyclopedia of Microbiology. (Moselio Schaechter, Editor), pp. 32-44 Oxford: Elsevier.
21. Mulec J, Podlesek Z, Mrak P, Kopitar A, Ihan A, Zgur-Bertok D (2003) A cka-gfp transcriptional fusion reveals that the colicin K activity gene is induced in only 3 percent of the population. J Bacteriol **185**:654-659
22. Moreno F, Gonzalez-Pastor JE, Baquero MR, Bravo D (2002) The regulation of microcin B, C and J operons. Biochimie **84**:521-529
23. Braun V, Pilsl H, Gross P (1994) Colicins: structures, modes of action, transfer through membranes, and evolution. Arch Microbiol **161**:199-206
24. Strauch E, Kaspar H, Schaudinn C, Damasko C, Konietzny A, Dersch P, Skurnik M, Appel B (2003) Analysis of enterocoliticin, a phage tail-like bacteriocin. Adv Exp Med Biol **529**: 249-251
25. Nakayama K, Takashima K, Ishihara H, Shinomiya T, Kageyama M, Kanaya S, Ohnishi M, Murata T, Mori H, Hayashi T (2000) The R-type pyocin of Pseudomonas aeruginosa is related to P2 phage, and the F-type is related to lambda phage. Mol Microbiol **38**:213-231

26. Klaenhammer TR (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**:39-86
27. Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. Nature Rev Microbiol **3**:777-788
28. Jung G (1991) Lantibiotics: a survey. In: Jung G, Sahl H-G (eds) Nisin and novel lantibiotics. ESCOM, Leiden, pp 1-34
29. McAuliffe O, Ross RP, Hill C (2001b) Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol Rev **25**:285-308
30. Garneau S, Martin NI, Vederas JC (2002) Two-peptide bacteriocins produced by lactic acid bacteria. Biochimie **84**:577-592
31. Buchman GW1, Banerjee S, Hansen JN (1988) Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J Biol Chem. **263**(31): 16260-6.
32. Siegers K1, Heinzmann S, Entian KD (1996) Biosynthesis of lantibiotic nisin. Posttranslational modification of its prepeptide occurs at a multimeric membrane-associated lanthionine synthetase complex. J Biol Chem. **271**(21):12294-301
33. van der Meer JR1, Polman J, Beerthuyzen MM, Siezen RJ, Kuipers OP, De Vos WM (1993) Characterization of the Lactococcus lactis nisin A operon genes nisP, encoding a subtilisin-like serine protease involved in precursor processing, and nisR, encoding a regulatory protein involved in nisin biosynthesis. J Bacteriol. **175**(9):2578-88
34. O'Shea EF, Cotter PD, Stanton C, Ross RP, Hill C, (2012) Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: Bacteriocins and conjugated linoleic acid. International Journal of Food Microbiology **152**:189-205
35. Scarpellini E, Cazzato A, Lauritano C, Gabrielli M, Lupascu A, Gerardino L, Abenavoli L, Petruzzellis C, Gasbarrini G, Gasbarrini A (2008) Probiotics: which and when? Dig Dis **26**:175-182
36. Pineiro M, Stanton C (2007) Probiotic Bacteria: Legislative Framework-Requirements to Evidence Basis. The Journal of Nutrition, pp 850-853
37. Wescombe PA, Hale JD, Heng NC, Tagg JR. (2012) Developing oral probiotics from *Streptococcus salivarius*. Future Microbiol **7**(12):1355-1371

38. Santagati M, Scillato M, Patanè F, Aiello C, Stefani S (2012) Bacteriocin-producing oral streptococci and inhibition of respiratory pathogens. FEMS Immunol. Med. Microbiol. **65**(1):23-31.
39. Burton JP , Wescombe PA, Moore CJ, Chilcott CN and Tagg JR (2006) Safety Assessment of the Oral Cavity Probiotic *Streptococcus salivarius* K12. Appl. Environ. Microbiol. **72**: 3050-3053
40. Burton JP, Wescombe PA, and Tagg JR (2010) Extended safety data for the oral cavity probiotic *Streptococcus salivarius* K12. Probiot. Antimicrob. Protiens **2**: 135-144
41. Burton JP, Cowley S, Simon RR, Mckinney J, Wescombe PA, and Tagg JR (2011) Evaluation of safety and human tolerance of the oral cavity probiotic *Streptococcus salivarius* K12: a randomized, placebo- controlled, double-blind study. Food Chem. Toxicol. **49**: 2356-2364
42. Zarco MF, Vess TJ , Ginsburg GS (2012) The oral microbiome in health and disease and the potential impact on personalized dental medicine. Oral Diseases **18**: 109-120
43. Burton JP, Chilcott CN & Tagg JR (2005) The rationale and potential for the reduction of oral malodour using *Streptococcus salivarius* probiotics. Oral Dis **11**(Suppl 1): 29-31.
44. Lederberg J, Mccray AT (2001) "Ome sweet"omics-a genealogical treasury of words. Scientist **15**:8-10
45. Ling Z, Kong J, Jia P et al. (2010) Analysis of oral microbiota in children with dental caries by PCR-DDGE and barcoded pyrosequencing. Microb Ecol **60**:677-690
46. Hooper LV, Wong M H, Thelin A, Hansson L, Falk PG, and Gordon JI (2001) Molecular analysis of commensal host-microbial relationships in the intestine. Science **291**:881-884.
47. Mazmanian SK, Liu CH, Tzianabos AO, and Kasper DL (2005) An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell **122**:107-118.
48. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, Lakshmanan A & Wade WG (2010) The human oral microbiome. J Bacteriol **192**: 5002-5017.

49. Garcia RI, Henshaw MM, Krall EA (2001) Relationship between periodontal disease and systemic health. Periodontol 2000 **25**:21-36
50. Wenzel V, Idris AH, Banner MJ, Fuerst RS and Tucker K.J. (1994) The composition of was given by mouth-to mouth ventilation during CPRt. Chest **106**:1806-1810
51. Aas JA, Paster BJ, Stokes LN, Olsen I & Dewhirst FE (2005) Defining the normal bacterial flora of the oral cavity. J Clin Microbiol **43**: 5721–5732.
52. Zaura E, Keijser BJ, Huse SM, Crielaard W (2009) Defining the health "core microbiome" of oral microbial communities. BMC Microbiol **259**:12
53. Bik EM, Long CD, Armitage GC et al (2010) Bacterial diversity in the oral cavity of ten healthy individuals. ISMEJ **154**:830-837
54. Parahitiyawa NB, Scully C, Leung WK, Jin LJ, Samaranayake LP (2010) Exploring the oral bacterial flora: current status and future directions. Oral Dis. **16**:10
55. Sanders CC, Sanders WE Jr. (1982) Enocin: an antibiotic produced by *Streptococcus salivarius* that may contribute to protection against infections due to group A streptococci. J Infect Dis. **146**(5):683-90.
56. Roos K, Hakansson EG & Holm S. (2001) Effect of recolonisation with 'interfering' alpha streptococci on recurrences of acute and secretory otitis media in children: randomised placebo controlled trial. BMJ **322**: 210-212.
57. Tano K, Grahn-Hakansson E, Holm SE, Hellstrom S (2002) A nasal spray with alpha-Haemolitic streptococci as long term prophylaxis against recurrent otitis media. International Journal of Pediatric Otorhinolaryngology. **62**(1):17-23
58. Favier CF, Vaughan EE, De Vos WM, Akkermans AD (2002) Molecular monitoring of succession of bacterial communities in human neonates. Appl. Environ. Microbiol. **68**:219-226
59. Tagg JR, Pybus V et al (1983) Application of inhibitor typing in a study of the transmission and retention in the human mouth of the bacterium *Streptococcus salivarius*. Arch Oral Biol **28**(10):911– 915
60. Wescombe PA, Heng NCK, Burton JP and Tagg JR (2010) Something Old and Something New: An Update on the Amazing Repertoire of Bacteriocins Produced by *Streptococcus salivarius*. Probiotics & Antimicro. Prot. **2**:37-45

61. Nobbs AH, Lamont RJ and Jenkinson HF (2009) *Streptococcus* adherence and colonization. Microbiol. Mol. Bio. Rev. **73**:407-450
62. Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson MW, Hale JD and Wescombe PA (2013) Influence of the probiotic *Streptococcus salivarius* strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. Journal of Medical Microbiology **62**:875-884
63. Afek S, Sperber AD, and Almog Y (2004) Carcinoma of the colon presenting as *Streptococcus salivarius* sepsis. J. Clin. Gastroenterol. **38**:86–87
64. Corredoira, JC, Alonso MP, Garcia JF, Casariego E, Coira A, Rodriguez A, Pita J, Louzao C, Pombo B, Lopez MJ, and Varela J (2005) Clinical characteristics and significance of *Streptococcus salivarius* bacteremia and *Streptococcus bovis* bacteremia: a prospective 16-year study. Eur. J. Clin. Microbiol. Infect. Dis. **24**:250–255.
65. EFSA (2005) Opinion of the Scientific Committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microrganisms used in food/feed and the production of food/feed additives. EFSA J **226**:1-12
66. Food and Drug Administration (2005) Agency Response letter GRAS Notice No.GRN 000171.US. Department of Health and Human Services. Available at http://www.cfsan.fda.gov/ ~rdb/opa-g171.htm.
67. Facklam R (2002) What happed to the streptococci: overview of taxonomic and nomenclature changes. Clin Microbiol Rev **15**: 613–630.
68. Mora D, Ricci G, Guglielmetti S, Daffonchio D & Fortina MG (2003) 16S–23S rRNA intergenic spacer region sequence variation in *Streptococcus thermophilus* and related dairy streptococci and development of a multiplex ITS-SSCP analysis for their identification. Microbiology **149**(Pt 3): 807–813.
69. Wescombe PA, Upton M, Dierksen K P, Ragland NL, Sivabalan S, Wirawan RE, Inglis MA, Moore CJ, Walker GV, Chilcott CN, Jenkinson HF, and Tagg JR (2006) Production of the lantibiotic salivaricin A and its variants by oral streptococci and use of a specific induction assay to detect their presence in human saliva. Applied and Environmental Microbiology. **72**(2):1459-1466
70. Hyink O, Wescombe PA et al (2007) Salivaricin A2 and the novel lantibiotic salivaricin B are encoded at adjacent loci on a 190- kilobase transmissible

megaplasmid in the oral probiotic strain *Streptococcus salivarius* K12. Appl Environ Microbiol **73**(4): 1107-1113

71. Wescombe PA (2002) Characterisation of lantibiotics produced by *Streptococcus salivarius* and *Streptococcus pyogenes*. Department of Microbiology and Immunology. Dunedin, University of Otago:324
72. Upton M, Tagg JR, Wescombe P, Jenkinson HF (2001) Intra-and interspecies signaling between *Streptococcus salivarius* and *Streptococcus pyogenes* mediated by SalA and SalA1 lantibiotic peptides. Journal of Bacteriology **183**:3931–3938
73. Tanzer JM, Kurasz AB, Clive J (1985) Inhibition of ecological emergence of mutans streptococci naturally transmitted between rats and consequent caries inhibition by *Streptococcus salivarius* TOVE-R infection. Infect. Immun. **49**(1):76-83
74. Tanzer JM, Kurasz AB, Clive J (1985) Competitive displacement of mutans streptococci and inhibition of tooth decay by *Streptococcus salivarius* TOVE-R. Infect. Immun. **48**:44-50
75. Guglielmetti S, Taverniti V, Minuzzo M et al. (2010) Oral bacteria as potential probiotic for the pharyngeal mucosa. Appl. Environ. Microbiol. **76**:3948-3958
76. Burton JP, Chilcott CN, Moore CJ, Speiser G, Tagg JR (2006) A preliminary study of the effect of the probiotic *Streptococcus salivarius* K12 on oral malodour parameters. Appl. Environ. Microbiol. **100**:754-764
77. Ishijima SA, Hayama k, Burton JP, Tagg JR (2012) Effect of *Streptococcus salivarius* K12 on the in vitro grown of *Candida albicans* and its protective effect in an oral candidiasis model. Appl. Environ. Microbiol. **78**:2190-2199
78. Adam E, Jindal M, Seney Set al. (2011) *Streptococcus salivarius* K12 and M18 Probiotics Reduce Periodontal Pathogen-induced Inflammation (IADR Paper 150126). American Association, Alexandria, VA, USA
79. Santagati M, Spanu T, Scillato M, Santangelo R, Cavallaro F, Arena V, Castiglione G, Falcone M, Venditti M and Stefani S (2014) Rapidly Fatal Hemorrhagic Pneumonia and Group A *Streptococcus* Serotype M1. Emerging Infectious Diseases **20**:98-101

80. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, et al. (2008) The RAST server: Rapid annotations using subsystems technology. BMC Genomics 9.
81. Fontaine L, Boutry C, Guédon E, Guillot A, Ibrahim M, Grossiord B and Hols P (2007) Quorum-Sensing Regulation of the Production of Blp Bacteriocins in *Streptococcus thermophilus*. J. Bacteriol. **189**(20):7195
82. Renye JA Jr1, Somkuti GA. (2013) BlpC-regulated bacteriocin production in *Streptococcus thermophilus*. Biotechnol Lett. **35**(3):407-12
83. Tagg, JR & Bannister, LV (1979) 'Fingerprinting' beta-haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. J Med Microbiol **12**: 397-411.
84. Pitcher DG, Saunders NA and Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidine thiocyanate. Lett Appl Microbial **8**:151-156
85. Truong TL, Me´Nard C, Mouton C and Trahan L (2000), Identification of mutans and other oral streptococci by random amplified polymorphic DNA analysis J. Med. Microbiol **49:** 63-71
86. Lee HY, Andalibi A, Webster P, Moon SK, Teufert K, Kang SH, Li JD, Nagura M, Ganz T, Lim DJ (2004) Antimicrobial activity of innate immune molecules against *Streptococcus pneumoniae*, *Moraxella catarrhalis* and nontypeable *Haemophilus influenzae*. BMC Infect Dis **4**:12
87. Rovers MM, Schilder AG, Zielhuis GA, Rosenfeld RM (2004) Otitis media. Lancet **363**(9407):465-473
88. Marom T, Nokso-Koivisto J, Chonmaitree T (2012) Viral- bacterial interactions in acute otitis media. Curr Allergy Asthma Rep **12**(6):551–558
89. Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, Morris PS, Leach AJ (2006) Measuring nasal bacterial load and its association with otitis media. BMC Ear Nose Throat Disord **6**:10
90. Bogaert D, de Groot R, Hermans PWM (2004) *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis **4**(3):144-154
91. Kadioglu A, Weiser JN, Paton JC, Andrew PW (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rev Microbiol **6**(4):288-301

92. Cole PJ (1986) Inflammation: a two-edged sword-the model of bronchiectasis. Eur J Respir Dis Suppl **147**:6-15
93. Power DA, Burton JP, Chilcott CN, Dawes PJ, Tagg JR (2008) Preliminary investigation of the colonization of upper respiratory tract tissue of infants using a paediatric formulation of the oral probiotic Streptococcus salivarius K12. Eur. J. Clin. Microbiol. Infect. Dis. **27**:1261-1263