Effects of oral streptococci and selected probiotic bacteria on the pathogen *Streptococcus pyogenes*: viability, biofilms, molecular functions, and virulence traits

Dissertation

zur Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat)
der Mathematisch-Naturwissenschaftlichen Fakultät
der Univesität Rostock

vorgelegt von
Catur Riani
geb. am 13.08.1976 auf Pulau Sambu
aus Indonesien

Rostock, Januar 2009

urn:nbn:de:gbv:28-diss2009-0087-1

Prof. Johannes Knobloch
(Gutachter / Reviewer)
Universitätsklinikum Schleswig-Holstein
Campus Lübeck Ratzeburger Allee 160
23538 Lübeck

Prof. Dr. Hubert Bahl
(Gutachter / Reviewer)
Uni Rostock
Institut für Biologie
Albert Einstein Str. 3
18059 Rostock

Prof. Dr. Regine Hakenbeck
(Gutachter / Reviewer)
Technische Universität Kaiserslautern
FB Biologie
P.-Ehrlich-Str.
67663 Kaiserslautern

Prof. Dr. Andreas Podbielski
(Gutachter & Betreuer / Reviewer & Supervisor)
Uni Rostock
Medizin
Abt. für Medizinische Mikrobiologie, Virologie und Hygiene
Schillingalle 70
18057 Rostock

Abgabedatum / date of submission: 30 Januar 2009

Verteidigungsdatum / date of defence: 4 Mai 2009

"Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes"

Table of content

Abbreviations

aad9	resistance gene for spectinomycin
ATCC	American Type Culture Collection
aqua dest.	aqua destillata
bar	pressure unit
BHI	Brain Heart Infusion
bp	base pair
BSA	bovine serume albumin
C	Coulomb, international unit for electric charge
°C	Celcius centigrade
$CaCl_2$	calciumchloride
cfu	colony forming unit
CLSM	Confocal Laser Scanning Microscopy
CO_2	carbondioxide
Col	collagen
cpa	gene encoding collagen binding protein
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTP	dideoxynucleosidetriphosphate
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EDTA	ethylene diamine tetraacetic acid
emm	gene encoding M protein
EPS	exopolysaccharide
EtBr	ethidiumbromide
fbp	gene encoding fibronectin binding protein
FCS	fetal calf serum
g/l	gram per liter
GAS	Group A *Streptococcus*
h	hour
HCl	hydrochloride

HEp-2 cell	human epithelial cell
Ig	immunoglobulin
isp	gene encoding immunogenic secreted protein
IVT	*in vitro* transcription
kb	kilo base pair
KCl	kalium chloride
kDa	kilodalton
kV	kiloVolt
l	liter
LB	Luria Bertani
luc	luciferase
M	molarity
mf	gene encoding mitogenic factor
Mga	multiple gene regulator of GAS
min	minute
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
ms	millisecond
MSCRAMM	microbial surface components recognizing adhesive matrix molecules
NaCl	natriumchloride
NaOH	natriumhydroxide
nm	nanometer
O_2	oxygen
ON	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pen/Strep	Penicillin/Streptomycin
pH	power of hydrogen
QS	Quorum Sensing
R	electrical resistance
RLU	Relative Light Unit
RNA	ribonucleic acid
rpm	revolutions per minute

RT	room temperature
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. mitis	*Streptococcus mitis*
S. mutans	*Streptococcus mutans*
S. oralis	*Streptococcus oralis*
S. parasangunis	*Streptococcus parasangunis*
S. pyogenes	*Streptococcus pyogenes*
S. salivarius	*Streptococcus salivarius*
*sag*A	gene encoding SLS
Sal	salivaricin
SEM	Scanning Electron Microscopy
ska	gene encoding streptokinase
SLO	streptolysin O
SLS	streptolysin S
speB	gene encoding streptococcal pyrogenic exotoxin
TAE	Tris Acetate EDTA buffer
TE	Tris-EDTA buffer
THB	Todd Hewitt Broth
THY	Todd Hewitt Yeast
Tris	tris (hydroxymethylaminomethane)
Tween 20	Polyoxyethylene sorbital monolaurate
U/mg	Units per milligram
UV	ultraviolet
V	volt
wt	wild type
µ	micro (10^{-6})
µF	micro-Farad, unit for capacitance
Ω	ohm, international unit for electrical impedance

I. Introduction

I.1 *Streptococcus pyogenes* as a human pathogen

Bacteria of the species *Streptococcus pyogenes* belong to the major agents causing purulent infections in humans. *S. pyogenes*-associated infections comprise frequent respiratory tract and skin diseases such as tonsillitis, pharyngitis, and pyoderma as well as occasional invasive diseases involving all organs and most tissue types of the human body (Cunningham, 2000; Rosenbach, 1884). From a phylogenetic standpoint, this is remarkable, because *S. pyogenes* is a member of the lactic acid bacteria (Friedemann, 1938), i.e., Gram-positive cocci which can not produce heme to generate energy-rich substances by the oxidative chain but exclusively by fermenting sugars to lactic acid. The majority of the lactic acid bacteria is not only non-pathogenic for humans but in addition, the basis for processing all kinds of human nutrition in terms of edibility and/or taste.

Like other streptococci, *S. pyogenes* is nutritionally fastidious, i.e., auxotrophic for several amino acids and complex substances. Also, it does not produce catalase and therefore, decreases the amount of intracellular oxygen radicals only by the activity of superoxide dismutase and transport of H_2O_2 to the extracellular environment.

S. pyogenes differs from other streptococci by growing in long chains due to cell division in a single plane and incomplete separation of the daughter cells. Its cell wall contains N-acetyl-β-D-glucosamine, which can be detected by specific antibodies and was used for its assignment to the serologic group A streptococci (Lancefield, 1933). Therefore, *S. pyogenes* is frequently addressed as group A streptococci (GAS). In addition to exported oxygen radicals in the form of H_2O_2, *S. pyogenes* secretes toxins and proteinases into its environment. If the growth medium contains full blood and thus, erythrocytes, the former compound transforms hemoglobin into methemoglobin, the latter completely lyse the erythrocytes and degrade the contained hemoglobin. When grown on a blood-containing semi-solid agar, methemoglobin formation in the vicinity of streptococcal colonies leads to a greenish color of the agar, while complete hemolysis results in clear zones around the colonies. The latter feature is typical for *S. pyogenes* colonies and was termed β-hemolysis by Smith & Brown (1915).

Like most other pathogenic bacteria, *S. pyogenes* produces a vast panel of virulence factors. The production of these factors is tuned by an interaction with the micro- and macro-environment of the bacteria, i.e., exclusively the human host and its diverse anatomical sites. Therefore, only a subset of virulence factors is expressed at a given time point according to specific needs of the individual bacteria and the current environmental conditions resulting in

maximum energy efficiency and least exposure to host defense mechanisms. This delicate balance is achieved by a regulatory network involving sensor molecules for specific chemical compounds and physical conditions as well as directly and indirectly associated regulator molecules (Kreikemeyer *et al*., 2003).

The virulence factors of pathogenic bacteria can be grouped according to their functions (Henderson *et al*., 1996). Like physiological bacteria, pathogenic bacteria need to firmly bind to a given anatomical site to exert further activities. This is achieved by adhesins. Unlike physiological bacteria, pathogenic bacteria then produce aggressins which enable them to lyse and digest their environment for their own sustenance. To gain access to other environments with potentially more thriving conditions, pathogenic bacteria produce invasins, which support internalization into host cells or generate breaches in host tissues allowing entry into deeper seated tissues and blood or lymphatic vessels. Since an immunocompetent host will not tolerate such activities and will fight invading pathogens by means of the innate adaptive immune system, pathogenic bacteria can either block such activities by inhibins or divert the activities by modulins in such a direction that they will hurt the host rather than the bacteria. The *S. pyogenes* genome, now completely sequenced for more than a dozen different strains, encodes several factors in each of the above mentioned classes adding up to overall more than 50 predominantly proteinaceous factors currently identified by experimental approaches (Olsen *et al*., 2008).

Examples of *S. pyogenes* adhesins are several fibronectin- and at least one collagen-binding surface proteins (e.g., protein F1, protein F2, and Cpa, respectively). To the class of aggressins belong secreted proteases (e.g., cystein protease SpeB) and membrane lytic factors such as the hemolysins streptolysin S (SLS) and O (SLO). Tissue is invaded by the action of Ska streptokinase and plasminogen binding protein, which is redirect human proteases to digest human intercellular matrix substances, as well as by the bacterial phospholipase A2 and SpyCEP protease. The phagocytic activity of granulocytes and macrophages is blocked by the inhibins (i.e., capsule proteoglycan and M surface protein) and complement activity by ScpA, a C5a protease. Host defense mechanisms are finally changed by modulins such as the SpeA, SpeC, SpeF, and MF superantigens as well as the human leukocyte β2-integrin homologue Mac1/EndoS (Cunningham, 2000; Hynes, 2004; Olsen *et al*., 2008).

The *S. pyogenes* M protein is peculiar since it has been identified before more than 70 years to be one of the determinants for invasive *S. pyogenes* infections because of its major contribution to phagocytosis resistance (Horstmann *et al*., 1988). Because of the M protein's importance for the bacterial virulence and its frequent exposure to antibodies, the resulting

selective pressure led to a high sequence variability of the predominantly exposed N-terminal section of the M protein sequence. This variability and the availability of specific antibodies have been used for a serologic typing system which identifies about 80 different M types among *S. pyogenes* isolates (Lancefield, 1928). Since the publication of the first M protein encoding *emm* gene sequences (Hollingshead *et al*., 1986), the typing scheme was changed to nucleic acid sequencing of the 5' ends of *emm* genes, now differentiating several hundred diverse *emm* types (Beall *et al*., 1996; Facklam *et al*., 1999).

As a complex potential virulence mechanism of *S. pyogenes* involving several of the above mentioned virulence factors such as fibronectin-binding proteins and proteases, the bacteria can internalize into eukaryotic cells, e.g., various epithelial and endothelial cells as well as macrophages, without being killed in phagocytic vacuoles or in turn, killing its host cell by its toxins (Wang *et al*., 2006). This feature apparently enables the bacteria to circumvent defense mechanisms from the innate and adaptive host defense and as a consequence to persist for extended periods in the specific human host. The persisting bacteria can leave their "safe haven" upon so far undefined stimuli and lead to recurrent infections.

Another complex mechanism of *S. pyogenes* - biofilm formation - also involves several virulence factors such as M protein and capsule plus many other so far ill defined compounds. The existence of *S. pyogenes* mono-species biofilms was shown by several *in vitro* experiments (Hirota *et al*., 1998; Takemura *et al*., 2004). A biofilm is defined as a layer of pioneer bacteria firmly attached to a solid support and several to many layers of bacteria bound to the pioneer bacteria. In between the bacterial layers, intercellular matrix substance both produced by the bacteria and absorbed from the environment contribute to interbacterial binding and to stabilization of the three dimensional structure of the biofilm. Formation and maturation of a biofilm is a stepwise process, which comprises attachment, microcolony formation, production of extracellular matrix, binding of secondary colonizers from the planktonic environment, and detachment of single cells or cell packages from the mature biofilm (Costerton, 1999; Watnick & Kolter, 2000; Stoodley *et al*., 2002, Hall-Stoodley *et al*., 2004). Opposed to other streptococcal species such as *S. mutans*, little molecular details are known concerning the factors of *S. pyogenes* biofilm maturation such as the adhesins facilitating interbacterial binding or the extracellular substance involved in structuring the *S. pyogenes* biofilm. At least in serotype M1 *S. pyogenes* strains, microcolony formation seems to involve M (-like) proteins (Frick *et al*., 2000; Rickard *et al*., 2003; Cho & Caparon, 2005), while formation of full-sized biofilms depends on the presence of pili-structures on the streptococcal surface (Manetti *et al*., 2007). The importance of extracellular proteins for

biofilm formation was underlined by demonstrating negative effects of the secreted cysteine protease SpeB on the accumulation of biofilm. Consistently, Srv, a negative regulator for SpeB production, was found to affect biofilm mass. At least at initial steps, extracellular DNA is also involved in biofilm formation (Doern *et al*., 2009). In the minority of *S. pyogenes* strains which harbor the genes for the *sil* signaling locus, this interbacterial communication apparently contributes to biofilm building (Lembke *et al*., 2006).

The *in vivo* formation of monospecies *S. pyogenes* biofilms was demonstrated in the affected epidermis during uncomplicated skin infections (Akiyama *et al*., 2003). At this anatomical site, the trait could contribute to evasion from host defense and/or to long term persistence of the bacteria in the individual human host. The presence of *S. pyogenes* biofilms during other types of infections or at other sites has not conclusively been demonstrated so far, although *S. pyogenes*-containing structures described as polymicrobial lawns were visualized on the epithelial surfaces of tonsillar fissures (Swidsinski *et al*., 2007).

I.2 The physiological microflora of the upper respiratory tract

S. pyogenes is transmitted between two humans by direct contact or by contaminated airborne droplets. Therefore, the skin and the mouth are its first contact sites on new human hosts (Fiorentino *et al*., 1997). Before reaching its epithelial target cells in the upper respiratory tract, precisely in the pharynx and on the tonsils, *S. pyogenes* has to pass the oral cavity. The whole anatomical space is physiologically inhabited by several hundred different bacterial species (Aas *et al*., 2005; Paster *et al*., 2001; Moore & Moore, 1994; Kolenbrander, 2000).

Depending on the unspecific defense mechanisms of the involved surfaces, i.e., saliva/mucus production and directed flow of the produced fluids as well as rapid turnover of the top cell layer, the physiologic bacteria live as single planktonic cells in the saliva and sulcus fluids or are organized in mixed species biofilms directly on the eukaryotic cell surface. Alternatively, biofilm can develop on the diet-derived microparticles captured in the viscous oral fluids or on mucin macromolecules in the periciliary layer of the airway surface liquid, which is replenished less rapidly than the mucus layer. Biofilms directly on eukaryotic surfaces of the upper respiratory tract are commonly encountered on the teeth, the subgingival crevice, the areas around the ducts of the salivary glands, the rear third of the tongue dorsum, and the crypts of the tonsils (Stenfors & Raisanen, 1991; Chole & Faddis, 2003). These biofilms initially form on the inert enamel or on non-ciliated epithelial cells. Once the layer of ciliated cells is damaged, biofilm formation drastically increases (Wilson, 2005).

The single species of the physiologic microflora from the upper respiratory tract also differ with respect to their anatomical microenvironment. In addition, only 20 to 50% of the species at a given site can be demonstrated by the means of culture systems, the remainder is only found by molecular techniques (Wade, 2002). However, at any site of the upper respiratory tract streptococci comprise at least half of the absolute bacterial numbers detected there (Carlsson, 1967; Liljemark & Bloomquist, 1996). Among the more than 50 currently known streptococcal species, *S. mitis*, *S. oralis*, *S. parasanguinis*, and *S. salivarius* predominate in varying constellations both in the planktonic and biofilm-organized fractions at the respective sites. These species belong to the invalid taxon of "viridans streptococci", i.e., streptococci growing in pairs or short chains and with methemoglobin-formation (β-hemolytic) or without changes on blood agar. The valid taxonomy assigns them to the *S. mitis*-, *S. sanguinis*- and *S. salivarius*-groups of streptococci (Coykendall, 1989; Facklam, 2002)

Less frequently, other streptococci, Gram-positive aerobic and anaerobic rods (e.g., *Actinomyces* spp., *Eubacterium* spp., *Rothia* spp.), Gram-negative aerobic and anaerobic cocci (e.g., *Neisseria* spp., *Veillonella* spp.), and Gram-negative facultative aerobic rods (e.g., *Aggregatibacter* spp., *Capnocytophaga* spp., *Eikenella* spp., *Fusobacterium* spp., *Haemophilus* spp.) are isolated from the saliva or the oral/pharyngeal surfaces. Depending on the geographical region and the season, also *S. pyogenes* is part of the upper respiratory tract flora in up to 30% of apparently healthy persons, the so called asymptomatic carriers (Österlund & Engstrand, 1997). In these carriers, *S. pyogenes* is predominantly isolated from pharynx and hardly from the oral cavity (Fox *et al.*, 2006).

The species distribution and bacterial numbers in the human upper respiratory tract are determined by host factors as well as by bacterial interactions. Host factors with general effects are nutrient and fluids supply with more specific effects resulting from pH and constituents contained in a given diet (Wilkins *et al*., 2003). Food passage periods, the way of breathing (through nose or mouth) as well as the rate of saliva/sulcus fluid/airway surface liquid production are other general determinants (Rafay *et al*., 1996). As substances of the innate and acquired immune system, saliva contains mucins, lactoferrin, lysozyme, (lacto)-peroxidase, phospholipase A_2, several types of antibacterial peptides, and sIgA, which all interact with fractions of the local microflora. Similarly, gingival crevice fluid contains antibacterial peptides, nitric oxide, different Ig-classes, complement and leukocytes (Uitto, 2003; Pollanen *et al.,* 2003; Griffiths, 2003; Delima & Van Dyke, 2003; Uitto *et al.,* 2003; Elley & Cox, 2003; Ebersole, 2003).

Factors of interbacterial interactions can be differentiated into such with cooperative and others with antagonistic functions. Cooperativity is based on several components: first on interbacterial adhesins, which contribute to both mono and mixed species biofilm formation. Often bacteria of a single species within a mixed species biofilm form aggregates that are surrounded by other aggregates or by zones of mixed individual bacteria (Cisar *et al*., 1997; Clemans *et al*., 1999; Cook *et al*., 1998; Palmer *et al*., 2003; Park *et al*., 2005, Yoshida *et al*.; 2008). Second, some bacteria produce components for which other members of the local microflora are auxotrophic (Jakubovics *et al*., 2008; Egland *et al*., 2004). Often these components are waste products of the producers and not specifically produced to sustain phylogenetically unrelated neighboring bacteria. Third, other bacteria are engaged in degradation of toxic substances either secreted by their neighbors or presented from the environment. Such detoxification processes include the inactivation of antibiotic substances, resulting in resistance of whole microbial communities which also include basically susceptible members (Brook & Gilmore, 1993; Brook & Gober, 2006). Fourth, interbacterial communication via signaling molecules can induce functions in the signal receiving species which would not have been active when living as single species (Egland *et al*., 2004; Kuboniwa *et al*., 2006; Simionato *et al*., 2006).

Antagonism also relies on several pathways. First, competitors can simply be overgrown and eventually been lost by more efficient usage of nutrients and higher rates of multiplication. Second, unspecific adhesion of rivals can be inhibited by secretion of detergents. Third, specific adhesion of opponents at favorable sites can be blocked by either covering the target sites or by blocking/destroying the adhesins on the cell surfaces of the opponents. Fourth, undesired competitors can be killed by the controlled production and secretion of toxic factors. These molecules can be unspecifically active such as H_2O_2 or act via specific pathways (Kreth *et al*., 2008). The specific factors are commonly addressed as bacteriocins and often work by forming pores in the cell membranes, leading to osmolysis of the attacked bacteria. Production of toxin normally includes the simultaneous production of one or several immunity factors, which protect the producer against its own toxin (Wang & Kuramitsu, 2005). Fifth, the interbacterial communication of other players via signaling molecules can be disturbed. When refined to the own species, this communication is commonly addressed as quorum sensing, i.e., the constant measurement of a given population size in each member of this population. Many behavioral functions in bacteria, among them adhesion to certain targets, biofilm growth, and virulence to eukaryotic cells, rely on quorum sensing adapted

regulation. In most instances, the interbacterial signaling molecules diffuse into the environment of its producer and thus, can act on distant cells (Egland *et al*., 2004).

The host control of bacterial species and numbers should guarantee a sufficient and adequately mixed mass of beneficial microflora to support the digest process, to protect from unfavorable intruders, and to stimulate the local immune system to a baseline level of necessary activity. The interbacterial control of species and numbers should help to keep up a symbiotic system and to protect this system both from overwhelming host defense mechanisms and from intruders that will destroy the balanced state of mutual well being.

Thus, aggressive interbacterial measures such as bacteriocin production are often directed against close relatives, because these bacteria compete for the same adhesion targets and nutrients, i.e., the same ecologic niche. As an example for this statement, streptococci are frequent producers of bacteriocins, which selectively act on other streptococci (Brook, 2005; Kreth *et al*., 2008). Because of their common occurrence and their potential economic impact as natural preservatives of processed food as well as medical therapeutics, streptococcal bacteriocins and their producing strains have been quite well studied in the past years.

I.3 Streptococci and their value as upper airways probiotics

The unwanted presence of pathogenic bacteria on human surfaces or in the gastro-intestinal tract can be fought via different avenues. If the affected site is accessible from the exterior, antiseptics can be applied. For bacteria located inside the human body, antibiotics are used. Both classes of substances kill a broad range of bacteria, both pathogenic and beneficial species. In addition, they often harm exposed host cells. Their combined effects on the physiological microflora and on the physiology of host cells result in a lasting dysbalance of the human surface ecology which often primes the attack of new pathogens or facilitates the recurrence of a potentially incompletely eradicated initial pathogen.

To overcome these flaws, probiotic microorganisms can be administered subsequently (or sometimes even simultaneously) to the application of antibiotics. Generally, probiotics are defined as viable microorganisms which have a beneficial effect in the prevention or treatment of specific pathological conditions (Fuller, 1989). So far, a lot of information has been accumulated concerning the effects of probiotics in treating inflammatory or infectious bowel diseases. Here, the beneficial effects of *E. coli* Nissle, *Lactobacillus rhamnosus* or *Saccharomyces cerevisiae* for reducing the duration of single episodes or preventing recurrences has unambiguously been demonstrated in several prospective clinical studies (Mombelli & Gismondo, 2000).

In the upper respiratory tract, so far probiotics have only been used for preventing repeated episodes of specific bacterial infections, i.e., otitis media and tonsillopharyngitis. The used microorganisms were isolates from the order of lactic acid bacteria, either as single streptococcal strains or as a mixture of three different strains such as lactobacilli, streptococci and *Bifidobacterium* (Dierksen *et al*., 2007; Glück & Gebbers, 2003; Roos *et al*., 2001; Roos *et al*., 1993; Tano *et al*., 2000; Tano *et al*., 2002; Thomas *et al*., 2000; Falck *et al*., 1999; Roos *et al.,* 1993; Rods *et al*., 1996; Hatakka & Saxelin, 2008). Although the clinical outcome of some studies was promising, only two products became commercially available - the *S. salivarius* strain K12, which is sold as BLIS™ lonzettes in New Zealand (Walls *et al*., 2003), and freeze-dried powders of *Lactobacillus rhamnosus* plus *Bifidobacterium casei* and *Enterococcus faecalis* sold as Symbiolact compositum® and Symbioflor1®, respectively, in Germany (Habermann *et al*., 2001; Habermann *et al*., 2002; Rosenkranz & Grundmann, 1994).

Using probiotics has several aims. First, antibiotic- or antiseptic-derived void spaces on human surfaces are filled by robust but harmless bacteria until the resident microflora is reconstituted from unaffected niches. Second, independent from the application of antibiotics or antiseptics, the host defense mechanisms are stimulated or modulated in favorable ways to eradicate persisting pathogens or to better resist future exposures to pathogens. The stimulation relies at least to some extent on the toll-like receptor recognition of bacterial surface molecules such as lipoteichoic acid (Chan *et al*., 2007; Hasegawa *et al*., 2007). Third, again independent from other therapies, the probiotic microorganism can outcompete and permanently replace pathogens. This aim is addressed as replacement therapy and is not generally achieved by probiotics, which more often only temporarily reside on exterior or interior human surfaces (Power *et al*., 2008; Tagg & Dierksen, 2003). In fact, since probiotics themselves can cause infectious diseases in predisposed humans, it seems prudent that they should be eventually eliminated from the human body like other therapeutics. In any case, the safety of probiotics needs to be thoroughly documented before and while their usage in humans (Burton *et al*., 2006).

The best studied probiotic bacterium for treatment of upper airway discomforts and diseases such as halitosis (Burton *et al*., 2006a) and recurrent tonsillopharyngitis is *S. salivarius* K12, the key ingredient of the commercial product BLIS™. Its probiotic activity is obviously exerted by several mechanisms.

First, the strain produces ample amounts of at least two bacteriocins, salivaricin A2 and B. The corresponding genes are harbored in a 190 kb megaplasmid, which could be

demonstrated to be present in 31% of tested *S. salivarius* isolates. The 2.37 kDa and 2.74 kDa peptides are both post-translationally modified and contain three intramolecular cystein-di-sulfide bridges. Both features are characteristic for lantibiotic bacteriocins. Lantibiotics plus the associated modification, secretion and immunity proteins with amino acid sequence homologies between 85% and 40% are encountered in some *S. pyogenes* strains (streptococcin A-FF22) as well as in the genera/species *S. mutans*, *Lactococcus lactis*, and *Ruminococcus* sp. (Hyink *et al*., 2007). The salivaricins have been demonstrated to predominantly exert bacteriostatic functions against every *S. pyogenes* strain tested so far (Dempster & Tagg, 1982). Of note, administration of a salivaricin-producing strain into the oral cavity induces salivaricin expression in resident bacteria carrying the appropriate genes probably because immunity and production genes are linked within an operon (Upton *et al*., 2001; Wescombe *et al*., 2006). Also the salivaricin production appears to depend on sugar availability. For example, co-culturing 10^7 cfu/ml *S. salivarius* K12 with an equally sized inoculum of *S. sanguinis* reduced the cell numbers of the latter strain by five orders of magnitude when the THB culture broth contained 0.5% sucrose as opposed to pure THB which supported equal growth rates of both strains (Russel & Tagg, 1981).

Second, *S. salivarius* K12 downregulates the innate immune responses of human epithelial cells and thereby promotes the host-microbe homeostasis. Especially the baseline secretion of IL-8 and IL-8 or Groα responses to the simultaneous presence of other pathogenic bacteria or their flagella, respectively, are attenuated by the K12 strain. Simultaneously, the NF-κB pathway was inhibited by the probiotic (Cosseau *et al*., 2008).

Third, *S. salivarius* produces an urease which balances unfavorable acidic pH in the bacterial environment and simultaneously, provides nitrogen via a specific pathway. This urease is especially induced when the bacteria grow within biofilms probably because of the low pH and shortness of nitrogen in such a milieu (Chen *et al*., 2000; Li *et al*., 2000). In addition, the production of the urease is coupled to carbohydrate availability especially when lactose or galactose instead of glucose is offered to the bacterium. Obviously, the pH-sensitive carbohydrate-specific phosphotransferase system exerts an indirect regulatory effect on the transcription of the urease gene (Weaver *et al*., 2000).

Taken together, the three features provide *S. salivarius* K12 with all means that qualify a potent probiotic bacterium. At least in some assays, other strains of viridans streptococci have been demonstrated to exert similar effects as the K12 strain, although the molecular basis of these effects is much less well understood (Hasegawa *et al*., 2007; Kreth *et al*., 2008). Because of the potential commercial value, major food producing/processing companies are

currently screening their own collections of oral bacteria trying to identify probiotic viridans streptococcal strains with promising qualities for stabilizing the health of the upper respiratory tract.

I.4 Aims of the present study

In order to establish itself as pathogen for acute and recurrent infections, *S. pyogenes* has to pass layers of resident microflora to gain access to its final target, the epithelial cells of upper human respiratory tract. The concept of an intimate and lasting interaction with the eukaryotic cells is fully accepted by the scientific community. Little efforts have so far been invested in understanding what is happening to *S. pyogenes* on its way to the final destination.

Recent *ex vivo* transcriptome analysis suggests that contact to saliva leads to upregulation of many key genes in *S. pyogenes*, thus defining this contact as the initial stage of host-pathogen interactions (Shelburne *et al*., 2005). Next, *S. pyogenes* obviously multiplies on the surface of epithelial cells before causing clinical symptoms, indicating that the bacteria obtain nutrients from other sources than lysed eukaryotic cells (Virtaneva *et al*., 2005). It is clear that the glucose content in the vicinity of the eukaryotic cells is too low to support *S. pyogenes* proliferation (Gough *et al*., 1996). Therefore, *S. pyogenes* has to use other sources which supply predominantly maltose. This compound could be derived via α-amylase degradation of salivary maltodextrins (Shelburne *et al*., 2008) or could be provided by activities of the resident microflora. At the same time, this microflora will produce unspecific and specific factors that interfere with the presence of *S. pyogenes*. Since both *S. pyogenes* and viridans streptococci have been found to form biofilms, these interactions most probably will take place between bacteria that form enduring and ordered macrostructures.

So far, studies on interactions between *S. pyogenes* and members of the physiological microflora have been confined to potentially probiotic *S. salivarius* strains. Even with these bacteria, the aspects of mixed species biofilm growth and the simultaneous presence of eukaryotic cells have not been addressed. Thus, the major goals of the present study are

i) the comparison of interactions between different *S. pyogenes* wildtype strains, the well characterized *S. salivarius* K12 strain, two *S. oralis* clinical isolates with so far undefined status as probiotics, an *Enterococcus faecalis* strain as an eventual commensal of the upper respiratory tract, and the *E. coli* Nissle strain as an intestinal probiotic which is administered via the oral route;
ii) the comparison of interactions between these bacteria in different culture media and as planktonic or biofilm-organized cells;

iii) the effects of these interactions on the simultaneous presence of eukaryotic cells.

In conclusion, we hope to establish new probiotic strains with already well characterized functions on pathogenic *S. pyogenes* isolates and to understand more details of the complex interactions of mixed species biofilms on the surface of eukaryotic cells.

II. Material and Methods

II.1 Material

II.1.1 Bacterial strains

The bacterial strains used in this study are listed in table 1.

Table 1: List and sources of bacterial strains

Strain	Relevant characteristic/function	Source/Reference
S. pyogenes M49 strain 591	Skin isolate	R. Lütticken, Aachen, Germany
S. pyogenes M6 strain 616 (S43/192/1)	Patient isolate	Podbielski *et al*., 1991
S. pyogenes M49 *sag*A-luc strain	Lab. collection, *sag*A transcription measurement	Kreikemeyer *et al*., 2001
S. pyogenes M6 *sag*A-luc strain	*sag*A transcription measurement	This study
Streptococcus salivarius K12	Lab. collection	Microbiology Dept. Rostock University
Streptococcus oralis DSM 20627	Reference strain	DSMZ (Braunschweig, Germany)
Streptococcus oralis 4087	Patient isolate	University Hospital Rostock
Enterococcus faecalis AC4376	Patient isolate, Group D *streptococcus*	Culture collection Technical University of Aachen
E. coli Nissle	Probiotic srain	Ardeypharm (Herdecke, Germany)
E. coli DH5α	For recombinant plasmid generation	GibcoBRL (Eggenstein, Germany)

II.1.2 Culture media for bacteria

All prepared media was autoclaved at 121 °C and 1 bar pressure for 15 minutes. The substance amounts indicated below always correspondence to 1 liter broth media. Agar medium was prepared by adding 15 g technical agar (Oxoid) per liter broth media.

Columbia agar:

Ready to used blood agar (Oxoid)

Todd Hewitt Yeast (THY):

34 g Todd Hewitt broth (Oxoid), 5 g yeast extract (Oxoid), aqua dest. ad 1 liter.

Brain Heart Infusion (BHI):

37 g BHI (Oxoid), aqua dest. ad 1 liter.

0.5% glucose supplemented BHI:

37 g BHI powder was resolved in 987.5 ml aqua dest. and subsequently autoclaved. 12.5 ml filter-sterilized 40% glucose was added to the cooled BHI broth.

Luria Bertani (LB):

20 g LB-broth-base (Invitrogen), 4.5 g NaCl, aqua dest. ad 1 liter.

Concentration of added antibiotics:

Addition of antibiotics into agar media was done after cooling the temperature close to 50 °C. 100 µg/ml of spectinomycin (Sigma) in LB agar was used for *E.coli* and 60 µg/ml in THY agar for *S. pyogenes*, when harbouring the pFW5-*sag*A-luc plasmid. 10 µg/ml nalidixic acid in THY agar was used for growing *S. pyogenes* in co-culture *S. pyogenes-E. coli* Nissle. 5 µg/ml erythromycin in THY agar was used for growing *S. pyogenes* in co-culture *S. pyogenes-S. salivarius* K12 or *S. pyogenes-S. oralis*.

Artificial saliva stock:

Table 2: Composition of artificial saliva stock

Substance	Weight (gram) for 1 l
Lab Lemco Powder (meat extract, Oxoid)	1
Yeast extract (Oxoid)	2
Proteose pepton (Oxoid)	5
Mucin (Sigma)	2.5
NaCl (Merck)	0.35
KCl (Merck)	0.2
KH_2PO4 (Merck)	0.476
K_2HPO4 (Merck)	0.34

Aqua dest. was added up to 990 ml, the pH adjusted to 6.7 and the artificial saliva was subsequently autoclaved. 10 ml filter sterilized urea 5% (w/v) was added aseptically after cooling.

For biofilm experiments, 750 ml artificial saliva stock was mixed aseptically with 250 ml of BHI broth which was supplemented with 0.5% glucose.

II.1.3 Eukaryotic cells and media for cell culture

For adherence/internalization and cytotoxicity assay, the human laryngeal epithelial cell line HEp-2 (ATCC, CCL23) was used.

Media for HEp-2 cell culture:

Dulbecco's modified Eagle's medium (DMEM; Gibco-Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS; PAA, Pasching, Austria) was used to culture the HEp-2 cells. If indicated, 1% (v/v) Pen/Strep stock solution (5000 U/ml Penicillin and 5000 µg/ml Streptomycin from Gibco) was added.

II.1.4 Plasmid

Plasmid pFW5-luc (Podbielski *et al*., 1999) was used for construction of the pFW5-*sag*A-luc plasmid, which was then used to monitor *sag*A gene transcription in the *S. pyogenes* M6 background. The size of the plasmid is 4.5 kb and the included spectinomycin resistance (*aad*9) cassette allows selection of recombinant strains.

II.1.5 Antibodies

The antibodies used in this study are listed in Table 3.

Table 3: List and sources of antibodies

Antibody	Supplier
Rabbit IgG anti *S. pyogenes*	Biodesign, Dunn Labortechnik GmbH., Asbach
Goat anti Rabbit-IgG-AlexaFluor 488	Molecular Probes, MoBiTec, Göttingen
Goat anti Rabbit-IgG-AlexaFluor 647	Molecular Probes, MoBiTec, Göttingen

II.1.6 Reagents and buffers

Reagents used in this thesis work were mainly purchased from Merck, Sigma, Boehringer, and Roth. Special reagents, enzymes and solutions are mentioned in the corresponding protocols. Some solutions frequently used in this thesis work are listed below:

PBS buffer: 0.8% NaCl, 5 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 7.4

EDTA: 0.5 M EDTA, pH 8.0

TE buffer: 1 mM EDTA, 10 mM Tris-Cl, pH 7.5

II.1.7 Instruments

Analytical balance (BP 4100S, Sartorius)

Centrifuge Biofuge pico (Heraeus)

Cooling centrifuge (5417R, Eppendorf)

Centrifuge (Varifuge 3.OR, Heraeus)

Incubator (B6060, Heraeus)

Shaking incubator (KTM 100RP HLC, Bovenden)

Water manteled incubator with regulated CO_2 and O_2 pressures (Heraeus)

Electroporator (EQUIBIO Easyject Plus, BioRad)

Electrophoresis device for agarose gels (BioRad)

Luminometer (Leader TM 50 Gen-Probe, Biomeriuex)

pH meter (MP220, Mettler-Toledo)
Spectrophotometer (SmartSpec TM 3000, BioRad)
Thermoblock PCR T3 (Biometra)
Thermomixer Thermostat 5320 (Eppendorf)
Gel documentation Intas Science Imaging Instrument GmbH
Fluoroscence lamp (U-RFL-T, Olympus)
Fluorescence microscope (BX60, Olympus)
Zeiss DSM 960A electrone microscope
Critical point drying instrument (Emitech)
Leica TCS SP2 AOBS laser scanning confocal imaging system
GenArray scanner (Agilent)
Affymetrix Fluidic Station 450

II.2 Methods

II.2.1 Bacterial culture condition

All bacteria, except *E. coli*, were grown at 37 °C under 5% CO_2, 20% O_2 atmosphere. For *E. coli* cultures 37 °C and aerobic conditions were used. Different conditions are otherwise indicated. Wild type bacteria were maintained and passaged every 5-7 days on Columbia blood agar plates. All *S. pyogenes* recombinant strains were grown on appropriate antibiotic containing-THY agar, and for all *E. coli* recombinant strains antibiotic-LB agar was used. For long term storage of stock cultures, 18 h-grown bacteria on agar plates were completely removed aseptically, suspended into a stock culture tube (Microbank, Prolab Diagnostics) and subsequently stored at -80 °C.

For most experiments all bacteria were cultured in BHI broth at 37 °C under 5% CO_2, 20% O_2 atmosphere or otherwise indicated. Overnight (ON) pre-cultures were prepared by growing the bacteria in broth medium for maximal 14 h. For DNA preparation, *S. pyogenes* wt strains were grown in THY broth and *S. pyogenes* recombinant strains were cultured in the presence of 60 µg/ml spectinomycin. For plasmid isolation from *E. coli* strain DH5α harbouring pFW5-luc or pFW5-*sag*A-luc, bacteria were grown in Luria Bertani (LB) broth supplemented with 100 µg/ml spectinomycin. Plasmids were prepared according to the instruction of the kit manufacturer (NucleoSpin-Plasmid kit, Macherey-Nagel).

Biofilm culture condition:

For biofilm assays, bacterial ON cultures in BHI broth were suspended in fresh BHI broth supplemented with 0.5% glucose at 37 °C under 5% CO_2, 20% O_2 atmosphere and incubated for 3 days as standing cultures. For quantification of biofilm masses using the safranin staining techniques, suspensions of 100 µl bacteria (10^4 cfu/ml) in BHI broth supplemented with 0.5% glucose were inoculated into 96-well polystyrene microtiter plates (Cellstar Greiner, Bio-one). The further setup depended on the type of biofilms experiment. For monospecies biofilms an additional 100 µl fresh medium was added prior to incubation. For the setup of mixed-species biofilm cultures the second species was added in a 100 µl suspension (10^4 cfu/ml) to the first culture prior the incubation. As a specific setup, also different ratios of the bacterial species under investigation were mixed together as indicated. For microscopic observations, biofilms were generated in 24-well polystyrene plates (Cellstar Greiner, Bio-one) with inserted round plastic coverslips (Nunc) or in glass-bottom 2/4-chambers (Nunc®). The number of bacteria inoculated in each well was 10^4 cfu/ml for monospecies biofilms or varying ratios of different bacterial species were used for the mixed-species biofilm-setup.

II.2.2 Culture condition and preparation of eukaryotic cell culture

HEp-2 cells were cultured in culture flasks (75 cm^2, Cellstar Greiner, Bio-one) with 25 ml DMEM supplemented with 10% (v/v) FCS at 37 °C, 5% CO_2 atmosphere. Cell harvesting was achieved with trypsin treatment of the monolayers and subsequently cells were counted under a microscope using a Bürker cell-counting chamber. Usually, a monolayer of HEp-2 cell in a 24 well-plate can be generated by cultivating $3.5x10^5$ cells/well ON. For the cytotoxicity assay, the cell monolayer was grown on glass coverslips (1 cm in diameter, BAA) which were aseptically added at the bottom of the well plate before inoculating the cells in the well. The cell culture was ready for the assay after changing medium with fresh DMEM.

II.2.3 DNA/RNA methods and manipulation

II.2.3.1 *S. pyogenes* DNA preparation

S. pyogenes chromosomal DNA for molecular cloning was isolated by a modified method of Martin *et al*. (1990). All centrifugation steps were done at 4 °C. Cells from 10 ml ON culture of *S. pyogenes* in THY broth were harvested by centrifugation at 4000 rpm for 5 minutes, then washed twice with 1 ml sodium acetate (0.2 M, pH 6.2), and centrifuged at 8000 rpm for 2

minutes. The pellet was suspended in 0.5 ml buffer A (100 mM Tris-HCl, pH 7.0; 0.1 mM EDTA, 25% glucose), and 150 µl lysozyme (2 mg/ml, Sigma) and 50 µl mutanolysin (5000 Unit/ml, Sigma) was added subsequently. After incubation for 2 hours at 37 °C, the cell suspension was centrifuged at 13000 rpm for 2 minutes. The pellet was resuspended in 0.5 ml lysis buffer (50 mM EDTA, 0.2% SDS; pH 8.5) and incubated at 70 °C for 30 minutes. After incubation, 50 µl potassium acetate (5M, pH 5.5) was added and the solution was again incubated for 30 minutes at -20 °C and centrifuged at 13000 rpm for 3 minutes. The DNA containing supernatant was collected and the DNA was purified by adding an equal volume of phenol. The mixture was vortexed and phase separated by centrifugation at 13000 rpm for 2 minutes. The upper phase was transferred into a new tube and the extraction process was repeated once again. Next, the aqueous solution was purified with a mixture of phenol-chloroform (1:1) and chloroform-isoamylalcohol (24:1), respectively, and each step was repeated twice. The DNA in the upper phase was precipitated with two volumes of absolute ethanol and pelleted by centrifugation at 13000 rpm for 15 minutes. The pellet was washed with 1 ml 70% ethanol. The final DNA pellet was resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) 5 µl RNAse (10 mg/ml) was added and the mixture was incubated at 37 °C for 30 minutes. All DNA solutions were stored at -20 °C.

II.2.3.2 Plasmid isolation from *E. coli*

For all cloning experiments, plasmid DNA was purified from *E. coli* using the NucleoSpin-Plasmid kit (Macherey-Nagel) according to the instruction of the manufacturer.

II.2.3.3 HEp-2 cells RNA isolation

For microarray assays, RNA from HEp-2 cells was isolated using Trizol® (Invitrogen) and purified using the RNeasy Kit (Qiagen). HEp-2 cells were cultured in 24-well plates (Cellstar Greiner, Bio-one) as described in section II.2.2. After 2 hours infection with *S. salivarius* and *S. oralis* DSMZ, all medium was removed from each well. HEp-2 cells were lysed directly by addition of 200 µl Trizol into each well and passing the cell lysate several times through a pipette tip. After transferring the cell lysate into a new tube, the lysate was incubated for 5 minutes at room temperature. After the incubation, 0.2 ml chloroform (for each ml of Trizol reagent) was added into the tube and mixed by shaking for 15 seconds. The mixture was incubated at room temperature for 2-3 minutes then centrifuged at 12000 rpm for 15 minutes at 4 °C. The aqueous phase was transferred into a new tube and the RNA in the aqueous phase

was purified using RNeasy Kit and processed according to the instructions of the manufacturer.

II.2.3.4 DNA/RNA concentration measurement

DNA/RNA concentration was estimated by measuring the absorbance at 260 nm (A_{260}) using a spectrophotometer (BioRad). An A_{260} of 1.0 is equal to 50 µg/ml DNA or 40 µg/ml RNA. The purity of DNA or RNA was estimated from the A_{260}/A_{280} ratio. A high-quality DNA or RNA sample is considered to have an A_{260}/A_{280} ratio between 1.8 and 2.0.

II.2.3.5 Polymerase Chain Reaction (Mullis *et al.*, 1986)

The Polymerase Chain Reaction (PCR) method was used to amplify DNA between two primers (oligonucleotide) *in vitro*. In this thesis work, PCR was used to amplify an upstream DNA fragment of the *sag*A gene of *S. pyogenes* M6 in order to construct a luciferase reporter gene plasmid and for confirmation of *E. coli* or *S. pyogenes* transformants. *S. pyogenes* chromosomal DNA or plasmid from *E. coli* was used as DNA template. Primers were ordered from Eurogentec. The PCR mixture composition is shown in the table below.

Table 4: Composition of PCR mixture

Substance	Amount
DNA template	*ca.* 100 ng
Primer forward & reverse (10 pM)	2.5 µl each
dNTP mixture (10 µM, Roche)	1 µl
Taq-polymerase (5 U/µl, Qiagen)	0.25 µl
10X reaction buffer (Qiagen)	5 µl
Aquabidest	ad 50 µl

Amplification of DNA was done using a thermocycler (Thermoblock (PCR) Typ T3 Biometra). Standard PCR conditions were as follows: (i) 5 minute DNA template denaturation at 95 °C, (ii) 30 cycles of 95 °C 1 min, 50 °C, 1 min, 72 °C 1 or 2 min, (iii) final extension at 72 °C for 5 min. The PCR reaction was cooled at 4 °C at the end of cycling. The PCR product was analyzed by running a small sample in agarose gel electorphoresis using 1% (w/v) gels. Purification of the PCR product was done by cutting out agarose gel slices containing the correct sized DNA band and continued as instructed by the manufacturer (NucleoSpin-Extract kit, Macherey-Nagel).

II.2.3.6 DNA restriction digest

Restriction reactions were used to prepare compatible ends of plasmid DNA and PCR products before the ligation step in the construction of recombinant plasmid, and to confirm the correct recombinant plasmid. Restriction conditions were chosen according to the manual of the enzyme restriction supplier (Roche). In general 1 U enzyme was used for digesting 1 µg DNA at optimal temperatures for the enzyme for a minimum of 2 hours in the presence of suitable supplied buffers. The size of the digested DNA fragments was estimated by electrophoresis on 1% agarose gels together with a DNA size standard marker. For further ligation reactions the fragments were purified using the NucleoSpin-Extract kit (Macherey-Nagel).

II.2.3.7 DNA ligation reaction

Prepared DNA insert fragments and linear plasmid DNA containing compatible ends were mixed together in 1.5 ml Eppendorf tube in a ratio of 4:1. 1 U of T4-ligase (Roche) and 2 µl of 10x ligation buffer were added into the mixture, aqua dest. ad 20 µl. Ligation was allowed to performe at 16 °C for a minimum of 12 hours.

II.2.3.8 Agarose electrophoresis for DNA (Sambrook *et al*., 1989)

DNA fragments can be separated with electrophoresis using agarose gels, and the different sizes can be determined by using defined DNA standard marker. Such DNA markers contain several DNA fragments of known size.

Preparation of agarose gel:

For 1% (w/v) agarose, 100 g agarose (PEqLab) was boiled with 100 ml 1x TAE buffer until the agarose is dissolved. After cooling down the solution (*ca*. 55 °C) the 1% agarose liquid was poured into an appropriate gel chamber and the gel comb was inserted at one side of the gel. The agarose gel is ready for use after solidifying and removal of the gel comb.

DNA electrophoresis:

The agarose gel was placed in an electrophoresis tank and soaked with 1x TAE buffer. The DNA sample was mixed with 1/6 volume of 6x DNA loading buffer. The mixture was carefully pipetted inside the gel well. For estimation of the DNA fragment size, a DNA marker was run in parallel to the sample under investigation. The electrophoresis was performed for 1 hour at a constant current of 100 V.

DNA visualization:

After electrophoresis, the agarose gel was soaked in an ethidium bromide (Et-Br) staining solution (1.5 µg/ml, Sigma) for 15 minutes, and after rinsing shortly in aqua dest., the gel was exposed to ultraviolet (UV) light using an UV-transilluminator. Ethidium bromide intercalates into the DNA double helix and is visualized when exposed to UV light. All gel pictures were photographed for documentation using an Intas gel documentation system.

50xTAE buffer stock:

242 g Trisma base, 57.1 ml glacial acetic acid, 100 ml EDTA (0.5 M, pH 8), aqua dest. ad 1 L. For preparation of a 1x TAE solution an appropriate dilution of the 50x stock in aqua dest. was done.

6x DNA loading buffer:

0.25% bromphenolblue, 0.25% xylene xyanol FF, 30% glycerol (in water)

II.2.3.9 Construction of the *S. pyogenes* M6 *sag*A-luc reporter gene strain

A 769 bp fragment upstream of the *S. pyogenes* M6 *sag*A gene was used for integration of the luciferase reporter box downstream of the promoter. The appropriate chromosomal DNA fragment was amplified by PCR with *S. pyogenes* M6 chromosomal DNA as template. These following primers were used for amplification: 5′ TTAAAACGGCCGATTAGTCTAGTGG ACTCATT 3′ (the forward primer) and 5′ AAATTTGGATCCAAGGTTTACCTCCTTAT CTA 3′ (the reverse primer). The fragment was ligated through *Ecl*XI and *Bam*HI restriction sites (underlined) into the pFW5-luc plasmid after the following steps: (i) The PCR generated DNA insert fragment was isolated from an agarose gel and purified as mention before, (ii) the plasmid pFW5-luc was isolated from *E. coli* and purified, (iii) in order to provide compatible ends for plasmid and DNA insert, both partners were digested via *Ecl*XI and *Bam*HI restriction (Roche) using Buffer B at 37 °C. Finally, both digested partners were purified as mention before and ligated as outlined above.

The ligation product was transformed into $CaCl_2$ competent *E. coli* DH5α cells (III.2.4). Integrity of the recombinant plasmid was confirmed by gel electrophoresis, restriction analysis and PCR. Subsequently, the correct recombinant plasmid was integrated by a site specific single-crossover event into the strain *S. pyogenes* M6 strain 616 genome through electroporation. The correct insertion was confirmed using PCR assays on genomic DNA from wt and transformant.

II.2.4 Preparation and transformation of *E. coli* DH5α competent cells

Bacteria can take up exogenous DNA from the environment through altering their cell wall. In this specific physiologic condition bacteria are called competent. Competent *E. coli* can be generated with $CaCl_2$ treatment and exogenous DNA can be taken up with a heat shock method according to a protocol by Cohen *et al*. (1972).

10 ml fresh LB broth was inoculated with 200 µl *E. coli* DH5α ON culture grown at 37 °C and shaken at 180 rpm until an OD_{600} of 0.4 was reached. Cells were harvested by centrifugation at 4000 rpm (Varifuge 3.OR) and 4 °C for 5 minutes and subsequently washed once with ice-cold sterile 50 mM $CaCl_2$. Washed cells were incubated with the same solution for 30 minutes on ice, centrifuged and then resuspended in 1 ml $CaCl_2$. Aliquots of 100 µl of the cell suspension can be stored for 3-4 days at 4 °C.

10 µl of the ligation mixture was added to 100 µl of competent cells and incubated on ice for 30 minutes. A heat shock was performed by placing the mixture at 42 °C for 1 minute and immediately on ice again for 3 minutes. 900 µl fresh LB broth was added and the mixture incubated again for 1 hour at 37 °C with shaking (180 rpm). Transformed *E. coli* was selected by plating 200µl aliquots of the bacterial suspension on 100 µg/ml spectinomycin containing LB agar plates which were incubated ON at 37 °C. All grown transformants were suspected to harbour the correct transformed plasmid. In order to confirm presence of the desired recombinant plasmid DNA, plasmids were re-isolated from the transformants and the integrity of the constructs was confirmed by gel electrophoresis, PCR and as restriction digest outlined in II.2.3.5, II.2.3.6 and II.2.3.8.

II.2.5 Preparation and transformation of *S. pyogenes* competent cells

S. pyogenes M6 competent cells were prepared in the presence of glycin, mutanolysin and hyaluronidase. Transformation of *S. pyogenes* was done via electroporation (Dunny *et al*., 1991; Caparon & Scott, 1991).

S. pyogenes was grown ON in 10 ml THY broth supplemented with 20 mM glycin, then 5 ml of preculture was added to 45 ml of THY supplemented with glycine (20 mM) and mutanolysin (10 U/ml) for ON incubation. Cells were harvested by centrifugation at 3000 rpm, 4 °C for 5 min and washed once with sterile PBS. Pelleted cells were suspended in 1 ml PBS containing 500 U hyaloronidase and incubated for 1-1.5 hours at 37 °C. The pellet was washed 2 times with ice cooled PBS and 2 times with ice cooled sterile sucrose (0.625 M). Subsequently, the pellet was resuspended in 1.5 ml sucrose (0.625 M) and 100 µl were

aliquoted in 1.5 ml Eppendorf tubes. The competent cells can be used directly or stored at -80 °C. For electroporation, 20 µl plasmid DNA (containing 10-30 µg DNA) was mixed together with 100 µl *S. pyogenes* competent cells in ice-cold 1 mm-gap electroporation cuvettes (PeqLab). Electroporation was done under 1.75 kV, 329 Ω, 25 µF and 8.2 ms conditions. After electroporation, the cuvette was placed on ice for 3 min, 1 ml warmed fresh THY broth was added, and the transformed bacteria were incubated at 37 °C under 5% CO_2, 20% O_2 atmosphere for 3 hours. After incubation *S. pyogenes* transformants were selected by plating on 60 µg/ml spectinomycin contained THY agar plates. Incubation for selection was done for 2 days at 37 °C under anaerobic conditions. Confirmation of correct *S. pyogenes* transformants was achieved through specific PCR analysis of chromosomal DNA isolated from single transformants.

II.2.6 Quantitative co-culture and transwell system

All bacterial strains were co-cultured in BHI broth either in tubes or 6- or 24-well plates with different combinations of bacterial cfu's of ON cultures. Pre-cultures of all bacteria were done in the same medium. Single and co-culture bacteria were grown ON at 37 °C under 5% CO_2, 20% O_2 atmosphere. Viable bacteria were determined through serial dilution of ON co-cultures which were plated on blood agar plates. For the transwell system setup, the same growth conditions and bacterial cfu combinations were used in 6 wells- or 24 wells-plate of polycarbonate with a membrane Transwell® Insert (Corning, Cat. No 3412 or 3413). *S. pyogenes* was always cultivated in the bottom well and test bacteria were cultivated in the upper compartment, both compartments being separated by a 0.4 µm size pore membrane. After ON incubation, viable *S. pyogenes* were plated on THY agar plates for enumeration. A picture of the transwell system that was used in this study is shown in figure 1 below.

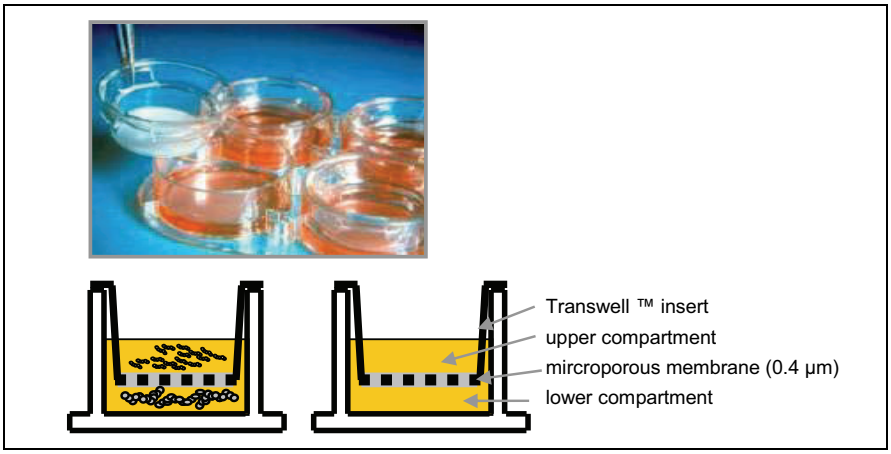


Fig. 1 Transwell™ system from Corning.

II.2.7 Bacteriocin assay

The bacteriocin assay was performed based on a modified "differed antagonism cross streak technique" on blood agar (Abbott & Shanon, 1958). Bacteria which are tested as bacteriocin producers were first streaked on blood agar and incubated for a minimum of 18 hours. Subsequently, the strain was killed with a vapour of chloroform for 4 minutes. Chloroform vapours were removed by passive ventilation of the agar plate for 10-15 minutes. *S. pyogenes* strains were streaked across the primary streak and incubated ON. Growth inhibition and beta-haemolysis inactivation were observed around the cross of bacterial streaking.

II.2.8 Growth curve measurement

The culture condition for growth curve analysis was 37 °C under 5% CO_2, 20% O_2 atmosphere. All cultures were allowed to start growing with OD_{600} 0.05 by diluting an ON culture of bacteria in broth medium. The optical density was measured at 600 nm (OD_{600}) using a spectrophotometer by aseptically removing an aliquot of 1 ml from the growing culture every hour for the period of 8-10 h. The growth curve was extrapolated using Microsoft EXCEL and the OD_{600} values were plotted semi-logarithmically over the time (h).

II.2.9 Quantitative assays for *sag*A-luciferase activity

One very important virulence gene in *S. pyogenes* is *sag*A which encodes streptolysin S, a hemolysin. In this thesis work, the influence of tested bacteria on the *sag*A expression was investigated at the transcription level through luciferase reporter measurements. Two serotypes of *S. pyogenes sag*A-luc transformants (*S. pyogenes* M49 and M6) were used for this experiment. To investigate the direct contact influence of tested bacteria, the experimental setup was such that *S. pyogenes* and tested bacteria were grown as co-culture in 6 well-plates (Cellstar Greiner, Bio-one). For experiments aiming at potential effects from indirect contact, the experiment was done using the 6 well-plate of polycarbonate with a membrane Transwell® Insert.

All bacteria were cultivated in BHI broth and grown at 37 °C 5% CO_2, 20% O_2 atmosphere. From ON pre-cultures, bacterial suspensions with defined numbers of bacteria (cfu) were prepared. For *S. pyogenes* strains 10^3 cfu/ml and for tested bacteria either 10^2 or 10^6 cfu/ml were used for the experiments. For co-culture, *S. pyogenes* and each tested bacteria was positioned together in 6 well-plates and allowed to grow for 9 hours. Aliquots from bacterial cultures were withdrawn at 1-h intervals for luminescence and OD_{600} readings. For measurement of luciferase activity, 100 µl aliquots were mixed with 100 µl 2.5 x assay buffer

(62.5 mM glycil-glycin, pH 7.8, 25mM $MgCl_2$) in a sample tube (Nunc). 330 µM D-luciferin was injected automatically into the mixture and the Relative Light Unit (RLU) was measured with a luminometer (Typ Leader™ 50 Gen-Probe, bioMérieux). Viable bacteria at 9 h and 16 h of incubation were determined by plating serial dilutions of the sample on blood agar plates. For transwell system experiments, the *S. pyogenes* strain was inoculated into the lower compartment and tested bacteria were seeded into the upper compartment chamber. Measurment of luciferase activity, OD_{600} readings and elucidation of viable bacterial counts were done as outlined above for the co-culture allowing direct contact, but sampling was exclusively done from lower part of the system cointaining the *S. pyogenes* strain.

II.2.10 Hemolysis assay

All bacteria were grown ON in THY medium at 37 °C 5% CO_2, 20% O_2 atmosphere. Spent medium (SM) was collected after centrifugation at 4500 rpm for 15 minutes at 4 °C and filtered (filter pore size 0.22 µm). An equal volume of SM from *S. pyogenes* and tested bacteria (450 µl) was placed in a 1.5 ml Eppendorf tube. THY medium was used together with each SM from all bacteria as a control. To all samples and appropriate controls (negative control-no lysis: PBS; positive control-100% lysis: water) 10% defibrinized sheep blood was added and the mixture was allowed to incubate for 1 h at 37 °C on an end-over-end shaker. For final quantification of the released amount of hemolysis, which is a direct measure for the streptolysin S (contained in SM of *S. pyogenes*) erythrocyte lysing capacity, the samples were centrifuged at 5000 rpm for 3 minutes and absorbance from the supernatant was measured at 543 nm in a spectrophotometer. The absorbance value (A_{543}) represented the hemolytic capacity.

II.2.11 Coaggregation assay (modified from Cisar *et al*., 1979)

All bacteria were grown ON in BHI medium at 37 °C under 5% CO_2, 20% O_2 atmosphere. Cells were harvested by centrifugation at 4000 rpm at 4 °C for 10 min and washed twice with coaggregation buffer (tris(hydroxymethyl)amino-methane 1 mM, pH 8.0; $CaCl_2$ 0.1 mM; $MgCl_2$ 0.1 mM; NaN_3 0.02%; and NaCl 0.15 M). The suspension of bacterial cells in coaggregation buffer was adjusted to an OD_{600} of 2.0. Coaggregation of *S. pyogenes* mixed with selected bacteria was done by mixing an equal volume (2 ml) of cell suspension from *S. pyogenes* and selected bacteria in a tube for at least 10 seconds on a Vortex mixer. Suspensions of single bacterial species in coaggregation buffer were used as a control. The

tubes were allowed to stand at room temperature for 1 to 2 h and were subsequently observed for coaggregation by simple visual inspection.

II.2.12 Biofilm quantification with safranin assay

Quantification for biofilm assays was done by safranin staining as outlined by Lembke *et al*. (2006). After 3 days incubation time, the remaining planktonic bacteria were removed by aspiration of the liquid. The wells were washed once with PBS and stained with 0.1% safranin for 10-15 minutes at room temperature. After an additional washing step with PBS all wells were air-dried. Quantification of staining was done by measuring absorbance at 492 nm using an ELISA reader (Tecan Spectra Reader Classic, Tecan Trading AG).

II.2.13 Microscopic observation and documentation of biofilms (Fluorescence, SEM, CLSM)

II.2.13.1 Fluorescence microscopy

Planktonic bacteria were removed from biofilm cultures by aspiration of liquid and the biofilms on coverslips were washed once with PBS. For visualization, biofilms were stained with the two components of a live/dead staining kit for bacteria (Baclight, Invitrogene). A 1:1 ratio of component A (SYTO 9) and B (Propidium iodide) was diluted 1:1000 in PBS, the mixture added to the coverslips in the wells, and incubated for 10 minutes at RT in the dark. Biofilms were subsequently inspected and visualized with a fluorescence microscope (BX60, Olympus).

II.2.13.2 Scanning Electron Microscopy (SEM)

After removal of remaining planktonic bacteria, biofilms on coverslips were washed once with PBS and fixed with 2.5% glutaraldehyde ON. The coverslips were washed 2-3 times with 0.1 M sodium phosphate buffer (pH 7.3) and dehydrated with a degraded series of ethanol (5 minutes in 30%, 5 minutes in 50%, 10 minutes in 70%, 10 minutes in 90% and two times 10 minutes in ethanol absolute). Subsequently, coverslips were dried with CO_2 through critical point method with a critical point dryer from Emitech as outlined by the manufacturer. Dried coverslips were covered with gold to a 10 nm layer and scanned with a Zeiss DSM 960A electron microscope.

II.2.13.3 Confocal Laser Scanning Microscope (CLSM)

The visualization of the *S. pyogenes* bacteria located in single- and mixed-species biofilms was done by immunofluorescence staining employing a specific anti- *S. pyogenes* polyclonal antibody together with an Alexa 488-coupled secondary antibody. Hexidine iodine was used for staining all gram-positive bacterial species. The staining procedure is outlined below: After removal of planktonic bacteria, biofilms were washed once with PBS and fixed with 3% paraformaldehyde for 15 min at 4 °C. The fixation solution was removed and 1% FCS was added into the chamber for blocking at RT for 30 min. The biofilm was washed three times with PBS. A 1:5000 PBS dilution of the rabbit IgG anti *S. pyogenes* antibody was added to the biofilm and allowed to incubate for 1 h at RT. After three washing steps with PBS the second antibody (Goat anti Rabbit-IgG-AlexaFluor 488) was added at a 1:500 dilution in PBS for 45 min at RT in the dark. Again three washing steps with PBS were performed. Hexidine iodine (1 µl in 1 ml PBS, Invitrogen) was added for 10 min at RT in the dark. The biofilm was finally visualized and inspected with a Zeiss inverted microscope attached to a Leica TCS SP2 AOBS laser scanning confocal imaging system with an Argon laser at a 480 and 488 nm excitation wavelenght.

II.2.14 Adherence and internalization assay

S. pyogenes adherence and internalization to eukaryotic cell was determined by an antibiotic protection assay (Molinari *et al.*, 1997). The human laryngeal epithelial cell line HEp-2 (ATCC, CCL23) was used in this assay. HEp-2 cells were prepared as outlined in section II.1.3.

Bacteria cells were prepared as follows. An ON culture of bacteria in BHI broth was washed with PBS, resuspended with fresh DMEM supplemented with 10% FCS, and added to a confluent ON grown HEp-2 cell monolayer. The multiplicity of infection (MOI), expressing the ratio of bacteria per single eukaryotic cell, was set to 10 for all experiments with *S. pyogenes* and 25-100 for all other tested bacterial species. Two wells were used for every strain combination. After initial 2 hours *S. pyogenes* infection, HEp-2 cells were washed with PBS, then 200 µl of 0.05% Trypsin/EDTA (Gibco-Invitrogen) solution was added to each well for 10 minutes. HEp-2 cells from the same strain combination were collected into one 1.5 ml Eppendorf tube and then lysed with distilled water. Attached bacterial numbers in the lysate were assessed by viable count through plating serial dilutions. To quantify internalized bacteria, DMEM was changed with DMEM supplemented with 1% Pen/Strep antibiotic (Gibco-Invitrogen) after the initial 2 hours incubation period and the cells incubated for 2

hours to kill attached bacteria. For quantification the same procedure like for adherence was used.

Three different seeding strategies were done for this assay, i.e., simultaneous seeding, *S. pyogenes* first seeding and *S. pyogenes* last seeding strategy. The *S. pyogenes* infection time for all seeding strategies was 2 hours. In simultaneous seeding experiments, *S. pyogenes* and tested bacteria were added to the HEp-2 cell at the same time. For experiments in which *S. pyogenes* was seeded as the first species, the bacteria were allowed to infect the HEp-2 cell monolayers for 1 hour prior to addition of the tested bacteria. This particular setup was tested without removing *S. pyogenes* from the HEp-2 cell monolayers. The infection was continued for 1 more hour. As another variation, *S. pyogenes* was added after the tested bacteria were allowed to infect the cells for an initial period of 2 hours.

Moreover, several modifications of the latter method were performed: (i) *S. pyogenes* was added directly to HEp-2 cells which were infected 2 hours with tested bacteria without performing any removal step, (ii) after 2 hours infection tested bacteria were removed by changing the DMEM medium prior to *S. pyogenes* infection, (iii) as a variation of (ii) monolayers were washed 3 times with sterile pre-warmed PBS preceding the *S. pyogenes* inoculation, (iv) the transwell system setup was used. Determination of adherent and/or internalized bacteria in all modifications was the same as for monospecies adherence/internalization quantification.

II.2.15 Eukaryotic cell viability assay

The “Live/Dead Viability/Cytotoxicity” kit for animal cells (Molecular Probes, Mobitec) was used to determine viability of HEp-2 cell in the presence of *S. pyogenes* and tested bacteria. This kit contains two fluorescence dyes, calcein AM and ethidium homodimer-1. Membrane-permeable calcein AM is cleaved by esterases in live HEp-2 cells to yield cytoplasmic green fluorescence. Membrane-impermeable ethidium homodimer-1 labels nucleic acids of membrane-compromised or dead cells with red fluorescence. With the following method, both toxicity caused by adherent and internalized bacteria, can be measured at once.

Preparation for HEp-2 cells is defined in section II.2.2. Bacterial cell preparation and all seeding strategies used are outlined in section II.2.14. The MOI for *S. pyogenes* and tested bacteria was set between 25 and 100. Infection time for *S. pyogenes* was 1 hour, followed by changing fresh DMEM and continued incubation for the next 4 hours. The staining solution was prepared by mixing 14 µl of calcein AM and 6 µl of ethidium homodimer-1 together in 6 ml PBS. Staining was done for 40 minutes at RT in the dark after washing HEp-2 cells with

PBS. Visualization and inspection of live or dead HEp-2 cells was done under a fluorescence microscope at 400 fold magnification. In each assay, 3 randomly chosen microscopic fields were documented as a picture. In order to express the results as quantitative data, living cells (green fluorescence) were counted and expressed as percentage of all cells (live and dead) visible in each picture.

II.2.16 Double-immunofluorescence assay

This assay was performed as alternative method and for visualization of *S. pyogenes* bacteria adherent and/or internalized into HEp-2 cells. With this method, adhered *S. pyogenes* were visualized with green fluorescence and internalized *S. pyogenes* with red fluorescence using a fluorescence microscope. HEp-2 cells were visualized with regular light microscopy. One microscopic field was first inspected with a filter setting for green fluorescence. The same microscopic field was documented under red fluorescence filter setting. Finally the unstained eukaryotic cells were visualized by light microscopic setting. Subsequently, the three different picture frames were overlaid to generate one single picture including all the information with Adobe Photoshop software.

HEp-2 cells preparation is described in II.2.15. Bacterial cell preparation and seeding strategies were already described in II.2.14. After 2 hours *S. pyogenes* infection, HEp-2 cells were washed with PBS and fixed with 3% paraformaldehyde for 15 min at 4 °C. Unspecific binding was blocked with 1% FCS in PBS. Incubation time for blocking was 30 min at RT. Cells were washed three times with PBS. The first antibody for *S. pyogenes* staining (Rabbit IgG anti *S. pyogenes*) was added at 1:5000 dilutions in PBS and incubated for 1 hour at RT. Now, cells were washed three times with PBS and the second antibody (Goat anti Rabbit-IgG-Alexa Fluor 488) was added at 1:500 dilution in PBS for 45 min at RT in the dark. With these steps of the staining procedure, all adhered *S. pyogenes* were specifically stained with green fluorescence. The procedure was continued to stain the internalized *S. pyogenes*. HEp-2 cells were washed three times with PBS. 0.1% Triton X100 was added for 5 min at RT to permeabilize the HEp-2 cells. After washing three times with PBS, again the antibody for *S. pyogenes* (Rabbit IgG anti *S. pyogenes*) was added at the same dilution and condition as before. Subsequently, cells were washed three times with PBS and a third antibody (Goat anti Rabbit-IgG-AlexaFluor 647) was added at 1:500 dilution in PBS for 45 min at RT in the dark. This part of the method labelled all internalized *S. pyogenes* bacteria with red flurorescence. The glass coverslip was carefully removed and inspected under the fluorescence and light

microscope. Three different microscopic fields were observed for one sample glass coverslip and the overlaid pictures saved in the attached computer system for documentation.

II.2.17 HEp-2 cells microarray

To compare host cell gene expression profile changes caused by *S. salivarius* K12 and *S. oralis* DSMZ, high density oligonucleotide microarrays were applied. Total RNA samples from infected HEp-2 cells were hybridized to Human Genome U133 plus 2.0 array (Affymetrix, St. Clara, CA), interrogating 47000 transcripts with more than 54000 probesets. HEp-2 RNA samples from 2 h infected cell with *S. salivarius*, *S. oralis* DSMZ and controls were isolated as mentioned in section II.2.3.3.

Array hybridization was performed according to the supplier's instructions using the "GeneChip^R Expression 3`Amplification One-Cycle Target Labeling and Control reagents" (Affymetrix, St. Clara, CA). In detail, the first-strand cDNA was synthesized using 5 µg whole RNA sample and Superscript II Reverse Transcriptase (RNaseH minus) introducing a T7-$(dT)_{24}$ primer. The second strand synthesis was done as strand replacement reaction using the *E. coli* DNA-Polymerase I complex, hybridstrandspecific RNA degrading RNaseH, and a ligase reaction (*E. coli* DNA ligase). Last step for second strand synthesis was an endpolishing with recombinant T4-Polymerase. Then, the second strand DNA was cleaned up and used for the labelling step. Biotin-16-UTP was introduced as label by a linear amplifying *in vitro* transcription (IVT) reaction using T7 polymerase ON (16 h). The required amount of cRNA produced by IVT was fragmented by controlled chemical hydrolysis to release the proportionality of cRNA molecule length and the amount of incorporated biotin derivate. The hybridization was carried out ON (16 h) at 45 °C in the GeneChip^R Hybridization Oven 640 (Affymetrix, St. Clara, CA). Subsequently, washing and staining protocols were performed with the Affymetrix Fluidics Station 450. For a signal enhancement, an antibody amplification was carried out using a biotinylated anti-streptavidin antibody (Vector Laboratories, U.K.), which was cross-linked by a goat IgG (Sigma, Germany) followed by a second staining with streptavidin-phycoerythrin conjugate (Molecular Probes, Invitrogen). The scanning of the microarray was done with the GeneChip Scanner 3000 (Affymetrix, St. Clara, CA) at 1.56 micron resolution.

The data analysis was performed with the MAS 5.0 (Microarray Suite statistical algorithm, Affymetrix), probe level analysis using GeneChip Operating Software (GCOS 1.4), and the final data extraction was done with the DataMining Tool 3.1 (Affymetrix, St. Clara, CA).

All microarray experiments were done by the Institute of Immunology, research group Molecular Immunology (Dr. Dirk Koczan) except for HEp-2 cells infection and total RNA isolation.

Differentially up-regulated and down-regulated genes from two independent experiments were clustered manually then analyzed to find out annotation of the genes and their molecular function, biological process and pathway using tools in NetAffx™ analysis center (http://www.affymetrix.com/analysis/index.affx). As comparison, the same analysis was also done using PANTHER (http://www.pantherdb.org) and InnateDB (http://www.innatedb.com) for differentially expressed genes that have a correlation with Entrez ID

III. Results

III.1 *S. pyogenes* co-culture experiments: direct and indirect contact

When two bacterial species grow together in the same environment, there are several possibilities how these bacteria can influence each other. The first potential relationship is "mutualism", which is a peaceful coexistence without harm or benefits for the partners. The second type of association is "commensalisms", which is defined as a beneficial relationship for at least one partner. In most cases one bacterial species provides essential nutrients which support the establishment of the second species. The third and most aggressive link between different species is "cannibalism", which is characterized by competing interest during presence in the same niche and which normally ends in killing/growth suppression and thus removal of one bacterial species from the ecological niche.

Co-culture experiments are the easiest way to elucidate which type of interaction two bacterial species perform when they grow together. So far nothing is known about the scenario once *S. pyogenes* interacts with other oral species, thus we used co-culture experiments between *S. pyogenes* and other oral bacteria in this thesis.

First, co-culture experiments were performed by growing *S. pyogenes* with each tested bacterial species in several combinations and with changing bacterial numbers together in tubes. Growth was allowed to occur before remaining bacterial cfu's were determined by plating on blood agar. High and lower bacterial cfu combinations were used under this experimental setup to test whether bacterial numbers play a role in this interaction. Based on the hemolysis property of *S. pyogenes* on blood agar plates both bacterial species can be differentiated. Another possibility to differentiate them was antibiotic selection. Therefore, the growth behaviour of all bacterial species under investigation was initially determined. The results are shown in Table 5 below.

Table 5: Bacterial growth under different antibiotics

Bacteria	Spectinomycin 60 µg/ml	Erythromycin 5 µg/ml	Nalidixic acid 10 µg/ml	Blood agar
S. pyogenes	-	-	+	β-hemolysis
S. salivarius K12	+	+	+	no hemolysis
S. oralis DSMZ	+	+	+	α-hemolysis
S. oralis 4087	+	+	+	α-hemolysis
E. faecalis	+	+	+	no hemolysis
E. coli Nissle	+	+	-	no hemolysis

+ = growth; - = no growth

The interaction of the bacteria in tubes, allowing direct contact, and in transwell plates, allowing exclusively secreted substance exchange, was investigated. In the tube setting, growth of both partners was quantified, whereas in the transwell system exclusively *S. pyogenes* development was monitored.

The results are shown in figures 2 and 3, however, only selected and meaningful cfu combinations are included in the figures. In general, from experiments using the transwell system, secreted substance from most bacteria did not decrease *S. pyogenes* strain numbers except for *S. salivarius* K12. The *S. salivarius* K12/*S. pyogenes* initial mixtures of 10^6 (cfu/ml)/10^3 (cfu/ml) lead to a 7 log decrease of *S. pyogenes* cfu compared to untreated controls (Fig. 2a & f). Furthermore, *S. oralis* DSMZ reduced *S. pyogenes* cfu by 1 log (Fig. 2b). No other strain combination or cfu variation revealed significant effects. The effect of *S. salivarius* on *S. pyogenes* M49 was more prominent than the effect on *S. pyogenes* M6. Thus, *S. salivarius* K12 apparently secretes a diffusible substance which could be the known salivaricin Sal A2 and Sal B, and *S. pyogenes* M6 is more resistant to the action of this substance, which could be due to a more massive capsule production as compared to *S. pyogenes* M49.

For the direct contact experiments it can be concluded that high initial cfu's of *E. faecalis*, *S. salivarius* K12, and *S. oralis* strains mixed with low *S. pyogenes* cfu's cause a repression of *S. pyogenes* M49 and M6 bacterial cfu's in the experiment (Fig. 3a-i). One exemption of this picture is *S. salivarius* K12 of which also low cfu's inhibited low initial cfu's of both *S. pyogenes* serotypes (Fig. 2a & f).

Interestingly, not in the transwell setting, but in the direct contact experiments *E. coli* Nissle suppressed growth and establishment of both tested *S. pyogenes* strains in all mixtures investigated (Fig. 2e & j). This is even more remarkable given to the fact that *E. coli* Nissle is not affected itself by any mixture with *S. pyogenes*, as it always grows to a very high final cfu in all combinations (Fig. 3e & j). In contrast to this observation, *E. faecalis, S. oralis* strains and also *S. salivarius* K12 revealed decreased cfu's in the mixture of high *S. pyogenes* numbers together with low number of their own species.

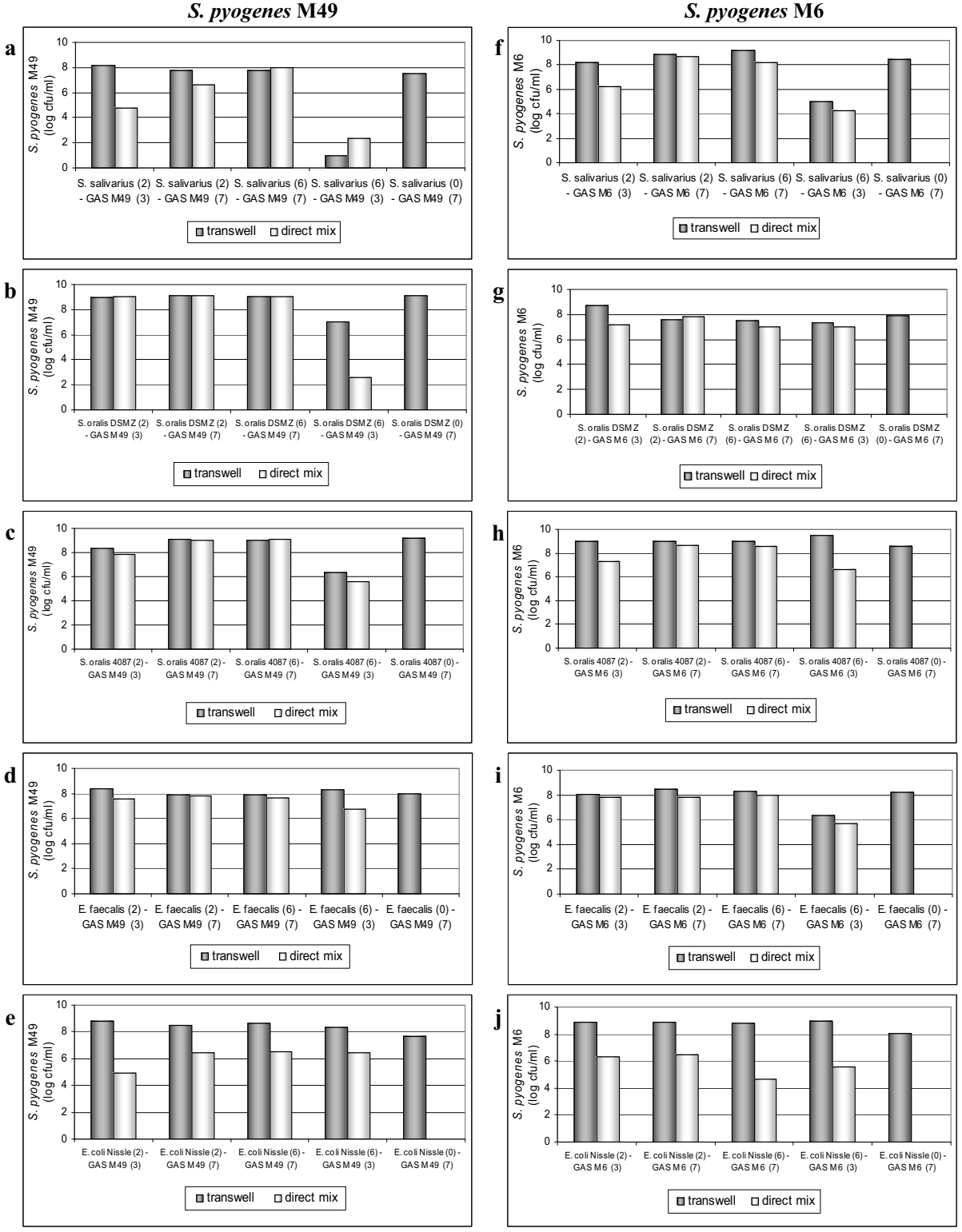


Fig. 2 *S. pyogenes* development after mixed-species growth with other oral bacteria and *E. coli* Nissle in direct contact and transwell experiments.

Parts a-e of the figure depict results obtained with *S. pyogenes* M49 and parts f-j illustrate results of experiments with the M6 serotype. *S. pyogenes* serotypes were co-cultured with *S. salivarius* K12 (a & f), *S. oralis* DSMZ (b & g), *S. oralis* 4087 (c & h), *E. faecalis* (d & i) and *E. coli* Nissle (e & j) using different initial cfu combinations in BHI medium. The varying initial cfu's are indicated as follows: For example, *S. salivarius* (2)-GAS M49 (3) points out an initial mixture of 10^2 cfu/ml for *S. salivarius* and 10^3 cfu/ml for *S. pyogenes* M49. GAS = *S. pyogenes*.

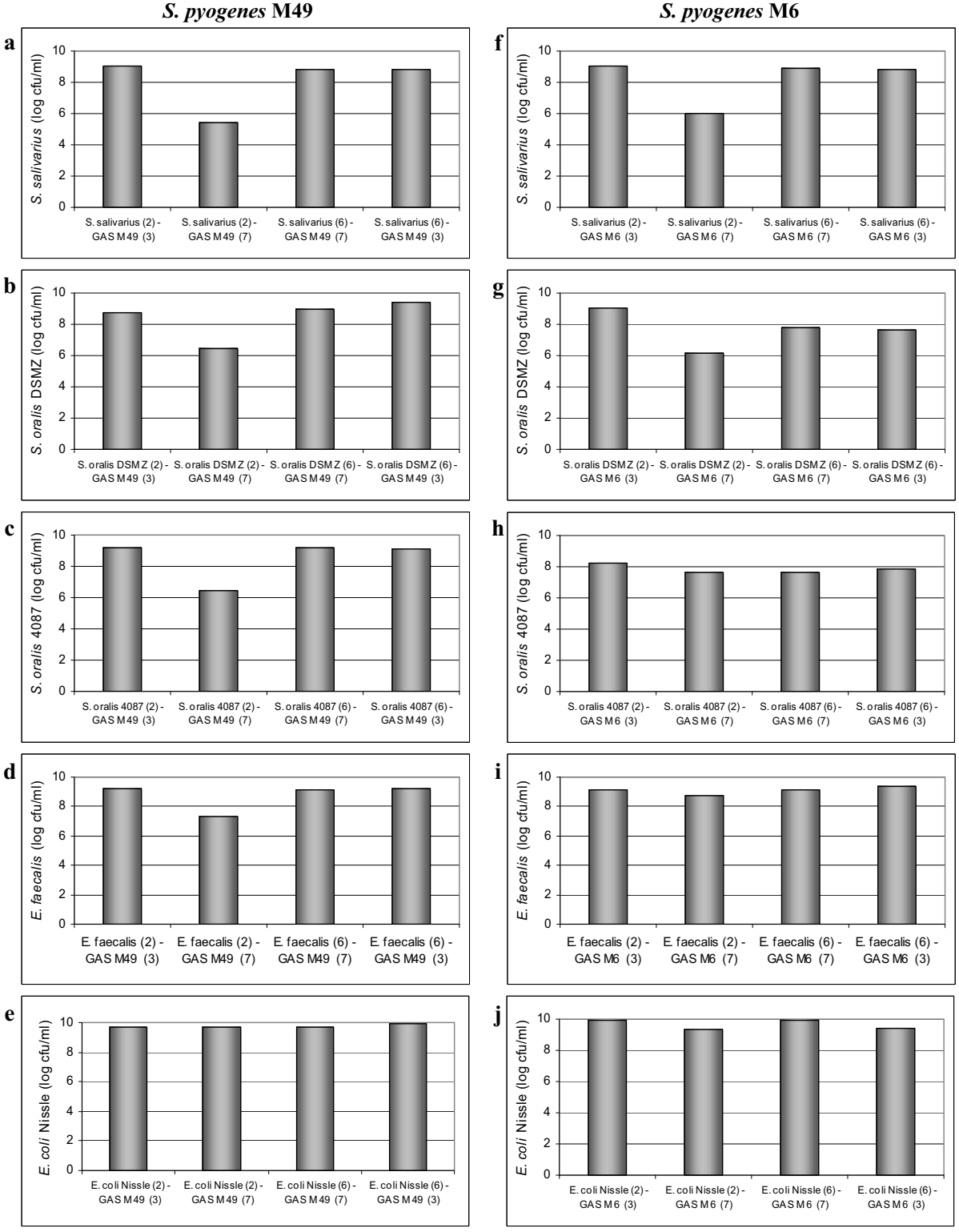


Fig. 3 Development of the tested bacterial species after mixed-species growth with *S. pyogenes* serotype M49 and M6 in direct contact experiments.

Parts a-e of the figure depict results obtained with *S. pyogenes* M49 and parts f-j illustrate results of experiments with the serotype M6. *S. salivarius* K12 cfu's after co-culture with *S. pyogenes* M49 and M6 are shown in a & f, respectively. *S. oralis* DSMZ cfu's are illustrated in b and g. *S. oralis* 4087 and *E. faecalis* cfu's are displayed in c/h and d/i, respectively. The *E. coli* Nissle development after co-culture with *S. pyogenes* serotypes is presented in e and j. Values in parenthesis hint to the cfu/ml which have been used: (2) indicates that of 10^2 cfu/ml were used for the co-cultures as starting point.

III.2 Bacteriocin assay

As already mentioned, one possible explanation for some of the results observed in the co-culture experiments (Fig. 2 & 3) could be the secretion of diffusible substances like bacteriocins. Thus, a simple bacteriocin assay on solid blod agar medium was performed in this thesis. By using blood agar plates, not just effects on *S. pyogenes* growth can be visualized and monitored, but additionally the effects on one important virulence trait of *S. pyogenes*, the β-hemolysis caused by streptolysin S expression, can easily be observed. For the bacteriocin assay used here, the first streaked bacteria (bacteriocin producer) were inactivated by chloroform vapour. Hence, any observed effect is caused by chloroform stable-difussible substances which are produced by the first streaked bacteria.

From all five tested bacterial species, only *S. salivarius* K12 can kill *S. pyogenes* (Fig. 4a). Even though *E. faecalis* can not kill *S. pyogenes*, this experiment revealed an effect on *S. pyogenes* hemolytic activity (Fig. 4b). Based on this assay, both *S. oralis* strains and *E. coli* Nissle have no effect on *S. pyogenes* viability and hemolytic activity. (Fig. 4c, d, e).

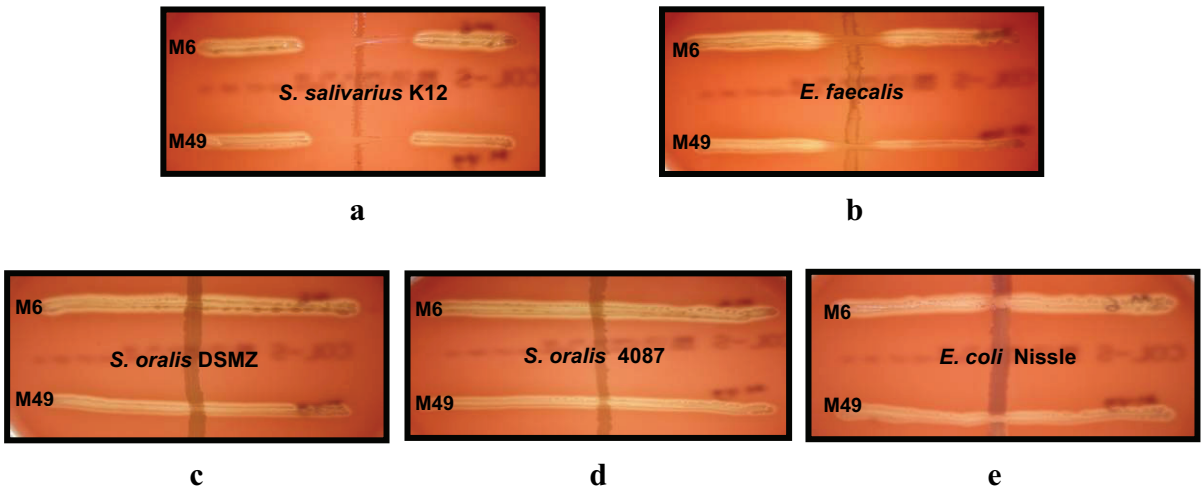


Fig. 4 Bacteriocin assay on blood agar plate.

The tested bacteria (bacteriocin producers) were first streaked in vertical direction. After 18 hours of growth producing strains were treated with chloroform vapour. Subsequently, *S. pyogenes* were cross streaked in horizontal direction and grown ON prior to plate inspection.

III.3 Effect on *S. pyogenes sag*A transcription

Streptolysin S of *S. pyogenes* is a secreted toxin and the protein is encoded by the *sag*A gene, located in a 9 gene *sag*-operon. Next to causing the hallmark phenotype of β-hemolysis on blood agar plates, streptolysin S is an important virulence factor during *S. pyogenes*-host interactions which acts on many different levels.

Gene expression on the transcription level can be monitored using a gene reporter system. For this study a *sag*A-luciferase (luc) reporter system for the *S. pyogenes* serotype M49 was already available. In this strain the luciferase activity which is expressed based on transcription from the *sag*A promoter was monitored by measuring the luminescence in the presence of luciferin and glycil-glycin using a luminometer.

III.3.1 Construction of an *S. pyogenes* serotype M6 *sag*A-luc reporter gene strain

A reporter gene fusion of the *sag*A promoter in the *S. pyogenes* serotype M6 was not available. Therefore, such a recombinant strain was constructed prior to the experiments.
The organization of the *sag*A upstream region is different in various M serotype strains, thus the existing plasmid that was used for *S. pyogenes* M49 could not be used. Figure 5 schematically illustrates the genomic organization upstream of the *sag* operon.

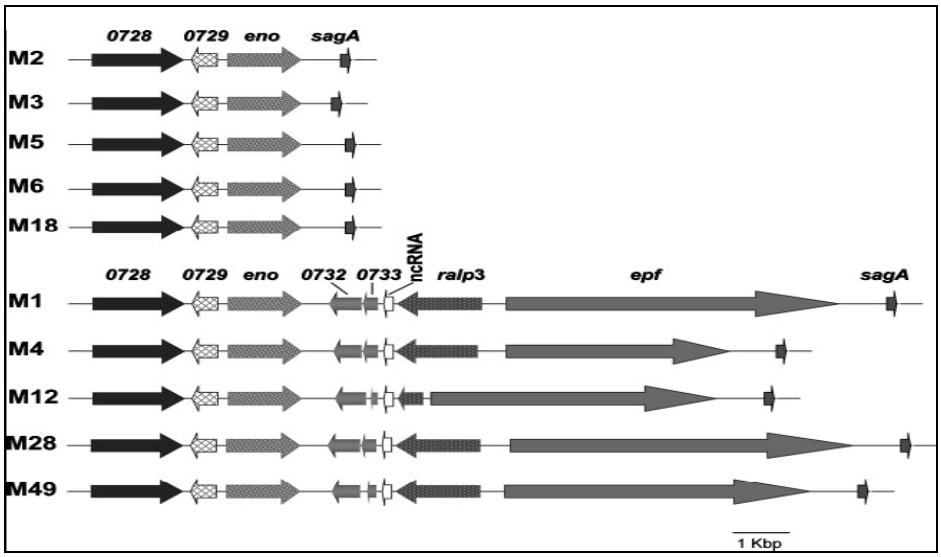


Fig. 5 Organization of the genomic region upstream of *sag*A in different *S. pyogenes* serotypes (Kreikemeyer *et al*., 2007)**.**

eno encodes enolase, *sagA* encodes streptolysin S, *ralp3* encodes RofA-like protein 3, *epf* encodes a novel plasminogen-binding protein, ncRNA is a putative untranslated small RNA species, numbers are other *S. pyogenes* open reading frames (SPy numbers based on the serotype M1 genome sequence).

The same luc reporter plasmid system utilized for the construction of the *sag*A-luc in *S. pyogenes* M49 was used. Briefly, a 768 bp upstream region of *sag*A from *S. pyogenes* M6 was inserted in MCS I (multiple cloning sites) of pFW5-luc through *Ecl*XI and *Bam*HI restriction sites and then transformed into *E. coli* DH5α. A picture of the pFW5-luc plasmid is shown in Fig. 6. The upstream region of *sagA* which was inserted in pFW5-luc is shown in Fig. 8.

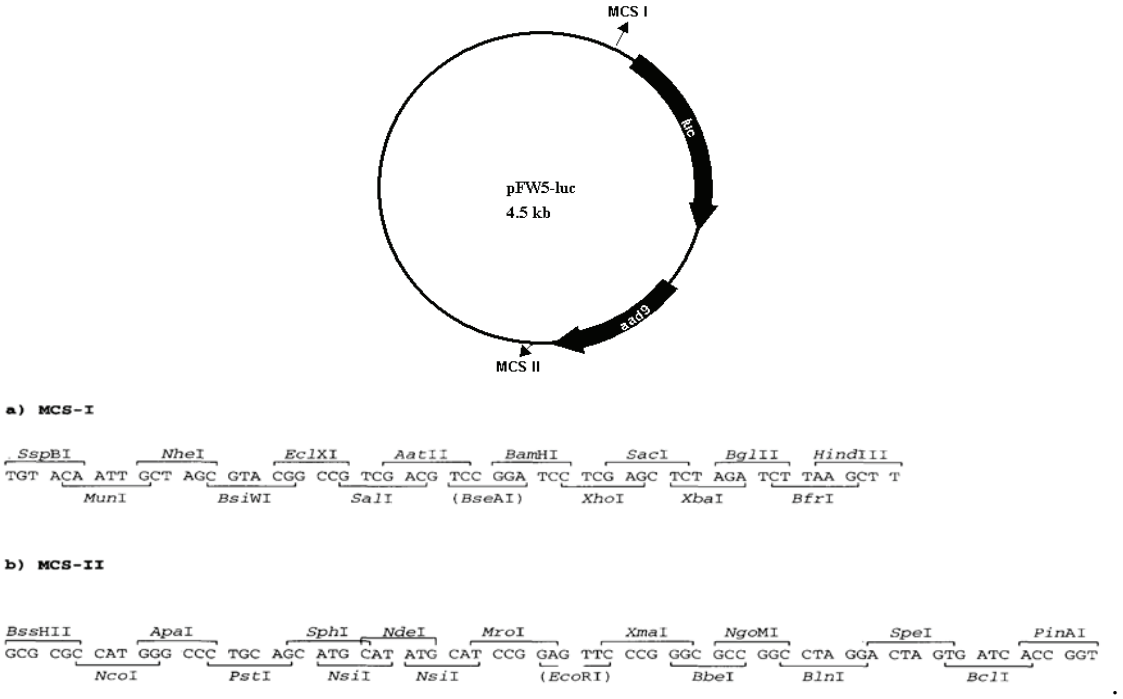


Fig. 6 The pFW5-luc plasmid and its MCS (Kreikemeyer *et al*., 2001; Podbielski *et al*., 1996)

The plasmids from two *E. coli* transformants were isolated and their integrity was confirmed by migration and restriction digest analysis. The recombinant plasmid from the transformant was compared with the empty pFW5-luc vector in agarose gel. Both recombinant plasmids migrated slower than the empty pFW5-luc (Fig. 7a). Restriction analysis with *Ecl*XI and *Bam*HI resulted in two fragments, one presenting the vector backbone (4.5 Kb in size, pFW5-luc) and the other corresponding to the insert (769 bp in size, promoter *sag*A region) (Fig. 7b).

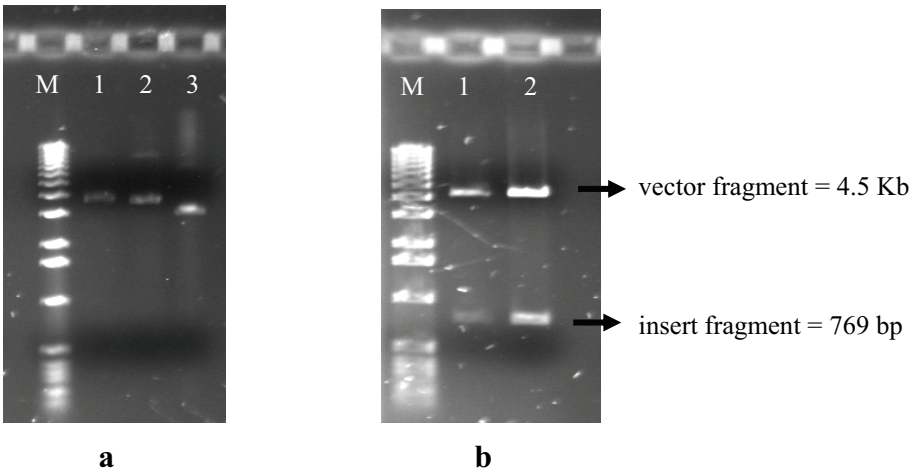


Fig. 7 Verification of the recombinant pFW5*sag*A-luc plasmid.

(a) Undigested samples, (b) recombinant plasmid after *Ecl*XI and *Bam*HI restriction digest. Lane 1&2: pFW5*sag*A-luc plasmid, lane 3: empty plasmid, M: 1 kb ladder DNA marker.

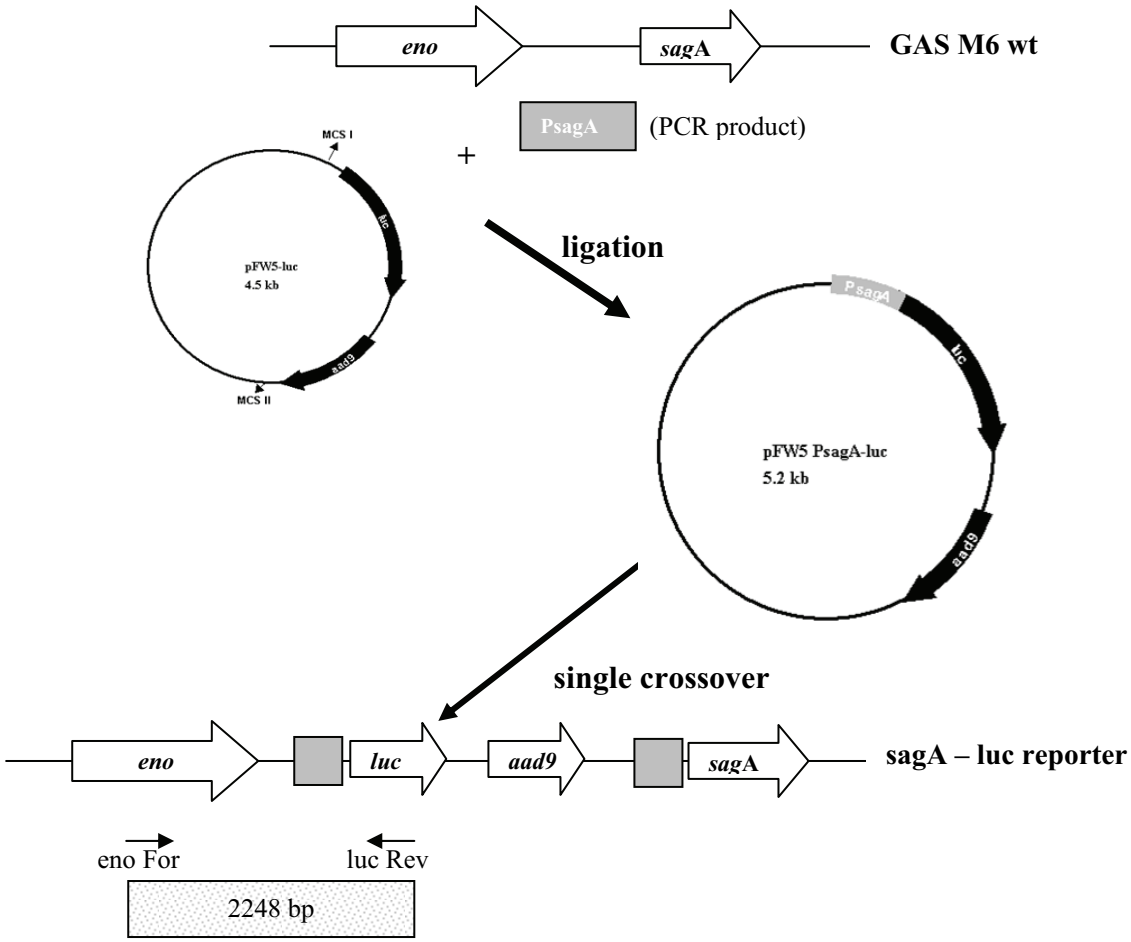


Fig. 8 Schematic drawing of the steps in the construction of the *sag*A-luc reporter system for *S. pyogenes* M6.

This figure is not drawn to scale. *eno*, encoding enolase; *aad*9, encoding spectinomycin resistance; PsagA, fragment containing *sag*A promoter; enoFor and lucRev, primer pair for *S. pyogenes* transformant integrity analysis.

The correct pFW5*sag*A-luc recombinant plasmid was electroporated into *S. pyogenes* M6 and through a single crossover event integrated into the *S. pyogenes* M6 genome. A PCR using primers enoFor (5'- CGGTGGATCACACTCAGATG-3') and lucRev (5'-TTAGGTAACCC AGTAGAT-3') was done on chromosomal DNA of the *S. pyogenes* M6 transformant to verify the correct position of integration (Fig. 8 & 9). Results shown in Fig. 9 confirmed the correct integration of the recombinant plasmid in the desired location, as a PCR fragment of 2.2 Kb was present in *S. pyogenes* M6 transformants but not in the wt.

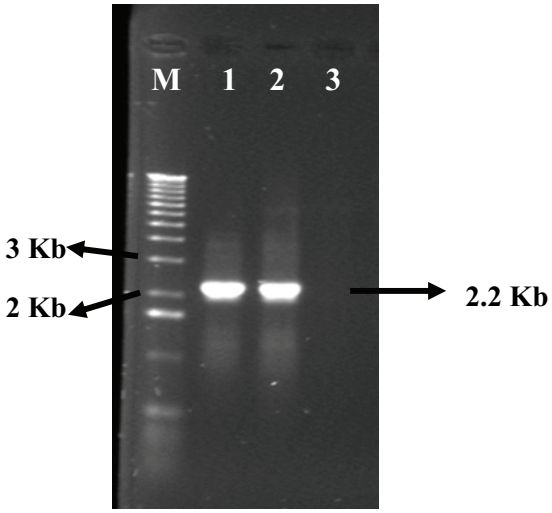


Fig. 9 PCR analysis of the *S. pyogenes* M6 *sag*A-luc transformants.

M: 1 kb ladder DNA marker, lane 1&2: *S. pyogenes* M6 *sag*A-luc transformants, lane 3: *S. pyogenes* M6 wt.

III.3.2 *sag*A-luc activity measurement in the presence of selected oral bacteria and *E. coli* Nissle

The recombinant *S. pyogenes sag*A-luc serotype M49 and M6 strains were subsequently used to investigate *sag*A transcription in the presence of tested bacteria using the already introduced two systems, the co-culture direct mix assay and the transwell system setup. Effects of direct and indirect contact were investigated with this approach. *S. pyogenes* reporter strains were co-cultured with low number (10^2 cfu/ml) and high number (10^6 cfu/ml) of selected bacteria to investigate the effect of different cfu mixtures. The number of viable *S. pyogenes* was determined to investigate the correlation between reduced luciferase activity and viable *S. pyogenes* in direct mixed cultures. This was necessary because OD_{600} measurement could not be performed due to the presence of two species in one culture. As a comparison, counting of viable *S. pyogenes* was also done in the transwell system setup. The details of the assay are described in section II.2.9.

From the experiments summarized in Fig. 10-12 it can be concluded that a general reduction of *sag*A transcription in all co-culture experiments occurred. However, this effect was paralleled by reductions in viable *S. pyogenes* numbers (Fig. 13). At the end of the experiment (16 hours) the *sag*A transcription, as measured by the luciferase reporter, was moderately reduced in the presence or low intial numbers of *S. oralis* DSMZ. With nearly no effect on the *S. pyogenes* viability, *E. faecalis* caused a remarkable reduction in *sag*A transcription in both *S. pyogenes* strains, thereby confirming the observations of the bacteriocin assay.

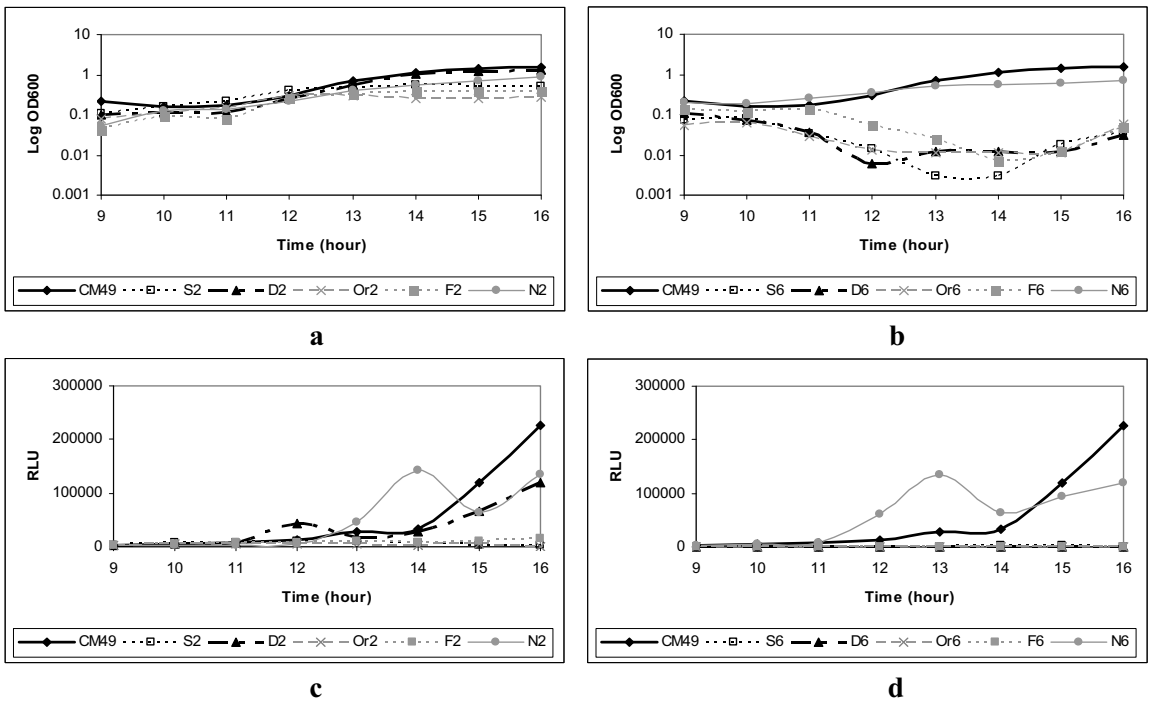


Fig. 10 Co-culture effect on *S. pyogenes* M49 luciferase activity in the transwell system.

Growth curve of *S. pyogenes* M49 *sag*A-luc (a, b) and luciferase activity (c, d) in the presence of 10^2 cfu/ml and 10^6 cfu/ml tested bacteria from the start of the culture, respectively. CM49, culture of *S. pyogenes* M49 alone as a control; S2, D2, O2, F2, N2 culture of *S. pyogenes* M49 in the presence of 10^2 cfu/ml *S. salivarius* K12, *S. oralis* DSMZ, *S. oralis* 4087, *E. faecalis* and *E. coli* Nissle, respectively. S6, D6, O6, F6, N6 culture of *S. pyogenes* M49 in the presence of 10^6 cfu/ml *S. salivarius* K12, *S. oralis* DSMZ, *S. oralis* 4087, *E. faecalis* and *E. coli* Nissle, respectively.

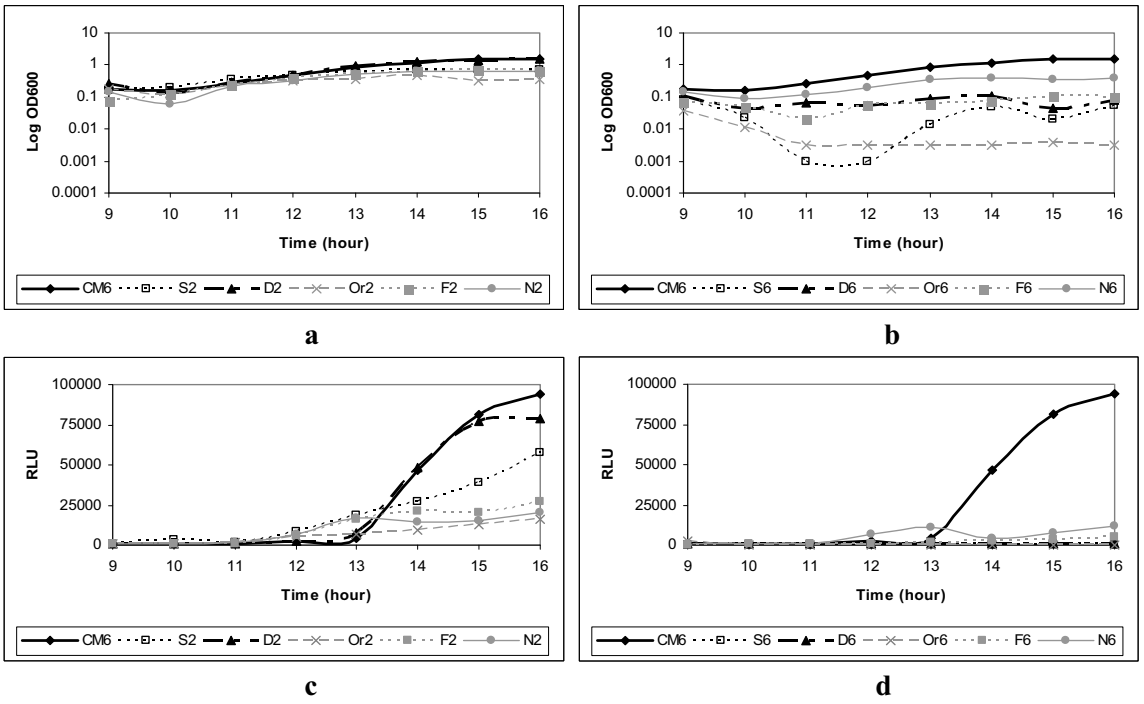


Fig. 11 Co-culture effect on *S. pyogenes* M6 luciferase activity in the transwell system.

Growth curve of *S. pyogenes* M6 *sag*A-luc (a, b) and luciferase activity (c, d) in the presence of 10^2 cfu/ml and 10^6 cfu/ml tested bacteria from the start of the culture, respectively. CM6, culture of *S. pyogenes* M6 alone as a control. For S2, D2, O2, F2, N2, S6, D6, O6, F6, and N6 designations please refer to Fig. 10.

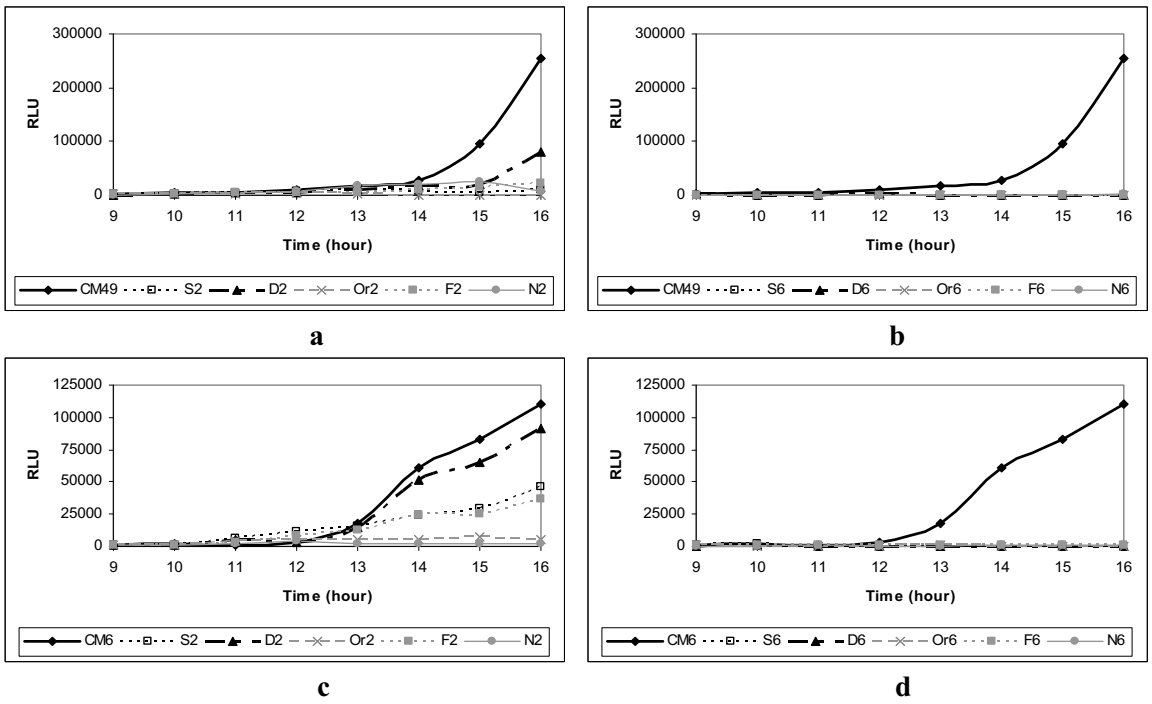


Fig. 12 Co-culture effects on *S. pyogenes* M49 and M6 luciferase activity in direct mix experiments.

Luciferase activity of *S. pyogenes* M49 *sag*A-luc (a,b) and *S. pyogenes* M6 *sag*A-luc in the presence of 10^2 cfu/ml and 10^6 cfu/ml tested bacteria from the start of the culture, respectively. For CM49, CM6, S2, D2, O2, F2, N2, S6, D6, O6, F6, and N6 designations please refer to Fig. 10.

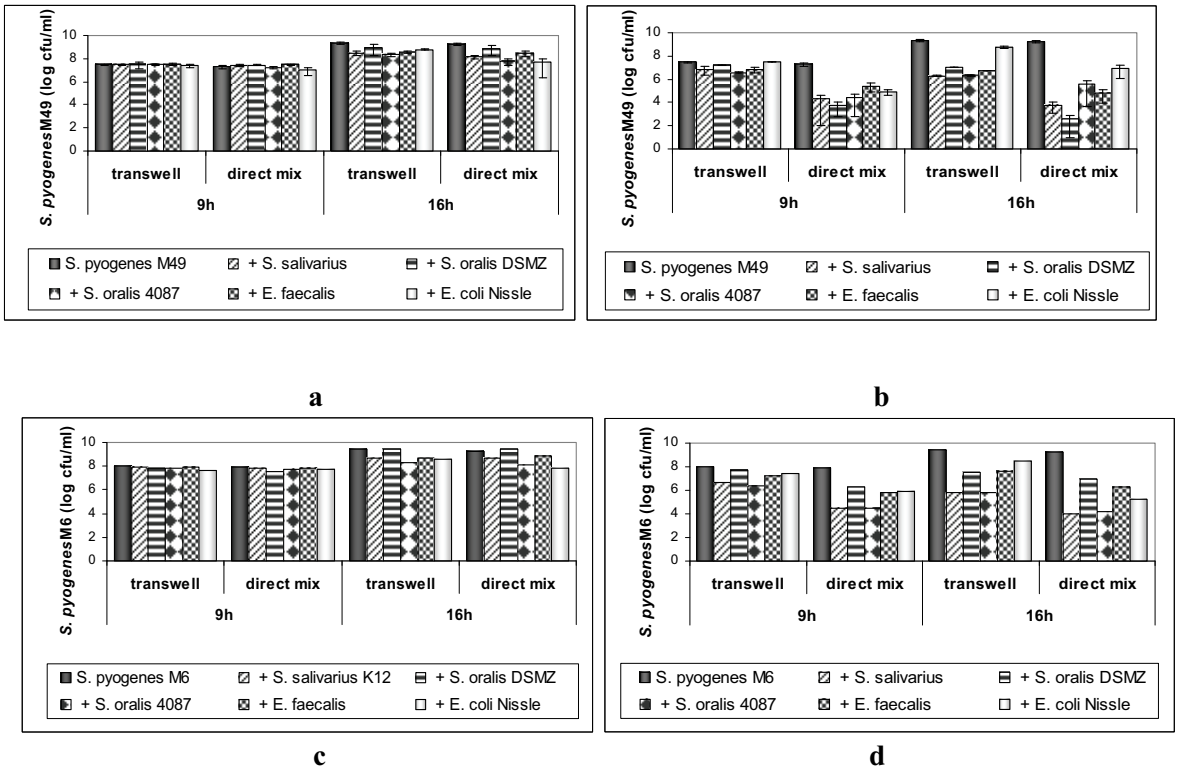


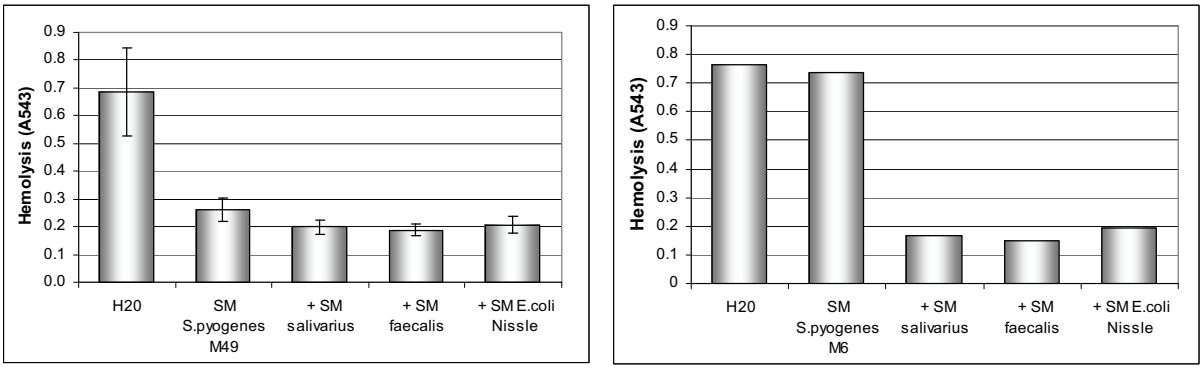
Fig. 13 *S. pyogenes* viable numbers (cfu/ml) in luciferase measurement experiments.

S. pyogenes M49 (a, b) and *S. pyogenes* M6 *sag*A-luc (c, d) viable numbers in the present of 10^2 cfu/ml (a, c) and 10^6 cfu/ml (b, d) tested bacteria from the start of the culture, respectively. Viable bacteria were counted after plating serial dilutions on agar plates at 9 and 16 hours incubation time.

III.4 Effect of spent medium on *S. pyogenes* hemolytic activity

In order to test whether spent medium of the oral species and *E. coli* Nissle, which after centrifugation does not contain bacterial cells anymore, also influences the streptolysin S activity of *S. pyogenes*, an experiment as outlined in section II.2.10 was performed.

Spent medium (SM) of *S. pyogenes* M49 and M6 was used to investigate its contained hemolytic activity in the presence of spent medium of the tested bacteria (Fig. 14). All spent medium of the tested bacteria led to reduction of the hemolytic activity of the streptolysin S contained in the *S. pyogenes* spent medium. Addition of the spent medium from *E. faecalis* gave a slightly higher reduction the A_{543} value of the spent medium from both *S. pyogenes* serotypes compared to spent medium from other tested bacteria. This observed reduction might be again an explanation for the effects of *E. faecalis* seen in the bacteriocin assay (Fig. 4b).



a **b**

Fig. 14 *S. pyogenes* hemolytic activity in the presence of spent medium from tested bacteria.

Spent medium (SM) from *S. pyogenes* M49 (a) and M6 (b) cultures was mixed together with SM of tested bacteria and subsequently used for hemolysis measurement.

III.5 Coaggregation of *S. pyogenes* with oral bacteria and *E. coli* Nissle

Previous work has documented that bacterial coaggregation plays important roles in the development of oral biofilms and during the interaction of bacteria with their respective host (Rickard *et al*., 2003; Lafontaine *et al*., 2004). The assay was done as outlined in the Material and Methods section (II.2.11). The results are shown in Fig. 15. None of the bacterial species investigated revealed any aggregation effect when incubated as a single species. From the combination experiments it is obvious that *S. oralis* 4087 coaggregates together with both *S. pyogenes* serotypes tested.

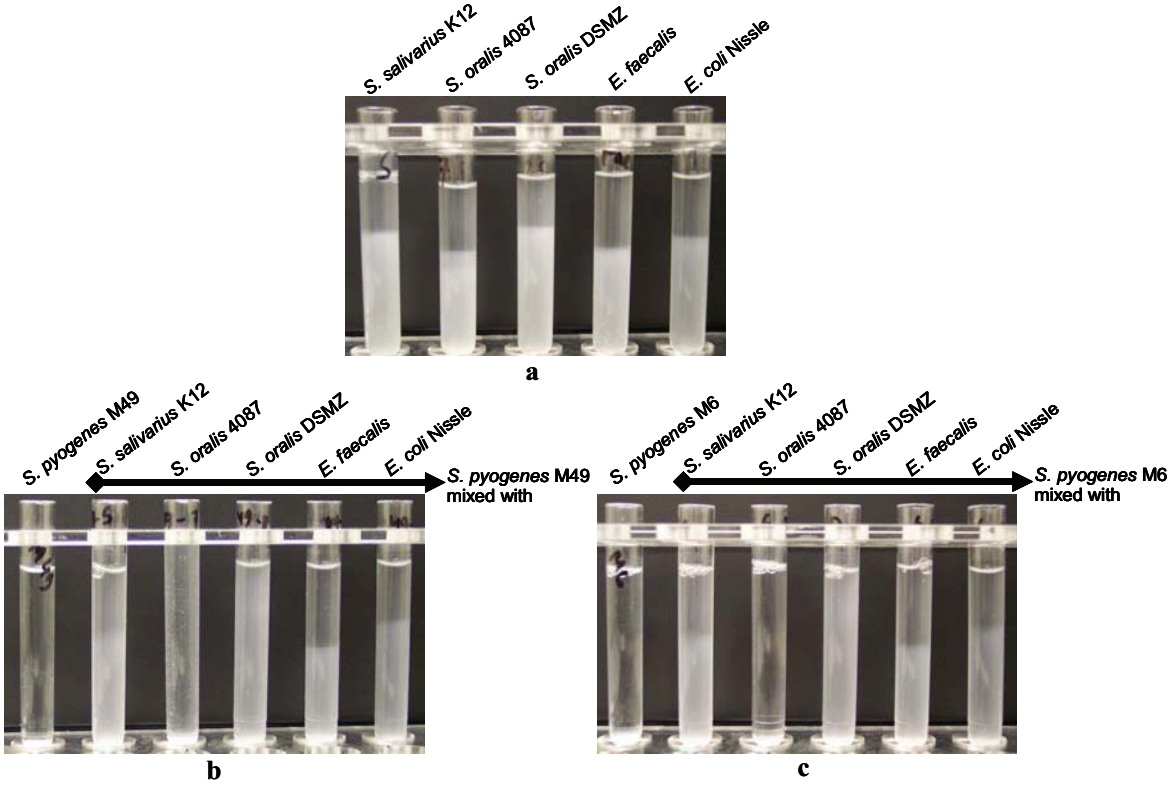


Fig. 15 Coaggregation of *S. pyogenes* M49 and M6 serotypes with selected oral bacteria and *E. coli* Nissle.

Cells suspension of equal volumes from each bacterial strain (2 ml at OD_{600} of 2.0) after 10 s vortexing and standing for 1-2 h at room temperature.

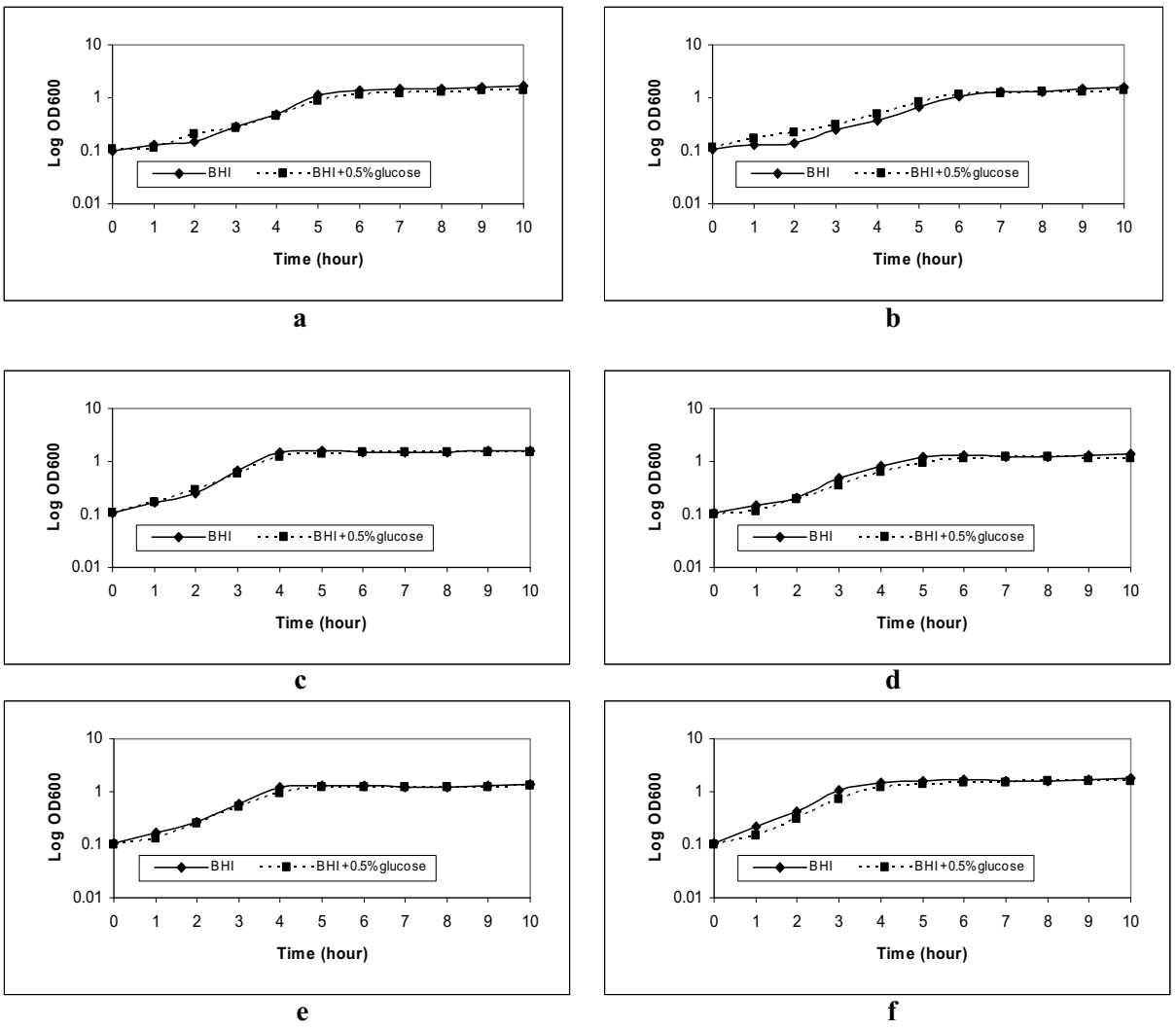
III.6 Effect of oral bacteria and *E. coli* Nissle on *S. pyogenes* biofilms

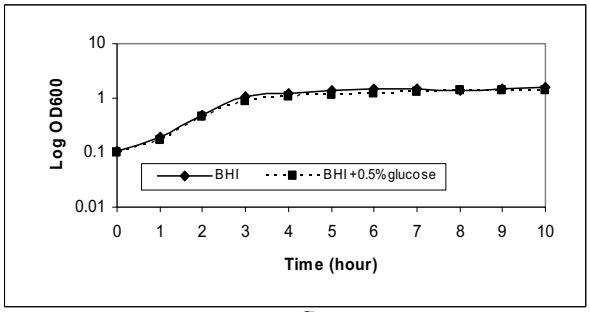
S. pyogenes is able to form biofilms *in vitro*. However, this ability is serotype dependent. Here, biofilm forming ability of two different serotypes, M6 (ubiquitous biofilm producer) and M49 (poor biofilm producer), was investigated in the presence of tested bacteria. These experiments were done to observe whether *S. pyogenes* can integrate into oral biofilms and whether its own biofilm ability is interfered by the presence of oral bacteria. Safranin assays were performed as outlined in II.2.12 in order to quantify biofilms. The average values of all tests and standard deviations were presented as quantitative measurements of biofilms. Of note, in mixed-species biofilms, the A_{492} measured is the absorption generated by the interaction of the mixed bacteria. Thus, it is not known from which bacteria the absorption was produced or dominated, but the changes in absorption can be easily quantified. Microscopic observation was done to visualize the biofilm structure. Technically, SEM is a simple method to observe biofilm structure. However, in some cases, it was difficult to differentiate *S. pyogenes* from the other closely related streptococcal species from the SEM

pictures. Consequently, immunofluorescence staining was chosen as additional method to differentiate *S. pyogenes* from tested bacteria in the mixed-species biofilm.

III.6.1 Evaluation of growth medium and monospecies biofilm behavior

Lembke *et al*. (2006) showed that *S. pyogenes* can form biofilms in BHI broth supplemented with 0.5% glucose. Thus, all biofilm assays were performed using this culture medium. In order to exclude growth inhibition in this medium, growth curve analysis of all tested bacteria was performed in BHI broth supplemented with 0.5% glucose. BHI was used as a control medium, since all bacteria grew well in BHI. The results of the bacterial growth test in BHI and BHI supplemented with 0.5% glucose are summarized in Fig. 16. No big differences were detected in the growth behaviour of the different bacterial species in both media.





g

Fig. 16 Bacterial growth curves in BHI and BHI sumplemented with 0.5% glucose.

S. pyogenes M49 (a), *S. pyogenes* M6 (b), *S. salivarius* K12 (c), *S. oralis* DSMZ (d), *S. oralis* 4087 (e), *E. faecalis* (f), *E. coli* Nissle (g).

Next, prior to the mixed species experiments we first determined the biofilm forming ability of all bacterial species using monospecies cultures and glucose supplemented BHI. From the safranin assays, only *S. pyogenes* M49 and *E. coli* Nissle emerged as poor biofilm producers (Fig. 17). This result was confirmed with scanning electron microscopy (Fig. 18).

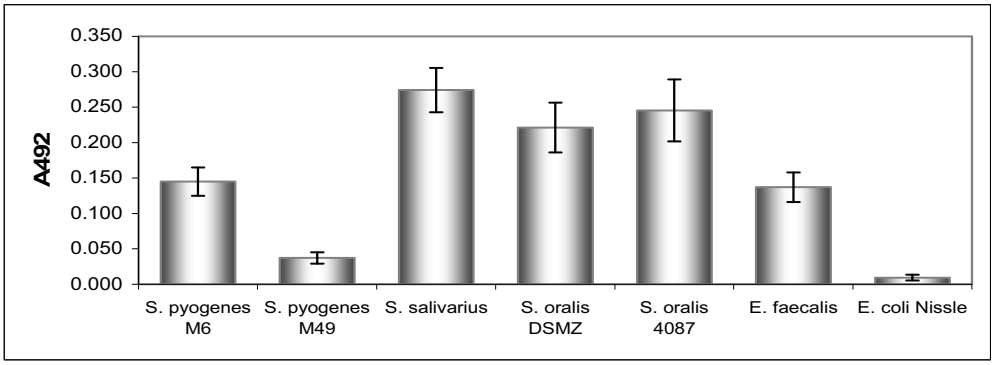


Fig. 17 Safranin assay on monospecies biofilms.

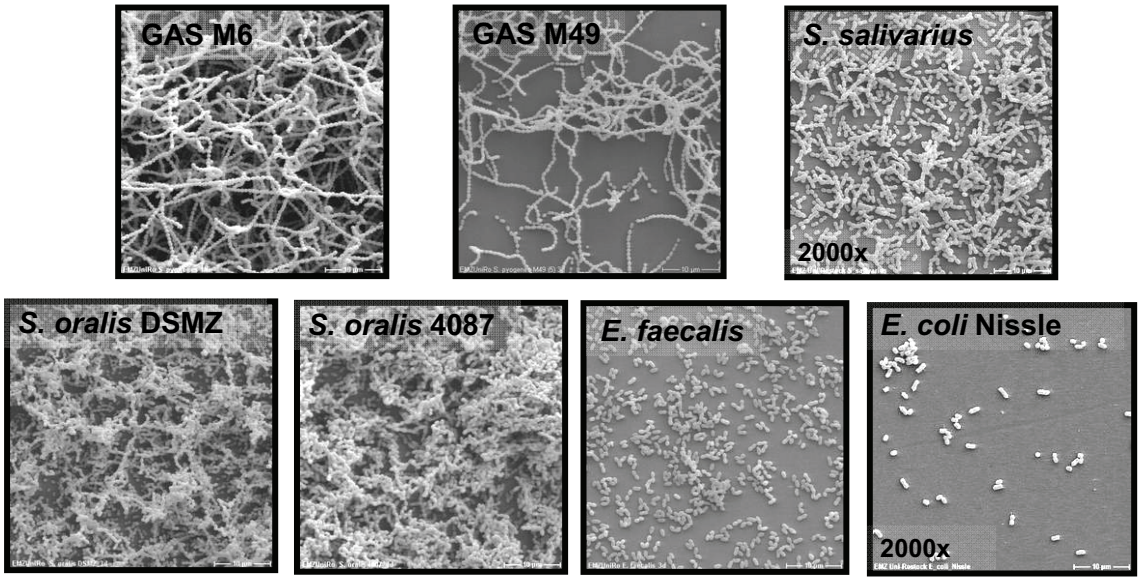


Fig. 18 SEM pictures of monospecies biofilms.

The next addressed question was how viable are the bacteria in 3 day cultures and after biofilm formation. Based on the microscopic observations using LIVE/DEAD staining (method in section II.2.13.1), most bacteria were alive in 3 days old biofilms. Only for *S. oralis* 4087 around 50% of the bacterial population was apparently dead after incubation (Fig. 19). However, this fact could not be confirmed by plating and cfu determination of biofilm cells. Biofilm cells of all tested bacteria did not give any detectable cfu once they are plated on BHI agar plates or blood agar plates.

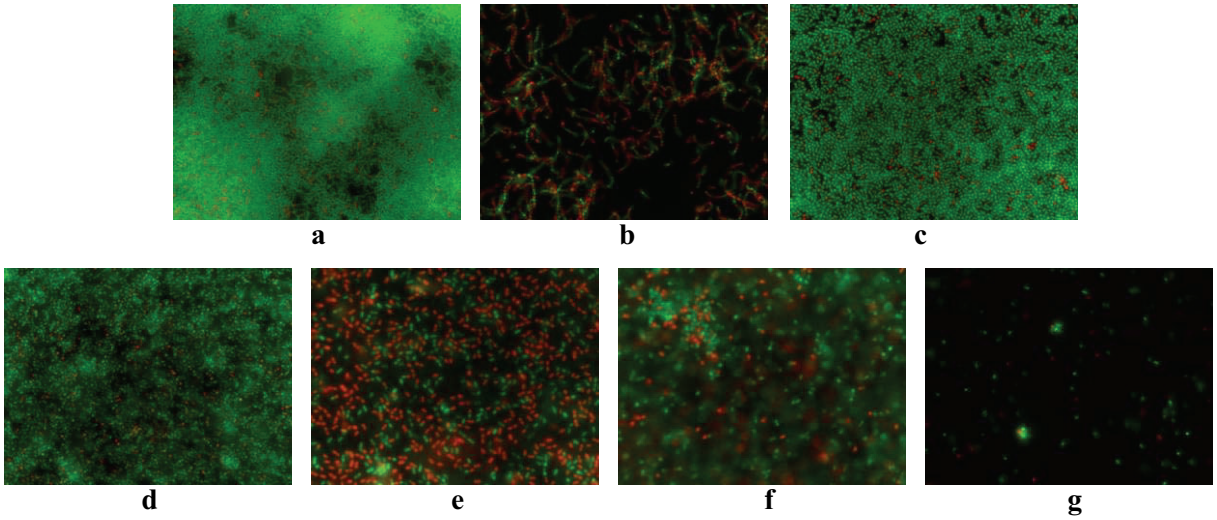


Fig. 19 Fluorescence microscopic observations of single biofilms.

Fluorescence microscope pictures of *S. pyogenes* M6 (a), *S. pyogenes* M49 (b), *S. salivarius* K12 (c), *S. oralis* DSMZ (d), *S. oralis* 4087 (e), *E. faecalis* (f), *E. coli* Nissle (g). Biofilms were grown for 3 days, green fluorescence indicates live bacteria and red fluorescence represents dead bacteria; magnification - 60x.

III.6.2 Investigation of mixed-species biofilms

After elucidation of the optimal conditions for the monospecies biofilms and establishment of all the different methods for biofilm quantification and observation, species behaviour in the mixed setting was now investigated as outlined in II.2.1, II.2.12 and II.2.13.

Mixed biofilms of both *S. pyogenes* serotypes and *S. salivarius* were dominated by *S. salivarius*. This is shown in SEM pictures (Fig. 20a for *S. pyogenes* M6 and Fig. 21a for *S. pyogenes* M49) and more specific in CSLM pictures using immunofluorescence staining (Fig. 22a for *S. pyogenes* M6 and Fig. 23a for *S. pyogenes* M49). For comparison, a picture of the single biofilms observed by CSLM can be seen in Fig. 24. Moreover, a change in A_{492} was observed in mixed *S. pyogenes*/*S. salivarius* biofilms (Fig. 25). In summary, *S. salivarius* K12 clearly dominated in biofilms if cultured together with *S. pyogenes*. Even *S. pyogenes* M6, which can be described as a ubiquitous and strong biofilm builder, is outcompeted by *S. salivarius* K12. These results hint to a rather cannibalistic relationship.

In the case of the mixed *S. pyogenes* biofilms with *S. oralis* (DSMZ and 4087 strain) a different picture emerged. *S. oralis* (short chain bacteria) was almost exclusively found on the bottom of the two species biofilm and *S. pyogenes* (long chain bacteria) was found in the upper layer of the biofilm (Fig. 20b, c and Fig. 21b, c). However, the biofilm was not as strongly dominated by *S. oralis* (Fig. 22b, c and Fig. 23b), as was the case for *S. salivarius*. Strikingly, *S. pyogenes* M49 alone can not be classified as a strong biofilm builder. This is obviously different in the presence of *S. oralis*. Apparently the bottom layer of *S. oralis* cells acts as a substrate for *S. pyogenes* M49, which is now able to grow as a multi-layered top coat in this two species biofilm. Thus, the relationship of *S. pyogenes* M6/*S. oralis* is more or less mutualistic, whereas the combination *S. pyogenes* M49/*S. oralis* exerts a rather commensalistic joint life style under the experimental conditions used in this study.

Both, *E. faecalis* and *E. coli* Nissle reduce the biofilm forming ability of *S. pyogenes* M6 (Fig. 20d, e and Fig. 22d). This was also verified in safranin assays, in which the A_{492} value of *S. pyogenes* M6 mixed biofilms with both bacteria was reduced, compared to single *S. pyogenes* M6 biofilms (Fig. 25a).

The interaction of *S. pyogenes* M49 with *E. faecalis* has no benefit or disadvantage for both species. In all experiments no changes were observed compared to the single species settings. This is totally different if we look at the *S. pyogenes* M49/*E. coli* Nissle combination. *E. coli* Nissle is apparently a very beneficial interaction partner for this serotype, since thick *S. pyogenes* M49 biofilms were visible (Fig. 21e).

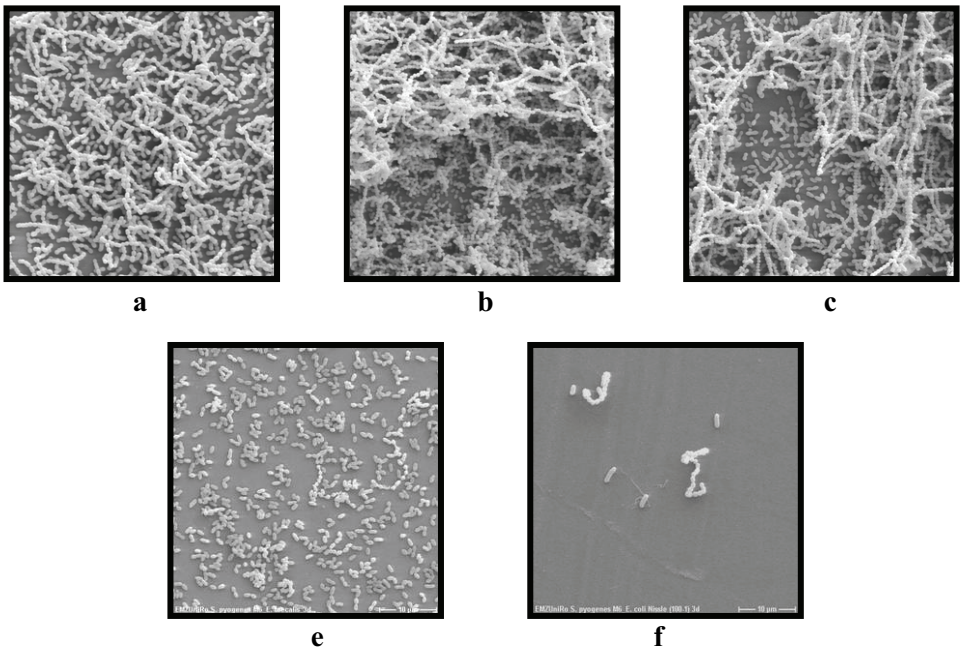


Fig. 20 SEM pictures of *S. pyogenes* M6 mixed-species biofilms.

SEM pictures of *S. pyogenes* M6 mixed species biofilms with *S. salivarius* K12 (a); *S. oralis* DSMZ (b); *S. oralis* 4087 (c); *E. faecalis* (d); *E. coli* Nissle (e). Biofilm was grown for 3 days; magnification of SEM-2000x.

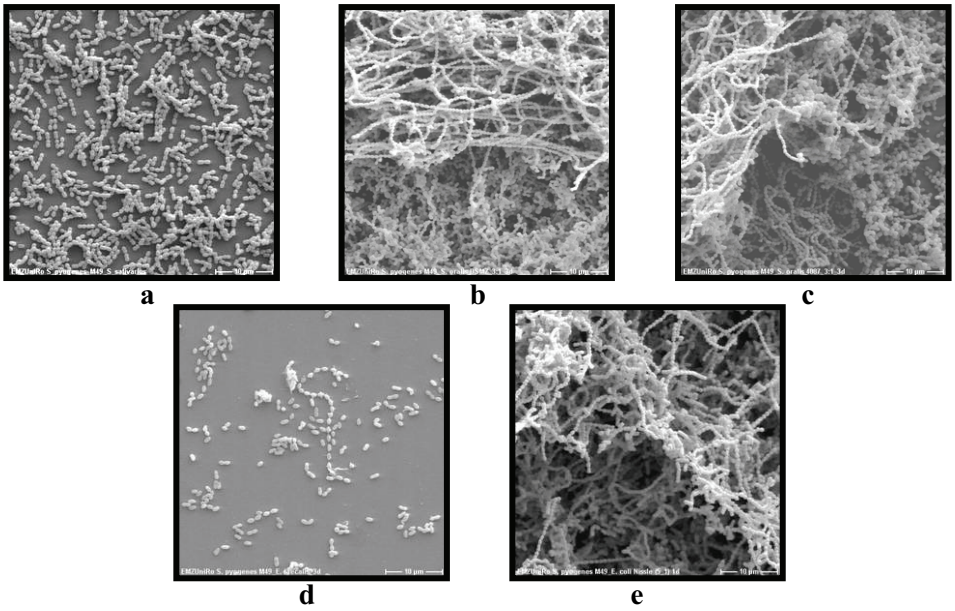


Fig. 21 SEM pictures of *S. pyogenes* M49 mixed-species biofilms.

SEM pictures of *S.pyogenes* M49 mixed species biofilms with *S. salivarius* K12 (a); *S. oralis* DSMZ (b); *S. oralis* 4087 (c); *E. faecalis* (d); *E. coli* Nissle (e). Biofilm was grown for 3 days; magnification of SEM-2000x.

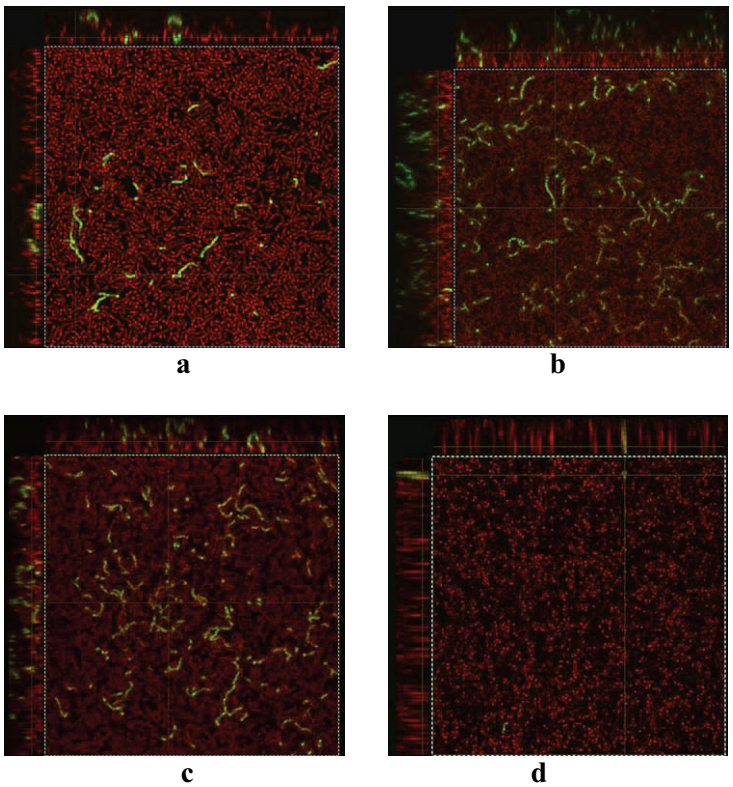
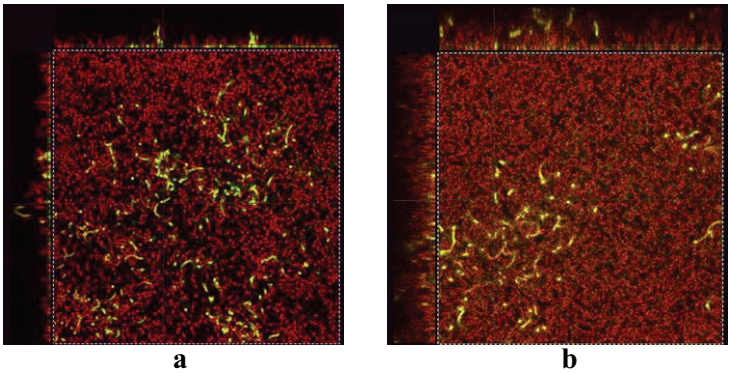


Fig. 22 CSL microscopic observations of *S. pyogenes* M6 mixed-species biofilms.

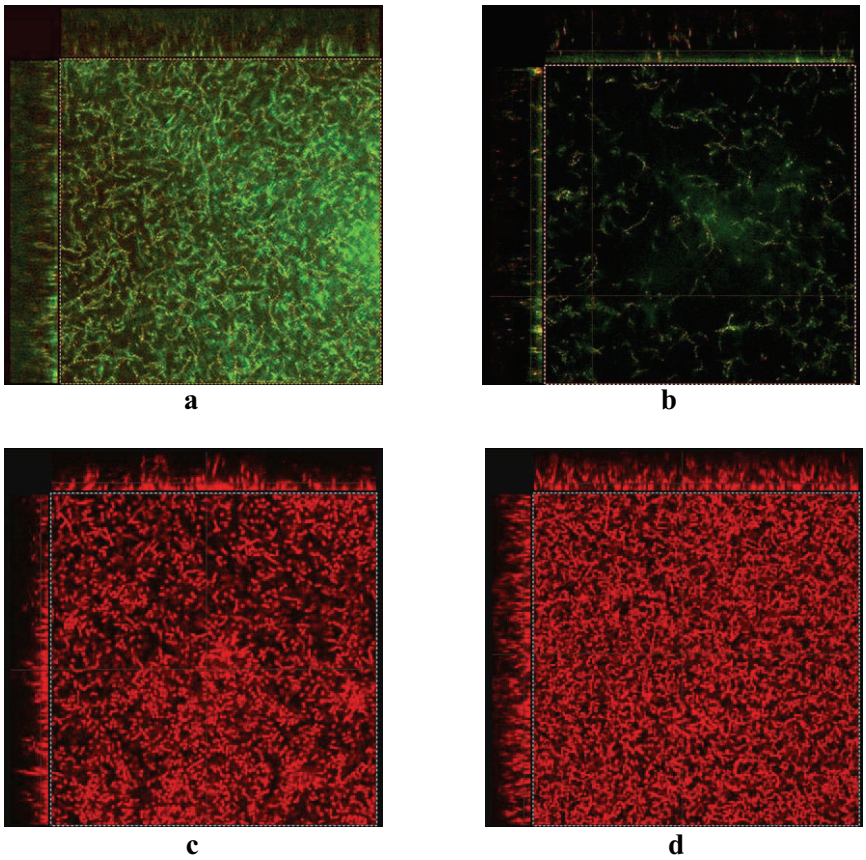
CSL microscope pictures of *S. pyogenes* M6 mixed species biofilms with *S. salivarius* K12 (a); *S. oralis* DSMZ (b); *S. oralis* 4087 (c); *E. faecalis* (d). Biofilm was grown for 3 days, *S. pyogenes* is visualized by green fluorescence and red fluorescence stained all Gram positive bacteria; magnification-60x.



a **b**

Fig. 23 CSL microscopic observations of *S. pyogenes* M49 mixed-species biofilms.

CSL microscope picture of *S. pyogenes* M49 mixed species biofilms with *S. salivarius* K12 (a); *S. oralis* 4087 (b). Biofilm was grown for 3 days; *S. pyogenes* is visualized by green fluorescence and red fluorescence was used to stain all Gram positive bacteria; magnification-60x.

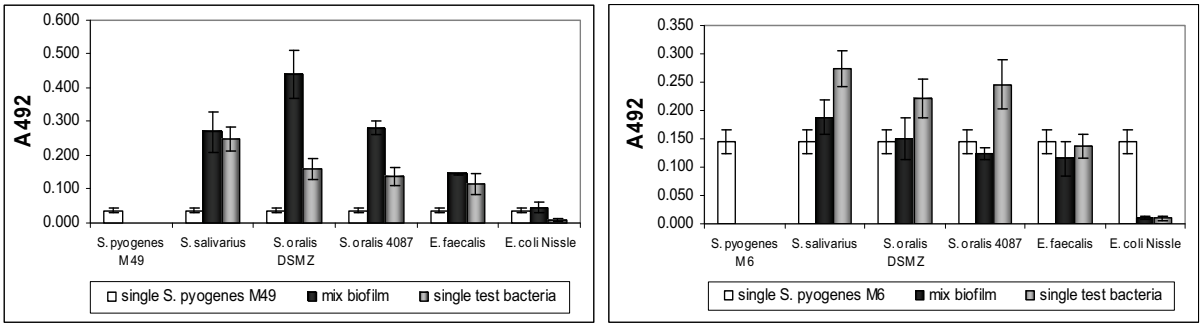


a **b**

c **d**

Fig. 24 CSL microscopic observations of monospecies biofilms.

CSL microscopic pictures of *S. pyogenes* M6 (a); *S. pyogenes* M49 (b); *S. salivarius* K12 (c); *S. oralis* 4087 (d); Biofilm was grown for 3 days; *S. pyogenes* stained in green and all of the Gram positive species stained in red; magnification-60x.



a **b**

Fig. 25 Safranin assay of mixed-species biofilms.

Absorption in safranin assay (A_{492}) from mixed species biofilms of *S. pyogenes* M6 (a) and M49 (b).

Taken together, this part of the study revealed quite astonishing, unexpected and diverse results from those co-existence experiments.

III.6.3 The effect of artificial saliva on the species interaction

For better mimicking of the condition in the oral cavity, artificial saliva was mixed in a ratio of 3:1 with BHI supplemented 0.5% glucose. The effect of artificial saliva was determined for monospecies biofilms and mixed species biofilms. All bacteria can still grew in this changed medium, which was observed from the turbidity in the wells used for this assay. Overall, artificial saliva reduced all monospecies biofilm A_{492} values except for *S. oralis* DSMZ (Fig. 26). This result was additionally confirmed by inspection of SEM pictures (Fig. 27).

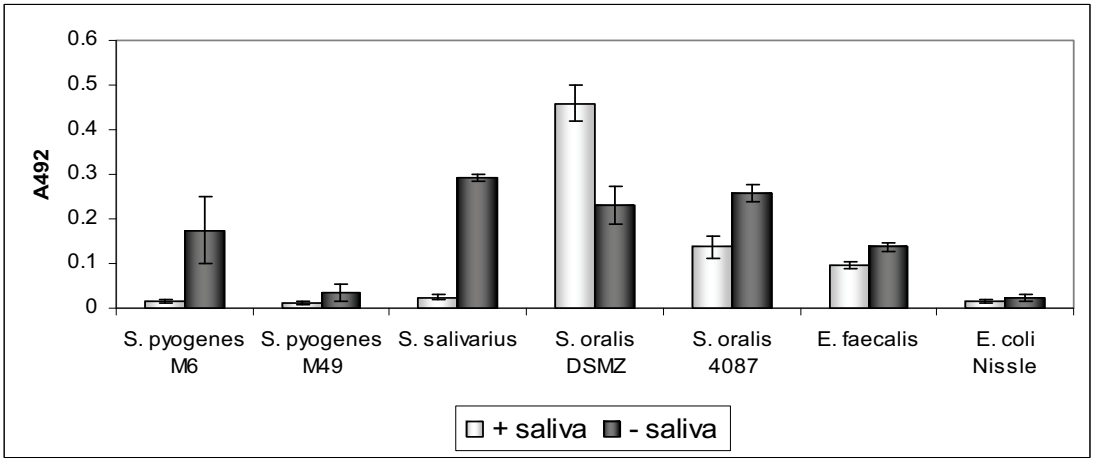


Fig. 26 Artificial saliva effect on monospecies biofilms.

Absorption of safranin assay (A_{492}) from monospecies biofilms in the presence of artificial saliva.

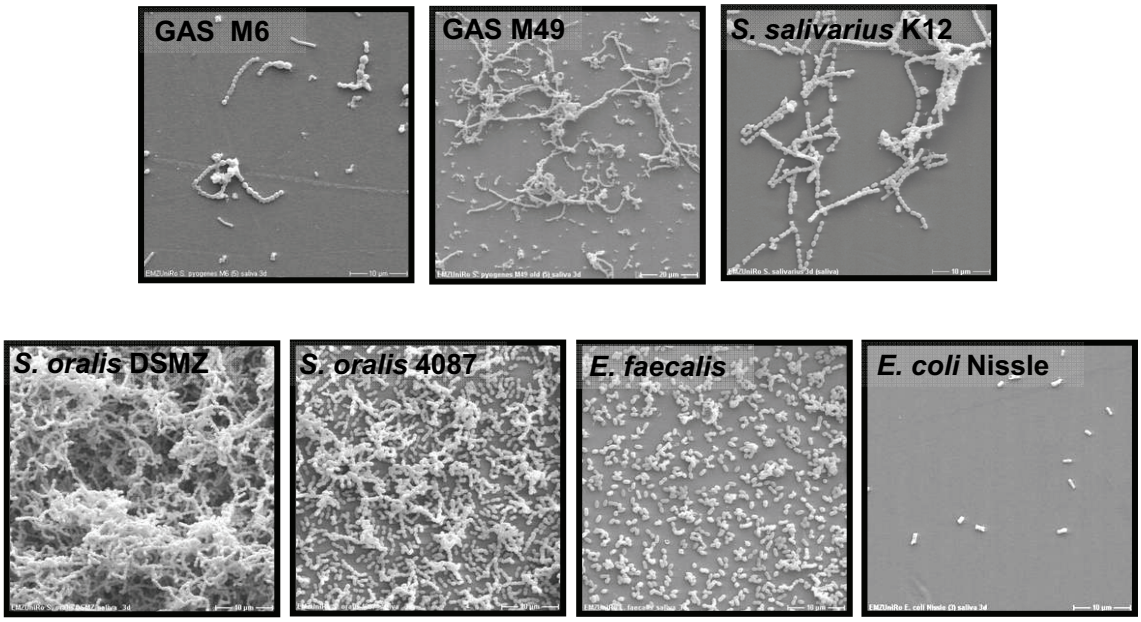


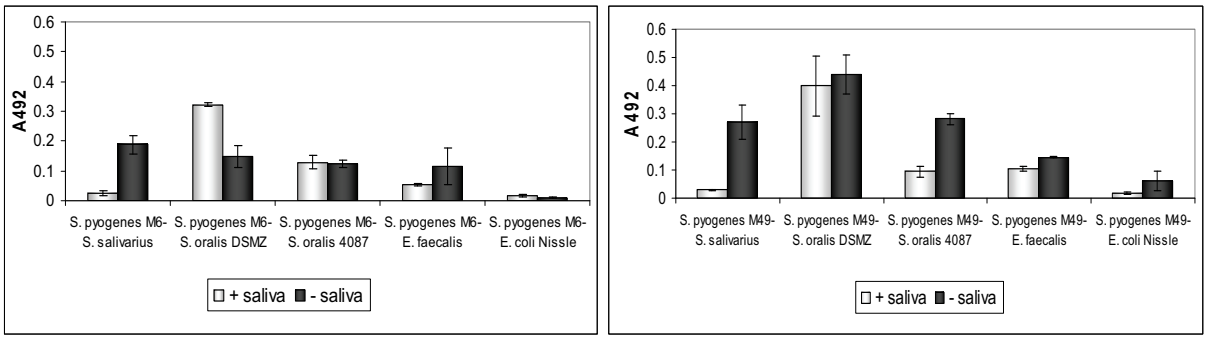
Fig. 27 SEM pictures for artificial saliva effect on monospecies biofilms.

The addition of artificial saliva to the experimental setup of the mixed species also reduced the measurable A_{492} values (Fig. 28a & b). However, this was not the case for the *S. pyogenes* M6/*S. oralis* DSMZ test, for which an increase of the A_{492} was observed (Fig. 28a). An apparent explanation could be the general physical domination of *S. oralis* DSMZ in the mixed-species setup and the increasing effect of the artificial saliva on *S. oralis* DSMZ as single species.

Supplementation of artificial saliva did not change the two discernible layers which were formed by the combination of any *S. oralis* with *S. pyogenes* (Fig. 29 b, c & Fig. 30b).

The domination of *S. salivarius* in the combination with *S. pyogenes* M6 was changed by saliva addition. Now *S. pyogenes* M6 was found as dominant species (compare Fig. 20a & 29a) illustrating the dramatic effect saliva components could have. Also *S. pyogenes* M49 out competed *S. salivarius* in the presence of salivary components, although the biofilm mass did not change (compare Fig. 21a; almost exclusively *S. salivarius* with Fig. 30a; almost exclusively *S. pyogenes* M49).

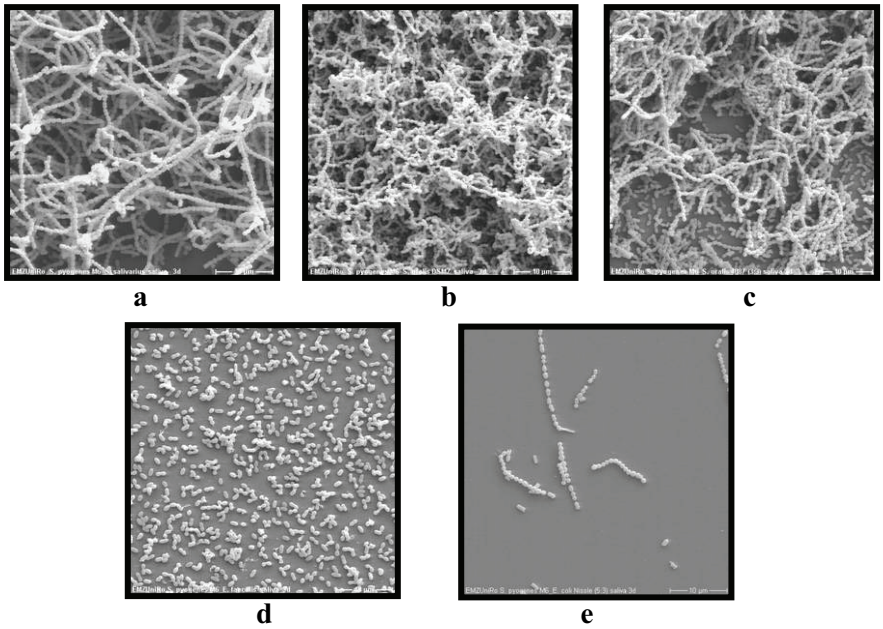
No crucial changes were observed by saliva supplementation in the interaction of *E. faecalis* and *E. coli* Nissle, as Nissle still turns *S. pyogenes* M49 into a good biofilm builder (Fig. 29d & e; Fig. 30c & d).



a **b**

Fig. 28 Artificial saliva effect on mixed-species biofilms.

Absorption of safranin assay (A_{492}) from mixed-species biofilms in the presence of artificial saliva. *S. pyogenes* M6 experiments (a); *S. pyogenes* M49 experiments (b).

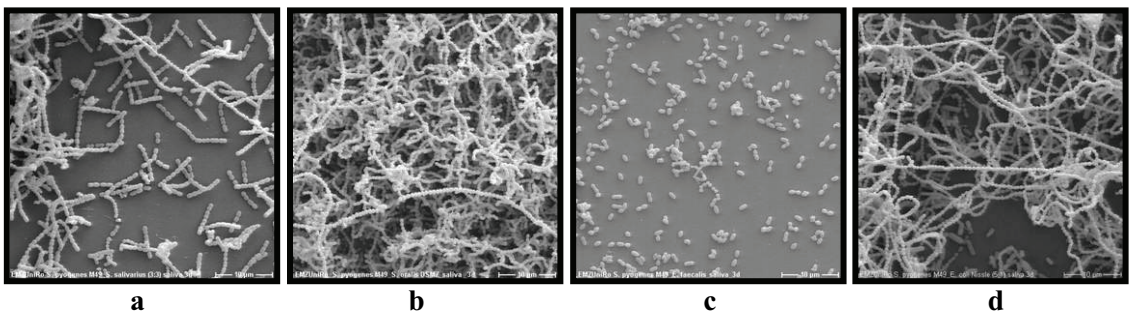


a **b** **c**

d **e**

Fig. 29 Artificial saliva effect on *S. pyogenes* M6 mixed-species biofilms.

SEM pictures of *S. pyogenes* M6 mixed species biofilms with *S. salivarius* K12 (a); *S. oralis* DSMZ (b); *S. oralis* 4087 (c); *E. faecalis* (d); *E. coli* Nissle (e). Biofilm was grown for 3 days in the presence of artificial saliva; magnification of SEM-2000x.



a **b** **c** **d**

Fig. 30 Artificial saliva effect on *S. pyogenes* M49 mixed species biofilms.

SEM pictures of *S. pyogenes* M49 mixed species biofilms with *S. salivarius* K12 (a); *S. oralis* DSMZ (b); *E. faecalis* (c); *E. coli* Nissle (d). Biofilm was grown for 3 days in the presence of artificial saliva; magnification of SEM-2000x.

III.7 Effect of oral bacteria and *E. coli* Nissle on *S. pyogenes* adherence to and internalization into host cells

S. pyogenes attachment to host cells is an important step to initiate an infection. Under certain conditions, *S. pyogenes* attachment is followed by internalization of bacteria into the host cell. As the results introduced in the previous sections have clearly established that oral bacterial species and *E. coli* Nissle in mixed-species communities interact with *S. pyogenes* it was now investigated how these interactions might influence the *S. pyogenes* host cell contact. For this purpose the *S. pyogenes* adherence to and internalization into HEp-2 cells was studied under the influence of the oral bacterial species *S. salivarius*, *S. oralis*, and *E. faecalis* as well as *E. coli* Nissle. Different experimental setups were chosen to mimick all possible interaction strategies: (i) *S. pyogenes* was first allowed to contact the HEp-2 cells prior to adding the other species to the host cell infection scenario. This strategy was expected to give hints whether the other species might support, delay or even cure harmfull effects which *S. pyogenes* can cause to host cells. (ii) Initially, the other bacterial species were allowed to make contact with the HEp-2 cells and only as a subsequent step *S. pyogenes* was introduced to the infection setting. This setup could give indications whether HEp-2 cells can be protected from *S. pyogenes* assault. (iii) As sort of an intermediate situation, both species were added to the host cells at the same time. In addition to quantitative assays, microscopic observations were also done for visualization of experimental results.

III.7.1 Quantitative assay

From all seeding strategies applied in this study, only the strategy (ii), in which *S. pyogenes* was added subsequent to the tested species had significant effects. The initial presence of the tested bacteria led to a marked reduction in adhered *S. pyogenes* bacteria (Fig. 31).

.

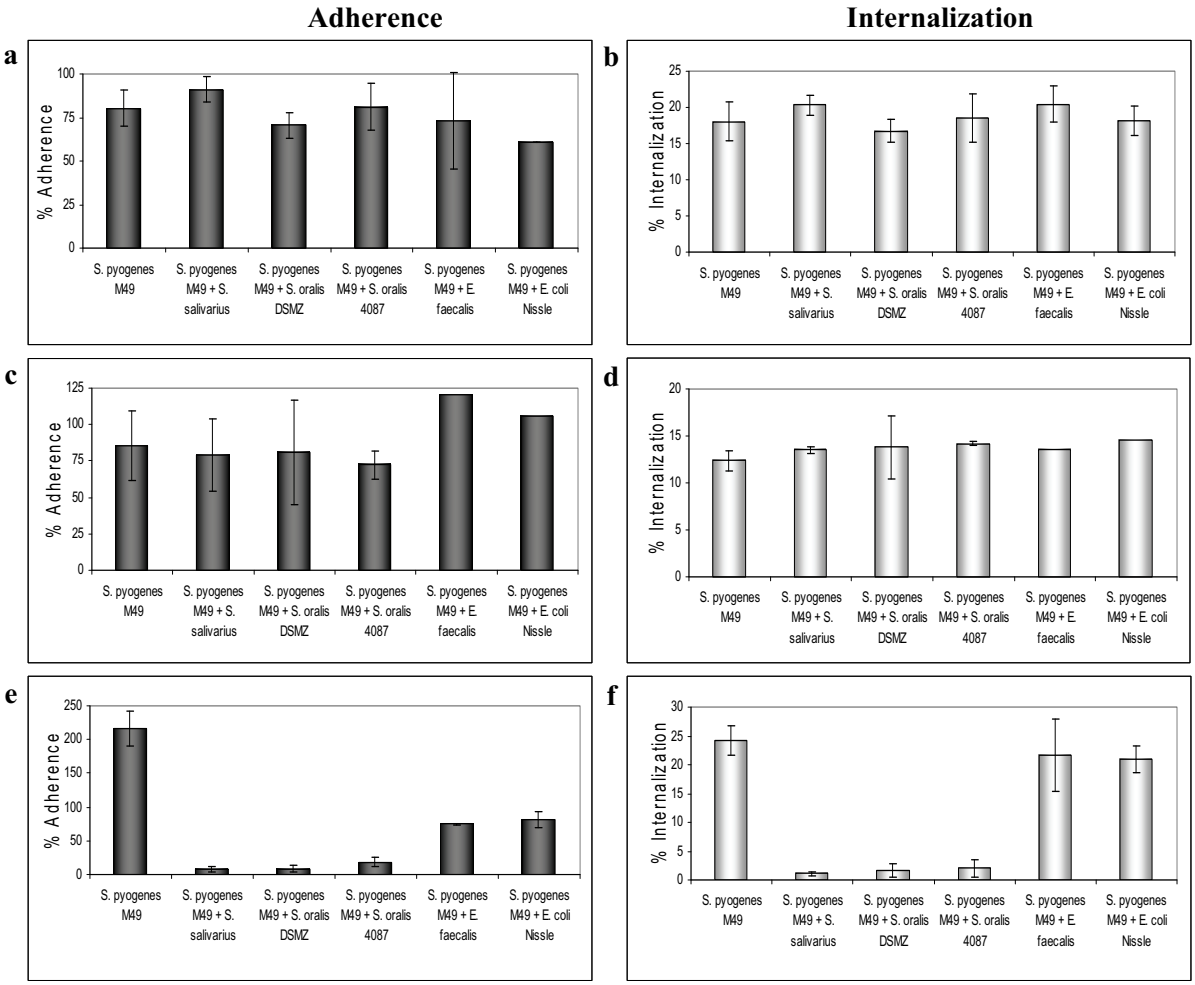


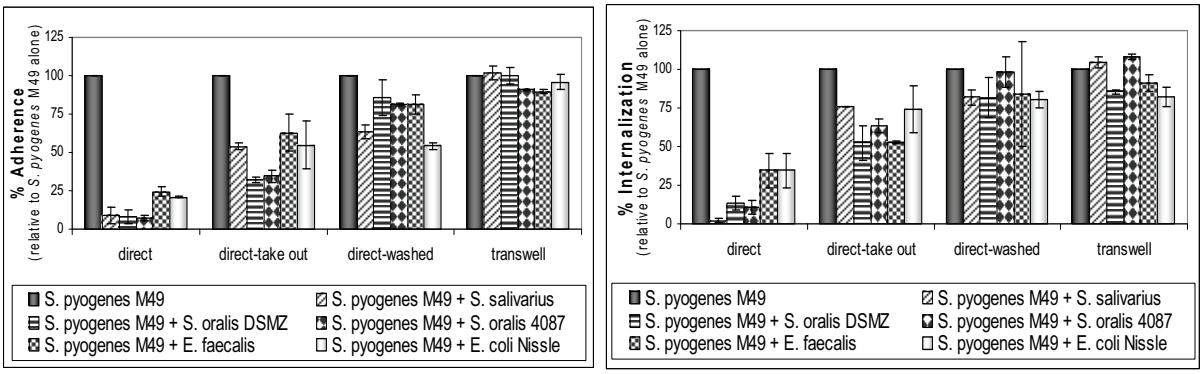
Fig. 31 *S. pyogenes* M49 adherence and internalization assay.

Quantitative assay of *S. pyogenes* M49 adherence to and internalization into HEp-2 cells with simultaneous (a, b); *S. pyogenes* first (c, d) and *S. pyogenes* last (e, f) seeding strategy.

This reduction in the number of adherent *S. pyogenes* M49 bacteria was also reflected in decreasing numbers of *S. pyogenes* which were found internalized into the HEp-2 cells. In conclusion, *S. salivarius*, *S. oralis*, and partially *E. faecalis* protected HEp-2 cells from *S. pyogenes* attack, however, exclusively if these species interacted first with the host cell monolayers.

These results raised the question about the mechanism behind this protection effect. In order to approach this query, the experimental setup was further modified. First, the initially added bacterial species were not removed from the HEp-2 cell monolayer prior to adding *S. pyogenes*. As a second variation, the tested bacteria were removed by a simple change in cell culture medium. The third modification introduced a vigorous washing step after test-bacteria removal. As a forth alteration, the experiments were done using the transwell system. These modifications were expected to give hints if the protection effect relies on direct contact of the

tested bacteria with the HEp-2 cell monolayers or if sterical hindrance and/or diffusible substances are enough to reduce the *S. pyogenes* host cell adherence and internalization.
Results shown in Fig. 32 evidently demonstrated that direct contact of the tested oral species is crucial for the protection effect, as all removal and washing steps apparently decreased the effect and, moreover, the transwell system did not lead to any reduced *S. pyogenes* adherence/internalization of the infected HEp-2 cell monolayers.



a **b**

Fig. 32 The influence of direct contact of the tested bacteria on the protection effect.

Adherence (a) and internalization (b) of *S. pyogenes* M49 using the *S. pyogenes* last seeding strategy.

A similar outcome of the experiments was observed if another *S. pyogenes* serotype, here M6, was used as a host cell infecting agent (Fig. 33). Although the adherence and internalization values of *S. pyogenes* M6 were found to be lower compared to *S. pyogenes* M49, the preincubation of the HEp-2 cells with *S. salivarius*, *S. oralis*, and partially with *E. faecalis* protected the host cells from *S. pyogenes* M6 infection (Fig. 33). This is a clear indication that this protection effect is a general feature of the oral species and is not dependent on the infecting *S. pyogenes* serotype. However, again the effectiveness of the protection phenomenon was found to depend on direct contact of the oral species with the host cells prior to *S. pyogenes* infection (Fig. 34).

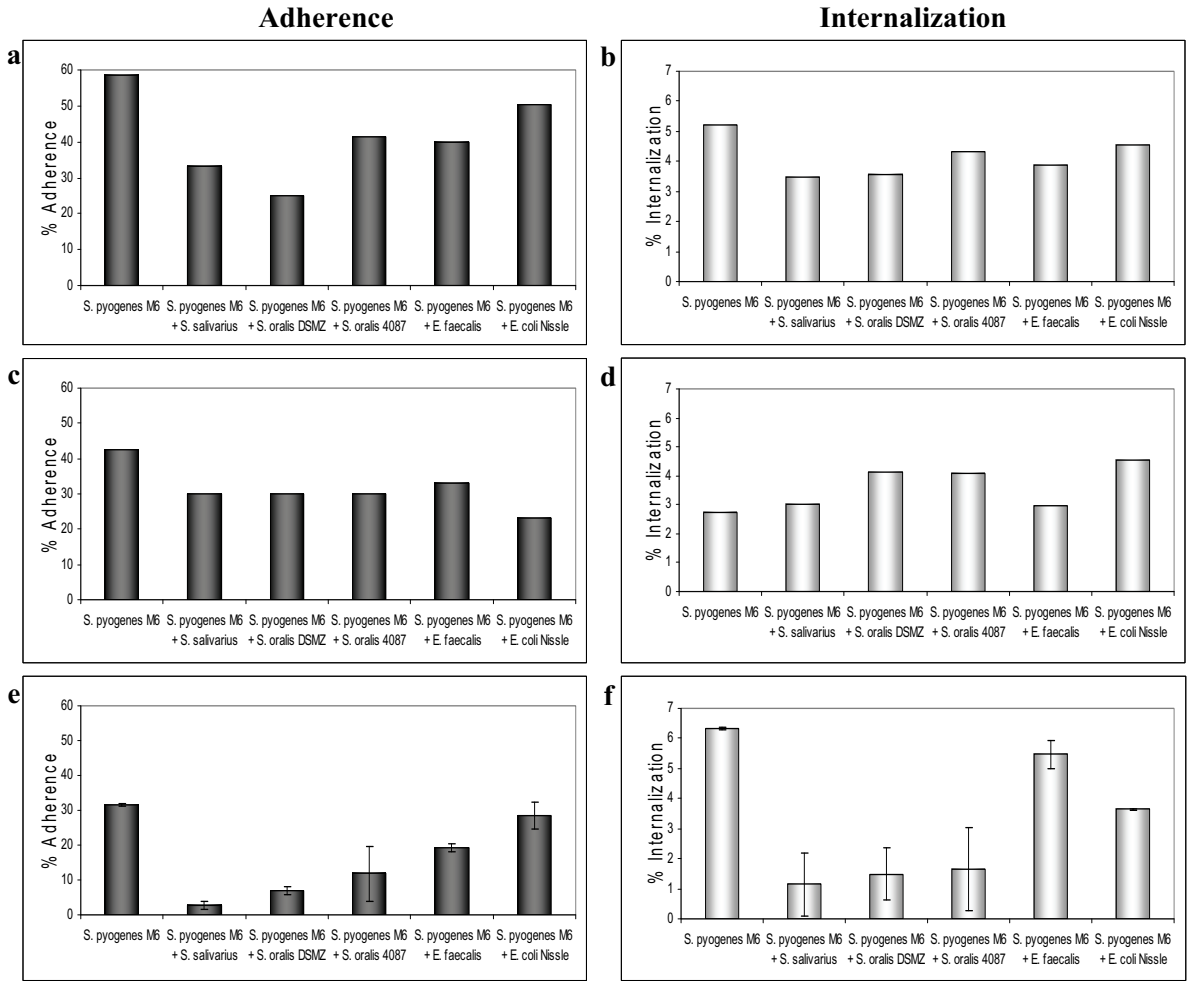


Fig. 33 *S. pyogenes* M6 adherence and internalization assay.

Quantitative assay of *S. pyogenes* M6 adherence to and internalization into HEp-2 cells with simultaneous (a, b); *S. pyogenes* first (c, d) and *S. pyogenes* last (e, f) seeding strategy.

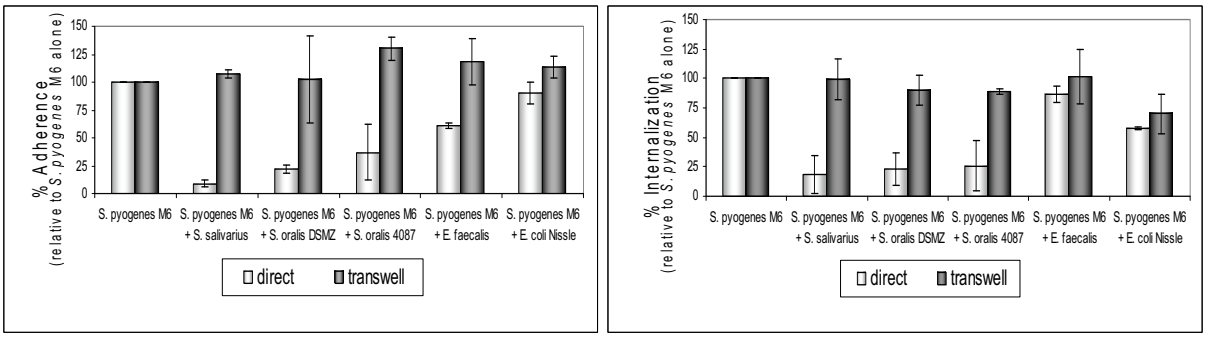


Fig. 34 The influence of direct contact of the tested bacteria on the protection effect.

Adherence (a) and internalization (b) of *S. pyogenes* M6 using the *S. pyogenes* last seeding strategy.

The next set of experiments allowed a better characterization of the protection effect. The number of adherent and internalized oral bacteria and *E. coli* Nissle to and into the HEp-2 cells was determined using the experimental setup where the tested bacteria were allowed to first interact with the host cells.

As shown in the Fig. 35a & b and 36a & b, particularly *S. salivarius* K12 adhered to and internalized into HEp-2 cells to the highest extent. The adherence effect was even more pronounced if *S. pyogenes* was later added to the host cell infection experiment. A nearly 3 to 8 fold increase in *S. salivarius* bacteria adhering to HEp-2 cells compared to the initial inoculum was noted. This can only be explained by massive growth and progression of the bacteria over the infection time.

Compared to the *S. salivarius* K12 behaviour all other tested species apparently did not adhere to or internalized into HEp-2 cells so efficiently (Fig. 35 & 36). Particularly *S. oralis* 4087 was inhibited in its HEp-2 cell adherence and internalization capacity if *S. pyogenes* M49 or M6 was added to the system (Fig. 35c & 36c).

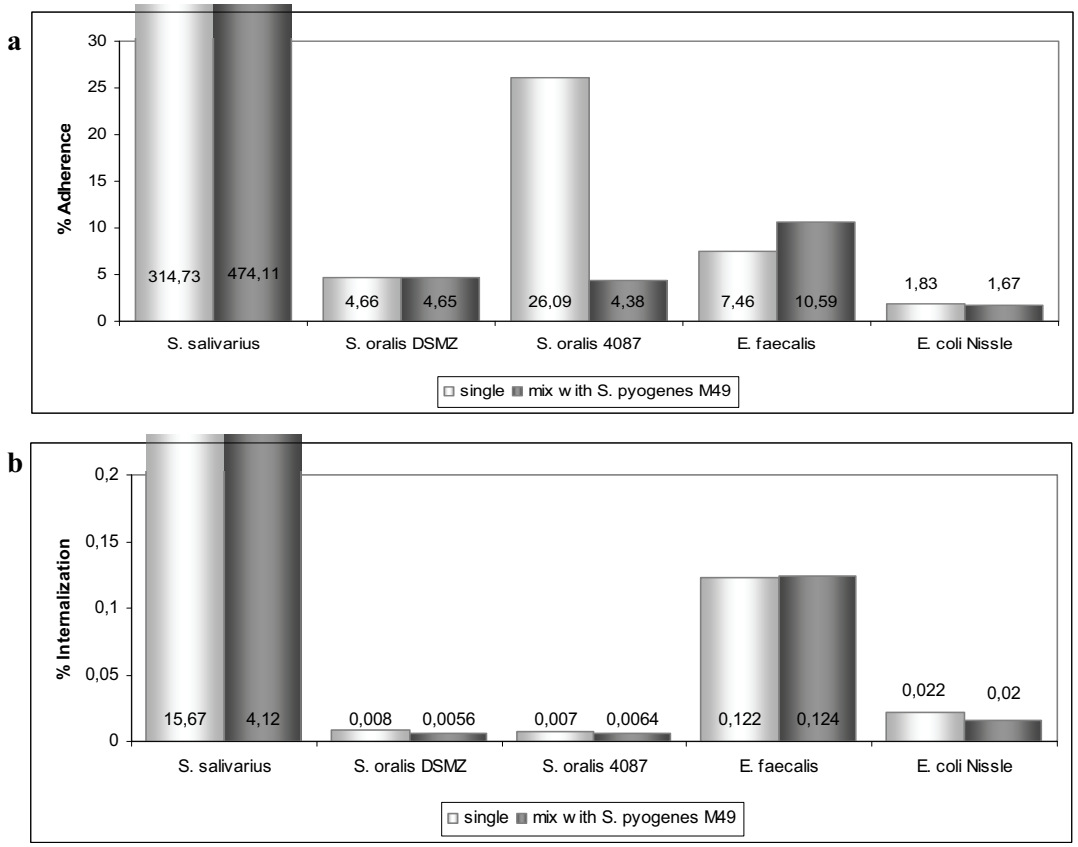


Fig. 35 Host cell adherence and internalization capacity of tested bacteria in the presence of *S. pyogenes* M49.

HEp-2 cells adherence (a) and internalization (b) capacity of selected oral bacteria and *E. coli* Nissle alone or mixed with *S. pyogenes* M49 in *S. pyogenes* last seeding strategy.

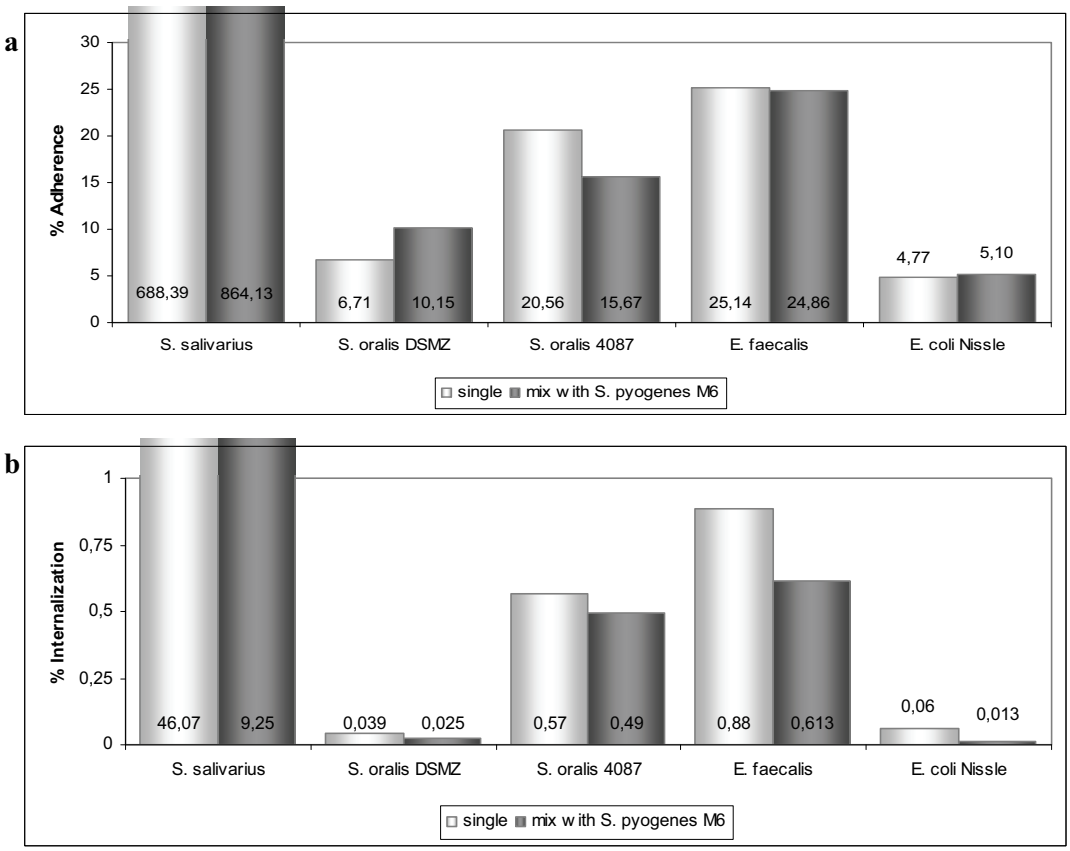


Fig. 36 Host cell adherence and internalization capacity of tested bacteria in the presence of *S. pyogenes* M6.

HEp-2 cells adherence (a) and internalization (b) capacity of selected oral bacteria and *E. coli* Nissle alone or mixed with *S. pyogenes* M6 in *S. pyogenes* last seeding strategy.

In order to confirm visually the previous results, SEM pictures were taken of the HEp-2 cell monolayer infected with the different bacterial species. As shown in Fig. 37, *S. salivarius* K12 was corroborated to attach to HEp-2 cells in large numbers. In fact, the complete host cell surface was physically covered with *S. salivarius* bacteria, which by pure definition apparently grow in biofilm-like structures on the HEp-2 cells. Thus, one potential explanation for the protection effect exerted by *S. salivarius* could be a sterical hindrance of *S. pyogenes* attachment to the cells, since all target structures for *S. pyogenes*-host cell interaction are physically blocked with *S. salivarius*.

The protection effect of *S. oralis* and also *E. faecalis* does most likely not exclusively rely on sterical hindrace since only few bacteria were found attached to the host cell surface. This microscopic result confirmed the previous adherence/internalization result.

Next to physical blockage of *S. pyogenes* adherence, most likely other mechanisms also contribute to the protection effect. Induction of specific transcriptional changes in the HEp-2 cells is one such possibility.

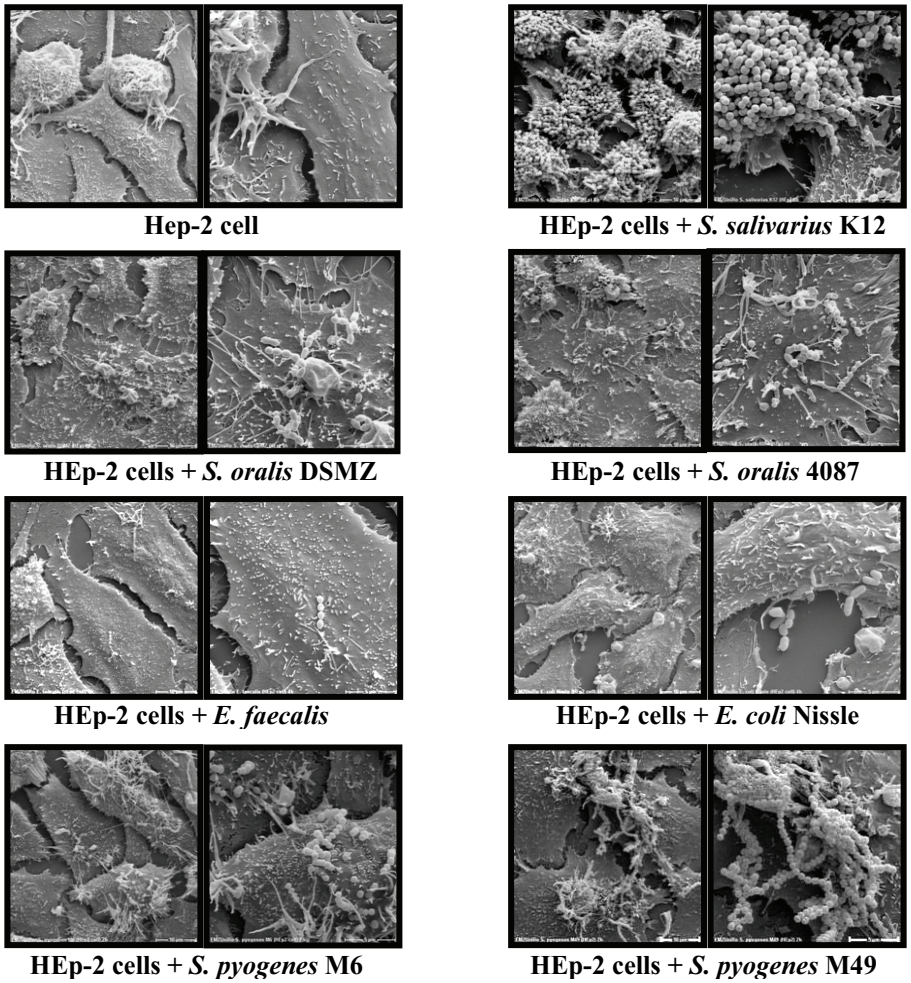


Fig. 37 SEM pictures of bacterial attachment on the HEp-2 cells surface.
SEM picture with 2000x (left) and 5000x (right) magnification. HEp-2 cells were infected for 4 hours with bacteria, except for *S. pyogenes*, where only 2 hours were used.

III.7.2 Double immunofluorescence

As an additional method to document the host cell protection effect of the oral bacteria and *E. coli* Nissle, a double fluorescence staining techniques were used. The method is described in the Material and Method section (II.2.16). Briefly, *S. pyogenes* bacteria attached to the surface of the infected HEp-2 cells were stained in green. Internalized bacteria were marked in red. The host cells were visualised by regular light microscopy. The results for *S. pyogenes* M49 are shown in Fig. 38. Results using *S. pyogenes* M6 are depicted in Fig. 39.

Taken together, the microscopic pictures confirmed the observed protection effect of *S. salivarius* and *S. oralis* on HEp-2 cells, if they were allowed to have contact with the cells prior to *S. pyogenes* infection. The quantitative data presented in Fig. 31-34 are nicely supported by microscopy. Of note, *E. faecalis* and *E. coli* Nissle also reduced the adherence of *S. pyogenes* M49 and M6 to the HEp-2 cells, however, this effect is not as pronounced as with *S. salivarius* and *S. oralis*. Moreover, the effect of both species is more prominent and strong on *S. pyogenes* M49 as compared to *S. pyogenes* M6.

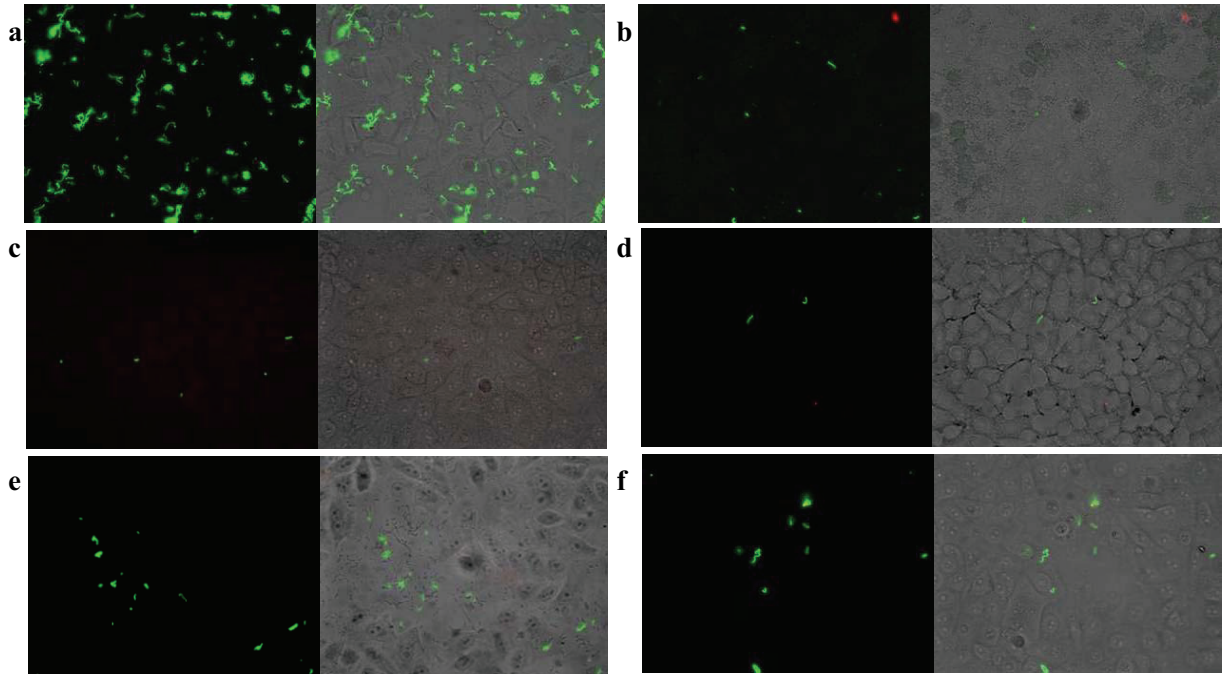


Fig. 38 Double immunofluorescence assay of *S. pyogenes* M49 in the oral bacteria and *E. coli* Nissle.

S. pyogenes M49 alone as a control (a); *S. pyogenes* M49 in the presence of *S. salivarius* K12 (b), *S. oralis* DSMZ (c), *S. oralis* 4087 (d), *E. faecalis* (e), *E. coli* Nissle (f). Left panel: fluorescence image; right panel: overlay of fluorescence image (visualizing *S. pyogenes* M49) with light microscopic picture (visualizing HEp-2 cells). Exclusively, results from experiments allowing oral bacterial contact with host cells prior to *S. pyogenes* infection are shown.

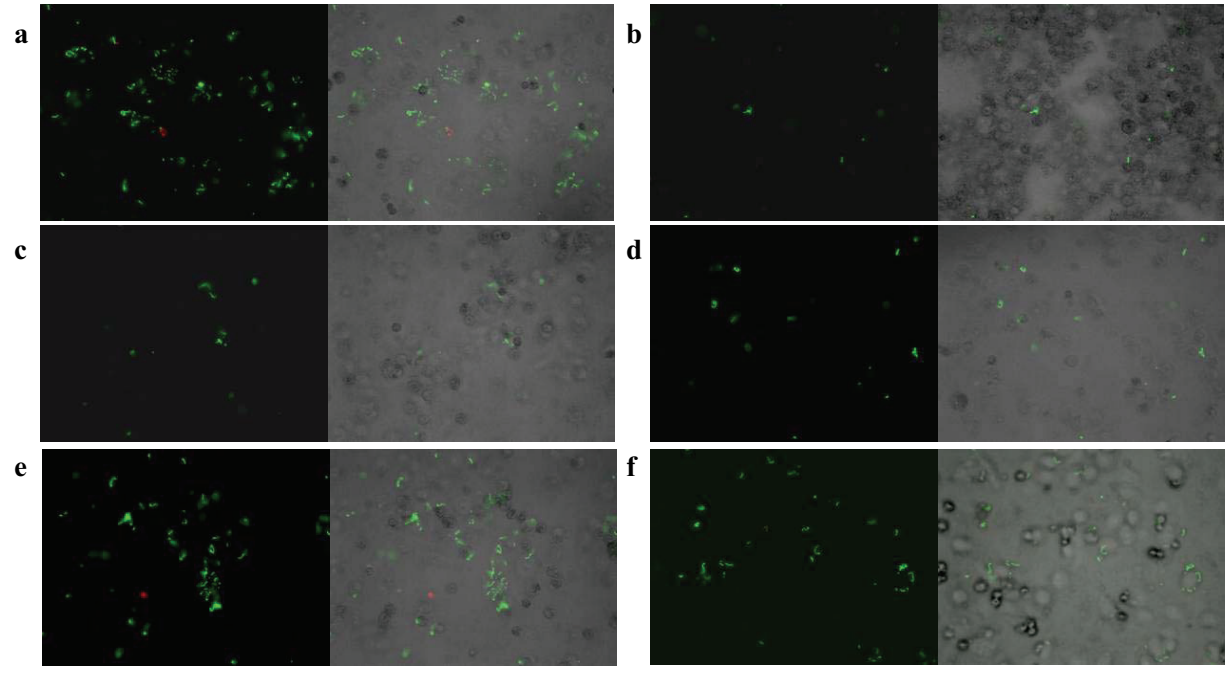


Fig. 39 Double immunofluorescence assay of *S. pyogenes* M6 in the oral bacteria and *E. coli* Nissle.

S. pyogenes M6 alone as a control (a); *S. pyogenes* M6 in the presence of *S. salivarius* K12 (b), *S. oralis* DSMZ (c), *S. oralis* 4087 (d), *E. faecalis* (e), *E. coli* Nissle (f). Left panel: fluorescence image; right panel: overlay of fluorescence image (visualizing *S. pyogenes* M6) with light microscopic picture (visualizing HEp-2 cells). Exclusively, results from experiments allowing oral bacterial contact with host cells prior to *S. pyogenes* infection are shown.

In conclusion, these series of experiments revealed a protection effect towards host cells by oral species, however, exclusively if they have direct contact to the cells and if they have the time to first interact with the cells prior to *S. pyogenes* infection.

Either sterical hindrance or transcriptional changes in the host cells emerge as potential mechanisms behind this protection effect. Most likely, a mixture of both mechanisms might act to protect host cells from pathogen attack.

III.8 Effect of oral bacteria and *E. coli* Nissle on *S. pyogenes* cytotoxicity

The last result section has shown that indeed oral bacteria can protect host cells by reducing the number of adherent and internalized *S. pyogenes*. However *S. pyogenes* is known to damage host cells via the action of secreted toxins and proteases, and this damage does not solely rely on direct *S. pyogenes*-host cell contact but can also occur over the distance.

Consequently, the cytotoxic effect of *S. pyogenes* on HEp-2 cells was monitored using all three different infection setups. For this purpose the eukaryotic Live/Dead staining kit was used to differentiate life from injured and damaged HEp-2 cells. The assays were performed as outlined in II 2.15. The results of these assays are illustrated in Fig. 40.

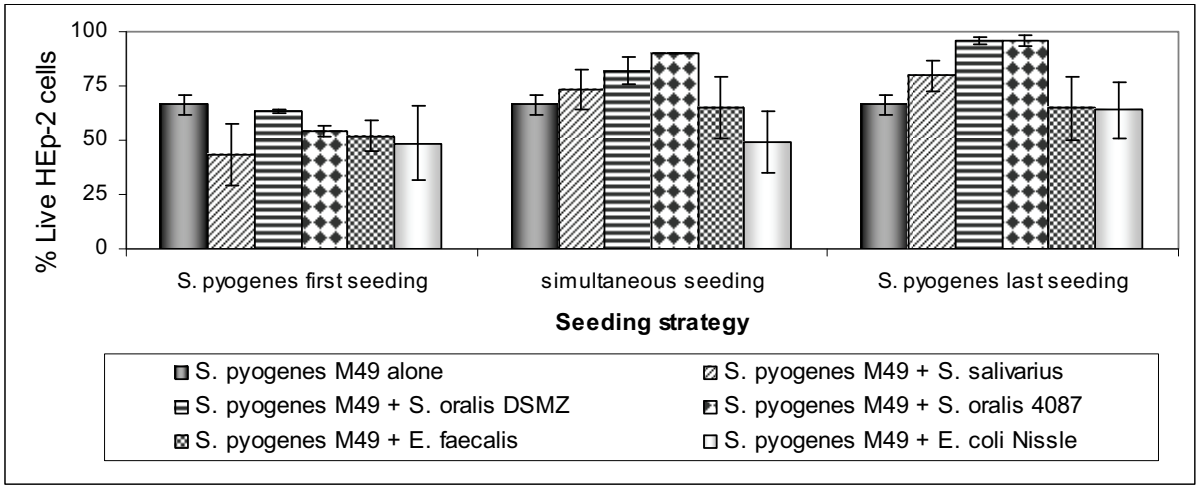


Fig. 40 Cytotoxicity assay.

Percentage of living HEp-2 cells after infection with *S. pyogenes* in the presence of tested bacteria using all three previously outlined different seeding strategies.

In this assay, *S. pyogenes* M49 alone can kill up to 66% of the infected HEp-2 cells. A protection effect was evident by additional of *S. salivarius* and *S. oralis* into the setup. Both species can increase the number of living HEp-2 cells by approximately 10% (*S. salivarius*) and 20-30% (both *S. oralis* strains) when they were seeded simultaneously or 2 hours before *S. pyogenes* was allowed to infect the cells (*S. pyogenes* last seeding strategy). This protection effect was species specific as it was not found with *E. faecalis* and *E. coli* Nissle in the same

seeding strategy. However, none of the tested species is able to protect or even reverse the damage done to HEp-2 cells by *S. pyogenes* infection (*S. pyogenes* first seeding strategy). Viability of Hep-2 cells was further visualized and inspected microscopically (Fig. 41).

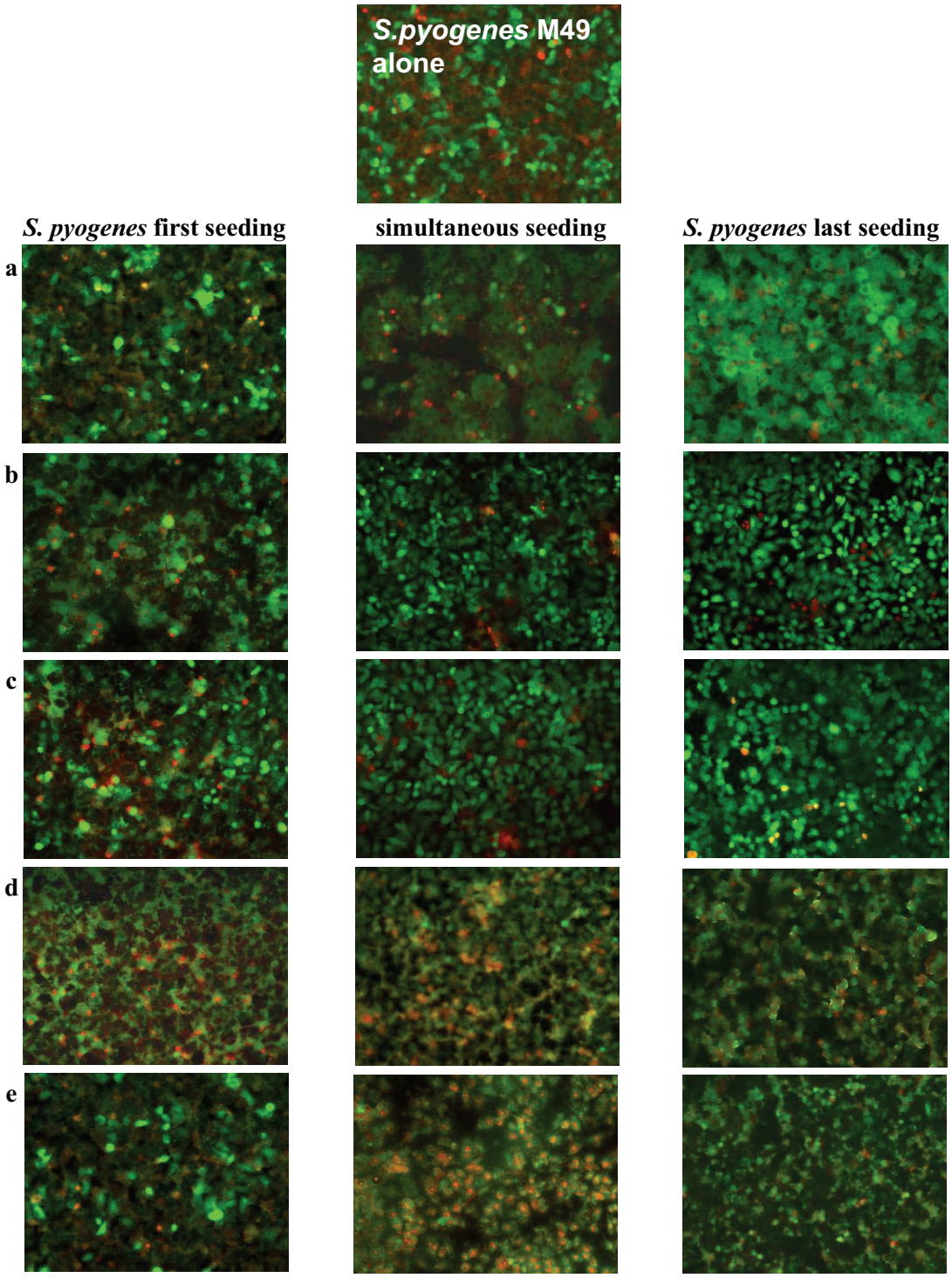


Fig. 41 Microscopic images of cytotoxic effects after infection of HEp-2 cells.

Fluorescence microscopic pictures of HEp-2 cells infected with *S. pyogenes* M49 from cytotoxicity assays in the presence of tested bacteria. HEp-2 cells infected with *S. pyogenes* alone were used as a control and compared with HEp-2 cells infected with *S. pyogenes* mixed with *S. salivarius* K12 (a); *S. oralis* DSMZ (b); *S. oralis* 4087 (c); *E. faecalis* (d) and *E. coli* Nissle (e) in three different seeding strategies. Live HEp-2 cells are stained green and dead HEp-2 cells are stained with red fluorescence.

III.9 Transcriptional response of HEp-2 cells in the presence of *S. salivarius* and *S. oralis*

Next to or in combination with the sterical hindrance exerted by oral bacteria during co-infection of HEp-2 cells with *S. pyogenes*, reprogramming of the host cell transcription upon contact with the oral bacteria could be a plausible explanation for the observed protection effect. Thus, the transcriptional response of HEp-2 cells after contact with *S. salivarius* K12 and *S. oralis* DSMZ was elucidated in comparison to those of non-infected cells using Affymetrix Technology (outlined in II.2.17).

From two independent experiments, *S. salivarius* K12 and *S. oralis* DSMZ were found to change the expression of 15 and 104 probe sets, respectively. From those probe sets, for 12 out of the 15 *S. salivarius* K12 differentially induced genes an Entrez gene ID was found, whereas gene IDs for 71 out of the 104 *S. oralis* DSMZ differentially induced genes were identified (Fig. 42).

Fig. 42 Venn diagram of overlap changed genes from HEp-2 cells (Oliveros, 2007)**.**

In order to extract information about differentially expressed genes, a tool in NetAff™ Analysis Center was used. As a confirmation or complement, PANTHER and InnateDB databases were used for data mining. As a result, functions, process terms and pathways of differentially expressed genes are shown in Table 9 (Appendix). The Entrez gene IDs, the gene symbols, the simplified category of gene product functions and the determined fold changes in transcription level are collectively shown in Table 6.

Table 6: Fold change of transcription level from infected HEp-2 cells

Entrez Gene ID	Gene Symbol	Category	Transcript level fold change
***S. oralis* DSMZ**			
54541	**DDIT4**	apoptosis	2.89 ± 1.23
112399	EGLN3		1.34 ± 0.06
10553	HTATIP2		1.32 ± 0.16
8870	**IER3**		1.80 ± 0.28
3725	JUN		1.36 ± 0.04
5292	PIM1		1.48 ± 0.08
7852	CXCR4	apoptosis/inflamatory response/cell migration	1.21 ± 0.06
388	RHOB	apoptosis/transport	1.55 ± 0.08
81	ACTN4	apoptosis/cytoskeleton	-1.27 ± 0.12
1490	CTGF	cell adhesion/cell signalling	1.60 ± 0.26
158158	RASEF	cell adhesion/transport	-1.37 ± 0.02
901	CCNG2	cell cycle	1.51 ± 0.39
1027	CDKN1B		1.18 ± 0.01
1906	EDN1	cell-cell signaling	1.37 ± 0.12
8614	STC2		1.59 ± 0.12
667	DST		-1.42 ± 0.04
133	ADM	cytoskeleton/cell-cell signaling	2.33 ± 0.33
4289	MKLN1	cytoskeleton/signal transduction	-1.38 ± 0.16
1847	DUSP5	dephosphorylation	1.54 ± 0.33
51302	CYP39A1	metabolic/oxidoreductase	1.40 ± 0.11
5507	PPP1R3C	metabolic/phosphatase	1.91 ± 0.25
55432	YOD1	peptidase activity	1.20 ± 0.08
80273	GRPEL1	protein folding	1.19 ± 0.09
4820	NKTR		-1.92 ± 0.7
5876	RABGGTB	protein modification process	1.19 ± 0.03
29923	HIG2	response to stress	1.42 ± 0.07
1843	**DUSP1**	response to stress/cell cycle/phosphatase	1.98 ± 0.07
10135	NAMPT	signal transduction	2.17 ± 0.06
5329	PLAUR		1.36 ± 0.19
7422	VEGFA		1.59 ± 0.14
3491	CYR61	signal transduction/cell adhesion	1.38 ± 0.27
8553	BHLHB2	transcription	2.06 ± 0.02
1106	CHD2		1.47 ± 0.04
1915	EEF1A1		-1.34 ± 0.01
2353	**FOS**		1.96 ± 0.36
3726	JUNB		1.58 ± 0.03
23764	MAFF		1.30 ± 0
4783	NFIL3		1.21 ± 0.01
64332	NFKBIZ		-1.64 ± 0.16
246721	POLR2J2		-1.69 ± 0.48
1827	RCAN1		-1.70 ± 0.23
9792	SERTAD2		1.29 ± 0.03
51616	TAF9B		1.55 ± 0.29
10628	TXNIP		-2.74 ± 0.85
150094	SNF1LK	transcription/immune response	1.32 ± 0

Entrez Gene ID	Gene Symbol	Category	Transcript level fold change
1195	CLK1	transferase activity	1.54 ± 0.37
25976	TIPARP		1.19 ± 0.02
1479	CSTF3	translation	1.34 ± 0.14
84919	PPP1R15B		1.25 ± 0.04
57222	ERGIC1	transport	1.42 ± 0.08
6515	SLC2A3		1.83 ± 0.44
5997	RGS2	transport/homeostasis	-1.48 ± 0.29
7037	TFRC	transport/homeostasis/cytoskeleton	-1.17 ± 0.02
353322	ANKRD37	unknown	1.62 ± 0.14
57561	ARRDC3		2.24 ± 0.03
64417	C5orf28		-1.62 ± 0.36
55744	C7orf44		1.97 ± 0.09
283846	DKFZp547E087		-1.40 ± 0.14
56986	DTWD1		-1.59 ± 0.22
56008	FLJ11236		-1.29 ± 0.06
401261	FLJ38717		-1.96 ± 0.52
153561	GUSBP1		-1.45 ± 0.29
283120	H19		-1.16 ± 0.02
9703	KIAA0100		-1.67 ± 0.59
100134282	LOC100134282		-1.35 ± 0.13
406991	**MIRN21**		1.57 ± 0.04
100130889	PSORS1C3		1.58 ± 0.1
200107	RP4-621O15.2		1.83 ± 0.03
81671	**TMEM49**		1.50 ± 0.07
283131	TncRNA		1.23 ± 0.03
80149	ZC3H12A		1.37 ± 0.29
***S. salivarius* K12**			
54541	**DDIT4**	apoptosis	1.29 ± 0.02
8870	**IER3**		1.39 ± 0.11
135114	HINT3	catalytic activity	3.01 ± 1.6
9685	CLINT1	cytoskeleton/matrix protein	-1.35 ± 0
1522	CTSZ	proteolysis	1.34 ± 0
1843	**DUSP1**	response to stress/cell cycle	1.24 ± 0.03
51306	C5orf5	signal transduction	-1.30 ± 0.04
2353	**FOS**	transcription	1.43 ± 0.11
4541	ND6	transport	-1.31 ± 0.04
284454	LOC284454	unknown	1.46 ± 0.18
406991	**MIRN21**		1.62 ± 0.19
81671	**TMEM49**		1.44 ± 0.15

All gene symbols for transcripts which were found in response to both *S. salivarius* K12 and *S. oralis* DSMZ were printed in bold. The list of genes name is shown in Table 8 (Appendix). The categories were simplified based on retrieved gene annotation from NetAff™. The detailed overrepresented function is shown in Table 9 (Appendix).

All identified differentially expressed genes were subsequently clustered manually into 5 clusters. Cluster 1 contained all overlapping up-regulated genes in response to both *S. oralis*

and *S. salivarius*. Cluster 2 and 3 contained non overlapping up-regulated and down-regulated genes in response to *S. oralis*, respectively. Cluster 4 and 5 contained non overlapping up-regulated and down-regulated genes in response to *S. salivarius* K12, respectively. The list of gene symbols in the 5 clusters is shown in table 7. The complete name of all gene symbols can be found in the Appendix (Table 8).

In cluster 1, *S. salivarius* and *S. oralis* both induced transcription of FOS. The FOS gene family consists of 4 members: FOS, FOSB, FOSL1 and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. In some cases, expression of the FOS gene has also been associated with apoptotic processes. DUSP1 plays an important role in cellular responses to environmental stress as well as in negative regulation of cellular proliferation. The dual specific protein phosphatase1 (DUSP1) is a non-receptor-type protein-tyrosine phosphatase and can inactivate mitogen-activated protein (MAP) kinase by dephosphorylation. DDIT4 is also described as apoptosis related and influences the mTOR-signalling pathway, which is involved in the precise regulation of cell growth and differentiation. Taken together, *S. salivarius* and *S. oralis* both influence HEp-2 cells on the level of cell proliferation and also apoptosis processes.

Table 7: List of associated genes differentially expresses in HEp-2 cells upon contact with *S. salivarius* K12 and *S. oralis* DSMZ

Cluster 1	Cluster 2		Cluster 3	Cluster 4	Cluster 5
DDIT4	ADM	JUNB	ACTN4	CTSZ	CLINT1
DUSP1	ANKRD37	MAFF	C5orf28	HINT3	C5orf5
FOS	ARRDC3	NAMPT	DKFZp547E087	LOC284454	ND6
IER3	BHLHB2	NFIL3	DST		
MIRN21	C7orf44	PIM1	DTWD1		
TMEM49	CCNG2	PLAUR	EEF1A1		
	CDKN1B	PPP1R15B	FLJ11236		
	CHD2	PPP1R3C	FLJ38717		
	CLK1	PSORS1C3	GUSBP1		
	CSTF3	RABGGTB	H19		
	CTGF	RHOB	KIAA0100		
	CXCR4	RP4-621O15.2	LOC100134282		
	CYP39A1	SERTAD2	MKLN1		
	CYR61	SLC2A3	NFKBIZ		
	DUSP5	SNF1LK	NKTR		
	EDN1	STC2	POLR2J2		
	EGLN3	TAF9B	RASEF		
	ERGIC1	TIPARP	RCAN1		
	GRPEL1	TncRNA	RGS2		
	HIG2	VEGFA	TFRC		
	HTATIP2	YOD1	TXNIP		
	JUN	ZC3H12A			

The host cell genes which were exclusively differentially regulated by *S. oralis*-HEp-2 cells interaction are listed in cluster 2 and 3. These genes were studied more deeply and were compared to the other clusters to extract molecular functions, biological processes and pathways in response to *S. oralis* which presumably have a role in decreasing *S. pyogenes* adhesion to HEp-2 cells. Induced genes apparently involved in cell adhesion in cluster 2 were CTGF, CYR61, RhoB, VEGFA, PBEF1, and EDN1. Down-regulated genes involved in cell adhesion, cytoskeleton formation and cell-cell tight junction formation in cluster 3 were RASEF, DST, MKLN1 and ACTN4.

Genes which were described to be involved in immune response processes in cluster 2 were ADM, CXCR4, NFIL3 EDN1, and SNF1LK. Further examples of genes involved in immune responses were JUN, PLAUR, CDKN1B, PBEF1, JUNB and RhoB. Down-regulated genes with such putative functions in cluster 3 were NFKBIZ, NKTR, RCAN1 and TRFC.

Genes involved in stress responses in cluster 2 were MAFF, VEGFA, CXCR4, HIG2, EDN1, DUSP5, EGLN3, and JUN. In cluster 3, only ACTN4 belongs to this category. Genes encoding proteins with protease activities were up-regulated in cluster 2 (PLAUR and YOD1 OUT) and in cluster 4 (CTSZ). A gene encoding a chaperone was exclusively present in cluster 2 (GRPEL1). Genes that were involved in homeostasis were present in cluster 2 (EDN1 and STC2) and in cluster 3 (TFRC).

Taken together, it is noteworthy that *S. salivarius* K12-HEp-2 cell interaction only lead to a limited number of differentially transcribed genes. This is amazing with respect to the fact that massive numbers of *S. salivarius* cover the HEp-2 cell surface. Exclusively *S. salivarius* affected genes belong to the cellular categories of proteolysis and signalling transduction.

Although only a limited number of *S. oralis* bacteria were found on the HEp-2 cells surface, the list of differentially transcribed genes is much larger and the list of cellular functional categories these genes belong to is thus more extensive.

It is quite obvious that processes like cytokine production, apoptosis, host cell cytoskeleton rearrangement, stress response and cellular differentiation are targeted by these oral bacteria and in final consequence lead to a protective effect toward *S. pyogenes* host cell attack.

IV. Discussion

IV.1 General considerations

On their way to their human target cells, bacteria of the species *S. pyogenes* pass the oral cavity and encounter other bacteria of the resident microflora. Many of these bacteria are streptococci and thus, close relatives to *S. pyogenes*. Irrespective of their phylogenetic status, most members of resident species are present in much higher numbers than the incoming pathogens. The initial interaction between the mass of physiological and few virulent microorganisms is one major determinant for the outcome of the exposure of humans to *S. pyogenes*, i.e., an spontaneous eradication of the pathogen before any harm is done or the development of an acute infection. If the pathogen can establish itself on the pharyngeal epithelium and multiplies to higher numbers, still the interaction with resident bacteria will support or prevent extended periods of *S. pyogenes* persistence although the numerical ratio now could favor the pathogen.

In order to study functional details of a *S. pyogenes* exposure to bacteria belonging to the physiologic microflora and simultaneously to eukaryotic cells the present investigation was performed. As major goals, the results concerning viability of every involved strain and the production of established virulence factors by the pathogen were to be determined. The influence of different culture conditions influencing the distance of the bacteria in mixed species cultures and their planktonic or biofilm status were to be tested. Finally, the effects of such interactions on typical epithelial target cells were to be examined. Every goal was extensively addressed, leading to several novel results and the identification of streptococcal strains potentially suited for usage in probiotic formulas.

The strains chosen as interactive partners for overall two *S. pyogenes* wildtype strains belonging to different serotypes were one *Streptococcus salivarius* and two *Streptococcus oralis* isolates as representatives of the dominant portion of the resident microflora as well as one *Enterococcus faecalis* and *Escherichia coli* isolate each as examples for the transient microflora of the upper respiratory tract. As a special twist, the *S. salivarius* and *E. coli* strains were established probiotics. Especially with these strains the consequences of interspecies battles were examined to answer the questions which isolate will stake the biggest claim in the favored environment and which one would be suitable as a novel therapeutics.

IV. 2 Changes of *S. pyogenes* numbers and viability in co-culture experiments

The numbers of species at different sites of the oral cavity as well as the numbers of bacteria from a given species in oral materials such as saliva or scrapings from the buccal surface, the tongue or the gingival crevice are well established by both culture and molecular techniques (Paster *et al*., 2001). Species distribution and bacterial numbers from pharyngeal materials are less well defined. However, streptococci as the predominant bacteria at every above mentioned locus are present in numbers ranging between 5 and 8 logs per ml fluid or mg material (Wilson, 2005). Much less is known about the numbers of *S. pyogenes* bacteria when introduced into the oral cavity or the pharynx. Most probably, *S. pyogenes* will not be ingested as single bacteria but in droplets or dried material containing packages of bacteria that could equal several logs.

As a first approach to assess numerical changes as a consequence of simultaneous presence of potential antagonists in a somewhat artificial setup, two *S. pyogenes* wildtype strains of serotypes M49 and M6 were mixed with oral bacteria in rich liquid media as ON standing cultures. To reflect the numerical variabilities of the natural environment, initial inocula between 10^1 and 10^7 cfu/ml were chosen and ratios varied between three logs more of the oral bacteria to two logs more of the *S. pyogenes* strains.

As a general result, growth of *S. pyogenes* at initial inocula exceeding 4 logs was not affected by any co-cultured bacterium (Fig. 2 and additional results not shown).

When combined with streptococcal strains, reduced viability (thus cell numbers below the initial inoculum) of *S. pyogenes* was only observed in the presence of the *S. salivarius* strain and additionally, when its inoculum was large while that of *S. pyogenes* was small (Fig. 2). As compared to pure cultures, decreased growth rates of *S. pyogenes* were measured when an equally small amount of *S. salivarius* or much larger amounts of the *S. oralis* isolates were simultaneously cultured. Effects to *S. pyogenes* viability also took place when a physical barrier (transwell system) separated the bacteria from the *S. salivarius* isolate. Oppositely, a growth rate reduction was predominantly observed during direct contact of the bacterial species and was bidirectional, i.e., it also occurred to the streptoccal test strains when mixed with much larger inocula of *S. pyogenes* (Fig. 3). Of note, direct contact between *S. pyogenes* and most streptococcal test strains did not include firm or lasting binding between the bacteria, since a coaggregation was only observed between *S. pyogenes* and the *S. oralis* strain 4087 (Fig. 15). Finally, in each of the above mentioned assays effects on the serotype M49 strain were more pronounced than those on the M6 strain.

Taken together, reduced *S. pyogenes* viability in the presence of *S. salivarius* was consistent with the concept of an a diffusible toxic substance produced by the probiotic bacterium (Hyink *et al.,* 2007; Wescombe *et al.,* 2006) – provided the *S. pyogenes* strain was susceptible to the substance and the cell numbers of the probiotic strain exceeded that of *S. pyogenes* by several logs. The present results extend the published data since so far growth inhibition testing of *S. pyogenes* was only conducted on solid media or in liquid assays employing (semi-)purified bacteriocin.

How do these results relate to the oral/pharyngeal situation? The *in situ* effects of salivaricin on *S. pyogenes* have been measured using the inductional effects of this bacteriocin for the production of related substances in other species. Thus, the SalA2 expression in *S. pyogenes* was induced by a minimum of $8x10^5$ *S. salivarius* cells per ml saliva (Wescombe *et al*., 2006). This number is above the levels of *S. salivarius* in saliva from healthy children, i.e., 10^5 cfu/ml (Carlsson, 1970) and 10^4 to 10^5 cfu/ml saliva from adults treated with the corresponding probiotics (Horz *et al.,* 2007). Therefore, the measured effects could be irrelevant for the natural encounter between both bacterial species in their human hosts.

Reduced *S. pyogenes* growth rates in the presence of large streptococcal inocula could be due to competition for nutrients (Ribble, 1967), especially since a similar effect on streptococcal growth rates was observed with an inverted ratio between *S. pyogenes* and the other streptococcal species. However, the effects on *S. pyogenes* growth appeared to be serotype-specific and were predominantly observed upon direct contact of the cells but not in the transwell system. Thus, these results could indicate the activity of a cell-associated factor similar to observations on co-cultures between *S. oralis* and *Haemophilus influenzae* or *Moraxella catarrhalis* (Bernstein *et al*., 2002) or *E. faecalis* and *S. pneumoniae*, *S. aureus*, or *Listeria ivanovii* (Bottone *et al.,* 1971; Galvez *et al*., 1998). However, the molecular nature of such a factor remains obscure.

The results from interactions between the *S. pyogenes* isolates and the *E. coli* Nissle strain indicated the presence of a similar phenomenon exclusively related to the presence of live *E. coli* bacteria, since *S. pyogenes* growth reduction was only noticed upon direct contact but not in the transwell system or in the subsequent bacteriocin assays. Contact-dependent inhibition (CDI) has been reported for uropathogenic *E. coli* EC93 when in direct contact with *E. coli* K12. The *cdi* genes were found to be involved in this feature, with *cdiAB* encoding the inhibiting factors and *cdiI* as well as *pilP-pilS* the immunity-conferring factor/pili (Aoki *et al*., 2005). Although the genome sequence of the *E. coli* Nissle strain displays high homology with that of another uropathogenic *E. coli* isolate, strain CFT073 (Sun *et al*., 2005), a BLAST

analysis in the present study identified only 39% of the *cdiA* and 2% of *cdiB* sequence to be present in *E. coli* Nissle while the *cdiI* and *pilP-pilS* genes were completely absent in our isolate. In addition, the *S. pyogenes* isolates did not coaggregate with *E. coli* Nissle when tested in the appropriate assays (Fig. 15). Thus, contact-dependent inhibition of *E. coli* Nissle for the *S. pyogenes* strains apparently relies on other mechanisms. Since growth inhibition was not observed in the transwell system, competition for nutrients is not a simple alternative explanation, although the *E. coli* duplication period is only half of the *S. pyogenes* doubling time under non-limiting culture conditions and the *E. coli* Nissle strain normally reached final cell densities two logs above the *S. pyogenes* levels.

IV. 3 Co-culture effects on *S. pyogenes* virulence factor expression

In parallel to the co-culture experiments, classical bacteriocin assays were performed on solid media utilizing the cross-streak technique (Fig. 4). Concerning growth inhibition of the two *S. pyogenes* indicator strains, the results of the co-culture in liquid media were confirmed for the *S. salivarius* K12 probiotic strain. No other isolate affected the growth of *S. pyogenes*, demonstrating that the potential contact-dependent inhibition exerted by the *S. oralis* and *E. coli* Nissle strains relied on viable cells.

However, the *E. faecalis* strain affected the hemolysin production or activity of both *S. pyogenes* serotype strains, revealing the underlying methemoglobin reaction exerted by H_2O_2 when being secreted by the streptococci (Fig. 4). This effect was tried to be confirmed by classical hemolysin assays in liquid media. Here, the *E. faecalis* supernatant decreased the *S. pyogenes* hemolytic capacity slightly more than other tested oral bacteria (Fig. 14). Therefore, in addition to effects on *S. pyogenes* viability, also the production or activity of one of the most important virulence factors can be governed by other bacterial species.

In order to discriminate between decreased production or increased inactivation of the *S. pyogenes* hemolysin in the presence of factors from co-cultured bacteria, a reporter gene was introduced into the promoter region of the streptolysin S gene *sagA* of the serotypes M49 and M6 *S. pyogenes* isolates. Both *sagA* gene expression and cell viability were measured in direct contact and physically separate co-cultures over periods of 16 h (Fig. 10-13). As in the above described co-cultures of wildtype bacteria, an inoculum- and time-dependent growth suppression of *S. pyogenes* isolates was visualized in the presence of the *S. salivarius* and *S. oralis* strains. In a similar fashion, the *sagA* reporter gene activity was reduced in such assays. Yet, there were at best small effects of the *E. faecalis* strain on *S. pyogenes* cell numbers, while the streptococcal *sagA* expression could be suppressed to zero levels (Fig. 10-13).

Interestingly, also the presence of the *E. coli* strain lowered the *sagA* gene expression to some extent and therefore, should also diminish the amount of translated hemolysin. Yet, the probably reduced hemolysin amounts still had sufficient activity to reach maximum effects in the classical hemolysin assays (Fig. 14). Thus, the effects of *E. faecalis* and *E. coli* culture supernatants on the hemolytic activity of *S. pyogenes* strains is explained by altered *sagA* streptolysin S gene expression rather than post-translational processes. The way by which the influence is exerted differs between both bacteria. According to the results from direct contact and transwell experiments (Fig.10-13), similar effects in both setups indicated the presence of a diffusible substance in the case of the *E. faecalis* strain. Small effects in the transwell system opposed to full effects during direct contact and simultaneously, unaltered viability of all test strains in the presence of the *S. pyogenes* reporter strains again indicated direct-contact inhibition as the responsible mechanism in the case of the *E. coli* Nissle strain.

Although the growth inhibitory effects prevailed when testing the *S. salivarius* or *S. oralis* strains, also these isolates apparently produced diffusible substances with negative effects on the *sagA* promoter activity. According to temporal measurements of both growth curves and *sagA* expression (Fig. 10-12), small initial inocula of the test strains allowed at least some growth of the *S. pyogenes* reporter strains while their *sagA* expression was already suppressed both in the direct contact and transwell formats. Of note, the classical bacteriocin assay with these bacteria did not show altered hemolysin production, indicating either a lower sensitivity of this test compared to liquid culture methods or a varying behavior of the tested bacteria in liquid vs. solid environments.

The molecular nature of the signaling substances influencing *sagA* expression was not elucidated. In the case of the *S. salivarius* and *S. oralis* strains, it could be the bacteriocin which at low concentrations interferes with the *sagA* promoter activity and becomes toxic only at higher concentrations. Signaling qualities affecting bacteriocin production in recipient strains have been described for salivaricin (Upton *et al*., 2001; Wescombe *et al*., 2006) and *sag*A itself was demonstrated to act as a signaling compound (Salim *et al.*, 2007; Li *et al.,* 1999). *E. faecalis* could secrete a similar bacteriocin that does not include *S. pyogenes* in its toxic target repertoire but still acts as a signaling molecule. A similar phenomenon has been described for an *E. faecalis – S. aureus* interaction, in which the β-hemolytic activity of *S. aureus* was suppressed by a diffusible, potentially proteinaceous substance from *E. faecalis* (Vitkova & Votava, 2005).

IV.4 Co-culture effects on *S. pyogenes* biofilms

S. pyogenes has unequivocally been demonstrated to form single species biofilms in vitro and in animal model infections involving zebra fishes and mice (Cho & Caparon, 2005; Lembke *et al*., 2006; Takemura *et al*., 2004). Growth in single species biofilms led to increased antibiotic resistance levels (Conley *et al.,* 2003). The general protective function of a biofilm and in addition, the increased antibiotic resistance level stimulated ideas that this behavior could be associated with long-term persistence in asymptomatic carrier persons (Baldassarri *et al.,* 2006). However, in the oral cavity and in the pharynx, *S. pyogenes* will encounter mixed species biofilms. Thus, it has to establish itself within and eventually to penetrate these biofilms. Based on data from other pathogens, it is conceivable that both establishment and penetration will be influenced by many factors produced by the resident microflora (Davies *et al*., 1998; Federle & Bassler, 2003; Stanley & Lazazzera, 2004; Stoodley *et al.*, 2002; Suntharalingam & Cvitkovitch, 2005).

The set of oral bacteria from the previous series of experiments was also used to study defined mixed species biofilms with the serotype M49 and M6 *S. pyogenes* strains. Based on published data (Lembke *et al.*, 2006; Riani *et al.*, 2007), the former strain formed only small amounts of biofilm, while the latter produced large masses on polystyrol surfaces. When testing the oral bacteria in similar single species assays by three different methods of quantification or visualization, all streptococcal isolates grew to significant biofilm masses, while *E. coli* Nissle was a poor biofilm producer (Fig. 17-19). Consistent with the latter observation, *E. coli* Nissle was recently described to form biofilms only on borosilicate glass but not on plastic material (Lasaro *et al.,* 2008).

Then similar bacterial numbers of the *S. pyogenes* isolates and individual oral test strains were simultaneously seeded and co-incubated for up to three days. When comparing the quantitative data on biofilm masses with those from single species biofilm assays, serotype-specific differences between the *S. pyogenes* strains were apparent.

For the serotype M6 strain, biofilm masses of mixed cultures were at the same levels as that of the M6 single species culture for all streptococcal test strains. Compared to the single species biofilms of the streptococcal test strains, the amounts of mixed species biofilms were reduced for the *S. salivarius* and *S. oralis* isolates. The situation was reversed in the serotype M6 – *E. coli* Nissle co-cultures. Here the mixed species biofilm mass was reduced to the very low levels of the *E. coli* Nissle single species biofilm (Fig. 20). As opposed to the serotype M6 isolate, co-cultures with the serotype M49 isolate led to increased biofilm masses in every test combination. The mixed species biofilm amounts did not only exceed the small amounts

of the M49 single species biofilm but also the much larger amounts of the biofilms formed by the single streptococcal test strains (Fig. 21).

Since measurements of mixed species biofilm masses do not tell about the fate of the single strains that were inoculated at the beginning of the experiments, also microscopic inspections were performed at days one to three of the incubation periods. Both scanning electron microscopy (SEM) and confocal laser scan microscopy (CLSM) gave consistent results. In combinations with either *S. salivarius* or *E. faecalis*, the streptococcal test strains dominated and few to very few *S. pyogenes* bacteria could be seen evenly distributed among the biofilm of test bacteria. In every combination with the two *S. oralis* isolates, almost equal amounts of the two species were present. The structure of the biofilm was remarkable, since the *S. oralis* strains formed the bottom layer and the *S. pyogenes* strains the top layer as especially discernible by SEM analysis. While hardly any bacteria could be seen in the combination of serotype M6 *S. pyogenes* and *E. coli* Nissle, serotype M49 *S. pyogenes* clearly dominated the picture in combination with *E. coli* Nissle (Fig. 20e & 21e).

The drastically reduced presence of *S. pyogenes* bacteria in *S. salivarius* and *E. faecalis* biofilms and simultaneously, the even distribution of the bacteria in such mixed species biofilms indicated the existence of similar processes to control *S. pyogenes* numbers with both co-culture partners. In the case of the *S. salivarius* co-cultures, the salivaricin activity would be the obvious explanation for the decreased *S. pyogenes* figures while the absence of any selective surface interactions could be the basis for the even distribution. However, both solid and liquid media assays for *S. pyogenes*-specific bacteriocins did not demonstrate such substances to be produced by the *E. faecalis* strain. Yet, the biofilm environment is a third test format which could selectively induce bacteriocin production or could keep a constantly produced bacteriocin for extended, effect-enhancing periods in the vicinity of its target cells. Alternatively, the *E. faecalis* signaling to *S. pyogenes* as documented in the hemolysin assays could also affect the streptococcal permanence in this specific mixed species biofilm.

The increased mass and layered appearance of mixed *S. oralis* - *S. pyogenes* biofilms could be due to the pioneer character of *S. oralis* also observed in *ex vivo* samples from human volunteers (Li *et al.*, 2004). The avidity of *S. oralis* for human cell surfaces seems to be associated with the activity of specific galactose- or sialic acid/N-acetyl-galactosamine-binding lectins (Murray *et al.*, 1982; Weerkamp & McBride, 1980). Also the interaction between *S. oralis* and *S. pyogenes* could rely on the activity of lectins and appropriate sugar moieties, since the latter species was shown to bind the lectin concanavalin A (Manetti *et al.,*

2007). The reason for the increased mass of these two species biofilms, however, cannot be explained so far.

Since oral and pharyngeal biofilms would be bathed in saliva, the experiments were repeated in the presence of mucin as the major component of saliva (Amerongen & Veerman, 2002). Generally, *S. pyogenes* should be able to deal well with saliva. It was shown to bind to mucin by its M protein (Ryan *et al.,* 2001). Simultaneously, the two component regulator SptRS is induced by the presence of saliva (Shelburne *et al*., 2005). This control circuit induces virulence factors such as Sic and SpeB, which inactivate antimicrobial peptides normally contained in saliva (Ferni-King *et al.*, 2002 & 2004; Frick *et al.*, 2003; Shelburne *et al.*, 2005). However, saliva decreases the ability of *S. pyogenes* to attach to eukaryotic epithelial cells (Courtney & Hasty, 1991).

When first testing single species biofilms, two patterns of behavior were revealed. While *S. oralis* and *E. faecalis* biofilms were not much affected by the artificial saliva, the *S. salivarius* and serotype M6 *S. pyogenes* biofilms were strongly decreased by this component. Consistently, in mixed species biofilms artificial saliva had comparatively small and rather augmenting effects in combinations with the *S. oralis* and *E. faecalis* isolates, while biofilm masses were minimized when combinations with *S. salivarius* were used. In the case of the serotype M49 – *E. coli* Nissle combination, artificial saliva had a negative influence on the resulting biofilm mass.

Decremental effects of saliva on *S. oralis* growth have been reported without showing the molecular basis of this phenomenon. Yet, co-incubation of this species with *Actinomyces naeslundii* also improved the mixed species biofilm forming capacity (Palmer *et al.*, 2001). The rather augmenting effects of artificial saliva on biofilm formation of both *S. oralis* single and mixed species biofilms is consistent with the species' capacity to use mucin or sialic acid as a mucin component as a carbon source by expressing secreted enzymes such as ß-D-galactosidase, β-D-glucosidase, and β-N-acetyl-D-glucosaminidase (Byers *et al*., 1996; Van der Hoeven *et al*., 1990; Van der Hoeven & Camp, 1991). Obviously, production of these enzymes is not sufficient to support biofilm formation since *E. coli* Nissle can metabolize fucose as another mucin component (Autieri *et al*., 2007) while building no biofilm in the present study.

IV.5 Co-culture effects on *S. pyogenes* interactions with eukaryotic cells

Specific and firm binding of *S. pyogenes* to its eukaryotic target cells is indispensable both for causing disease and for persisting in its human host (Courtney & Podbielski, 2004;

Kreikemeyer *et al.,* 2004). The influence of simultaneously present bacteria on this interaction is not well studied. So far, only for *Moraxella catarrhalis*, another nasopharyngeal pathogen, co-aggregation with *S. pyogenes* was reported leading to increased eukaryotic cell adherence but decreased internalization (Brook & Gober, 2006; Lafontaine *et al*., 2004). In turn, type IV pili-mediated biofilm formation apparently contributes to *Moraxella* airway colonization (Luke *et al.,* 2007).

To collect more data on the interaction of two bacterial and one eukaryotic partner, HEp-2 respiratory epithelial cells were exposed to combinations of the two *S. pyogenes* strains and the series of test strains from above. The seeding strategy varied in order to mimic different situations in the human host. Besides simultaneous seeding which compared to the natural situation is a less probable option, *S. pyogenes* cells were added two hours before ("*S. pyogenes* first seeding") and two hours after seeding the test strains ("*S. pyogenes* last seeding"). The former strategy should address a potential therapy based on live bacteria, while the latter should mimic a prevention approach by employing potentially protective bacteria.

When testing the growth of single species on the eukaryotic cells by SEM inspection, the *S. salivarius* isolate formed large masses on the cell surface, while the *S. pyogenes* strains formed structures resembling microcolonies. The remainder of the test strains only occasionally adhered to the eukaryotic cells as single cells or pairs (Fig. 37). While the molecular basis for *S. pyogenes* eukaryotic cell attachment and microcolony formation is well established (Akiyama, 2003; Maneti *et al*., 2007), for *S. salivarius* so far only surface antigen C organized as fibrils has been determined as responsible adhesin (Weerkamp & Jacobs, 1982; Weerkamp *et al.,* 1986).

Irrespective of their individual binding capacity for eukaryotic cells, the test strains could affect *S. pyogenes* interactions with such cells. Therefore, various combinations of two strains were exposed to the eukaryotic cells employing the described seeding strategies and subsequently, adherent and internalized *S. pyogenes* bacteria were quantified by viable counts. Co-cultures of serotype M49 *S. pyogenes* with the test strains showed no significant differences for both *S. pyogenes* adherence to and internalization into the eukaryotic cells when the β-hemolytic streptococci were added simultaneously or even before the test bacteria. However, with the "*S. pyogenes* last seeding" strategy, especially the *S. salivarius* and *S. oralis* strongly reduced *S. pyogenes* eukaryotic cell adhesion. Less pronounced, but still significant, also the *E. faecalis* and *E. coli* isolates reduced *S. pyogenes* adherence. While *S. salivarius* and *S. oralis* also efficiently prevented *S. pyogenes* internalization into the HEp-2

cells, *E. faecalis* and *E. coli* had no effect on that parameter (Fig. 31). Co-cultures of the serotype M6 strain led to similar results with the GAS last seeding strategy and even displayed some inhibition of *S. pyogenes* eukaryotic cell binding with the simultaneous seeding strategy (Fig. 33). When following the fate of the co-cultured test bacteria, their behavior towards the eukaryotic cells was unaltered in most cases. The only consistent and extensive change was observed for *S. salivarius* internalization into the HEp-2 cells, which was dramatically diminished by the "*S. pyogenes* last seeding" strategy (Fig. 35b & 36b).

In order to confirm these remarkable results by a second independent method and to visualize the events on and in the eukaryotic cells, the bacteria were subjected to double immuno-stains and were inspected by fluorescence microscopy. With all combinations there was an extremely high correlation between the quantitative data from the viability counts and the data from the microscopic analysis (Fig. 38 & 39). Of note, adherence of *S. pyogenes* to eukaryotic cells could convincingly be documented by an electronic overlay of pictures obtained by light and fluorescence microscopy.

Obviously, the established probiotic *S. salivarius* K12 strain and the two *S. oralis* isolates could efficiently protect eukaryotic cells from becoming targets of adherent *S. pyogenes* bacteria provided the streptococcal test strains have sufficient time to establish themselves before *S. pyogenes* entered the scene. Since *S. salivarius* was demonstrated by microscopy and quantitative measurements to form biofilm-like structures on the eukaryotic cells while *S. oralis* hardly bound to this target, the protection had to be based on different mechanisms. One obvious explanation would be the production of diffusible substances by at least the *S. oralis* strains to exert the observed effects. Therefore, the co-culture experiments were repeated with some modifications, i.e., the first seeded test bacteria were either mechanically removed or additionally washed away from the eukaryotic cells before adding the *S. pyogenes* strains or alternatively, kept at some distance from the eukaryotic cells throughout the observation period by utilizing the transwell system.

The clearest results were obtained with the transwell system. Irrespective of the used test bacterium, no protective effects concerning *S. pyogenes* adhesion or internalization could be documented (Fig. 32 & 34). Thus, the eukaryotic cell protection of the test strains relies on direct contact of the test strains with the eukaryotic cells and/or the *S. pyogenes* bacteria during the co-culture experiments. Consistent with that conclusion, reduced numbers of test bacteria due to more or less intense ways of removal displayed gradual protective effects in between the boundaries measured upon direct exposure to the first inocula and in the transwell system assays. Thus, the protective principle in the eukaryotic cell adherence tests

does not rely on the diffusible substances from *S. salivarius* and *E. faecalis* demonstrated in former experiments.

IV. 6 Co-culture effects on the integrity and metabolism of eukaryotic cells

Infection in a *S. pyogenes*-exposed human begins when eukaryotic cells are damaged and inflammatory responses are triggered by both bacterial components and factors from lysed eukaryotic cells (Casadevall *et al*., 1990). Thus, prevention of *S. pyogenes* binding to its human target cells will not necessarily interfere with every type of infection trigger mechanism. Therefore, the fate of the HEp-2 cells simultaneously or subsequently exposed to *S. pyogenes* strains and potentially protective bacteria was followed by fluorescence double staining and microscopy. The stain will detect membrane damages that lead to loss of transmembrane potential and expose the nucleic acids to intercalating substances.

Again using the various two species combinations and different seeding strategies for co-culture experiments, the metabolic status and membrane integrity of the exposed eukaryotic cells was assessed by microscopic inspection. When employing the *S. pyogenes* first seeding strategy about 50% of the eukaryotic cells were damaged or dead irrespective of any co-cultured bacterium. These figures were even worse than the damage levels achieved by exposure to *S. pyogenes* alone. With simultaneous seeding, improved survivor rates among the HEp-2 cells were apparent upon co-culture with the *S. salivarius* and *S. oralis* strains. Finally, utilizing the GAS last seeding strategy, co-culture with *E. faecalis* or *E. coli* resulted in similar percentage of damaged as the *S. pyogenes* control. The survivor rates in co-cultures with the *S. salivarius* and especially, the *S. oralis* strains were much higher than the control, reaching nearly 100% with the latter isolates (Fig. 40 & 41).

Obviously, both *S. salivarius* and *S. oralis* strains can protect eukaryotic epithelial cells from binding *S. pyogenes* bacteria and successively, from being damaged by the bound β-hemolytic streptococci. Other bacteria which prevent *S. pyogenes* attachment to its eukaryotic target cells less efficiently than the two viridans streptococcal species do not exert these protective effects. This finding is consistent with the published data on the activity of *S. pyogenes* streptolysin O. The gene for this hemolysin is a member of a regulon that also contains the genes for the fibronectin-binding protein F1, the NADase and a specific NADase inhibitor (Nakata *et al*., 2005). The fibronectin-binding protein enables the bacteria to firmly and closely bind to eukaryotic cells. Obviously the vicinity of the eukaryotic cell is sensed by the bacterium and leads to a sequence of actions. Next, streptolysin O is produced and released by the bacterium. The hemolysin will attack the nearby eukaryotic cell membrane and form large

pores in the membrane. Then the complex of NADase and its inhibitor is produced and secreted. Outside of the bacterium, the inhibitor dissects from the NADase molecule and the latter enters the nearby eukaryotic cell via the holes formed by streptolysin O. Inside the eukaryotic cell, the NADase will adenylate the ribosomal RNAs and thereby induce either cell necrosis or a strong inflammatory response (Ghosh & Caparon, 2006; Madden *et al*., 2001; Meehl & Caparon, 2004; Meehl *et al.*, 2005; Ruiz *et al.*, 1998).

The protection of the eukaryotic cells from *S. pyogenes*-induced damages exerted by *S. salivarius* could be due to simple steric effects, i.e., the biofilm-like structure by which these bacteria cover the cells. For the *S. oralis* strains, which guard the HEp-2 cells even more efficiently, another mechanism must be active. Only such small amounts of *S. oralis* bacteria bind to the host cells that this species cannot act via steric hindrance. Potentially, regulatory and metabolic pathways in the eukaryotic cells could be influenced by the presence of the *S. oralis* strains that renders the cells less susceptible to the attack by *S. pyogenes*. Such beneficial effects on eukaryotic cells as well as the opposite have been described for several viridans streptococci (Cosseau *et al*., 2008; Hasegawa *et al.,* 2007; Stinson *et al.*, 2003). Therefore, the HEp-2 cells were exposed to *S. salivarius* and *S. oralis* bacteria under circumstances also chosen for the co-culture experiments and then, the eukaryotic transcriptome was measured by appropriate DNA array hybridizations.

Opposed to the findings of Cosseau *et al*. (2008) with human bronchial epithelial cell line 16HBE14O, astonishingly few transcripts showed altered abundances in the exposed HEp-2 cells – 12 in the case of *S. salivarius* and 71 in the case of *S. oralis*. Yet consistent with that publication on *S. salivarius*, the presence of both bacterial species predominantly led to up-regulation of gene expression. Now consistent with observations from the present study, exposure of HEp-2 cells to *S. salivarius* does not require the activation of many genes to protect the eukaryotic cells because of the physical barrier formed by the bacteria.

When analyzing details of the altered transcriptomes (Table 7), exposure to both bacterial species induced the message for FOS, an activator protein. Members of the FOS and JUN families (JUN and JUNB were up-regulated in response to *S. oralis*) form a complex with the transcription activator AP-1 in response to cell damage or tissue invasion of pathogens. JUN itself can build complexes with other regulator proteins (such as MAFF and TAF9b in cluster 2; NFKB1Z in cluster 3) or with itself. A summary of interactions between FOS-JUN and other regulator proteins is shown in Fig. 43. Regulation of AP-1 includes phosphorylation of proteins and changes in transmembrane redox potential. In cluster 2 many of the up-regulated

genes have such functions (i.e., DUSP5, CDKN1B, NFIL3, SNF1LK, CLK1, CYP39A1, EGLN3, PIM1) (Chinenov & Kerppola, 2001; Foletta *et. al*, 1998).

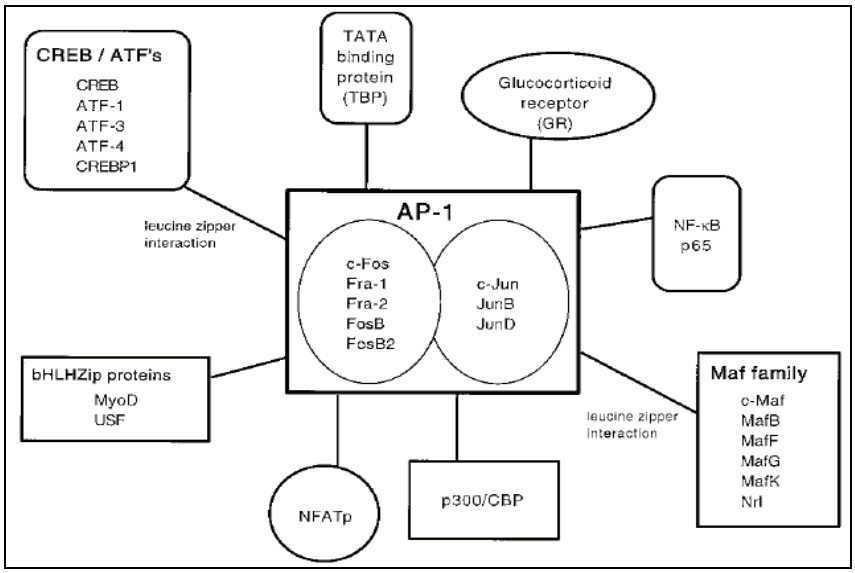


Fig. 43 Interaction of FOS, JUN and other transcription factor (Foletta *et al*., 1998).

The AP-1 complex has an important role in controlling immune reactions such as the activation of T and B cells and the production of immunoglobulins. Activation of T cell is exerted through IL-2 bound to the complex of TcR and MHC. Formation of the complex involves kinases such MAPK and PKC and changes in intracellular calcium levels (Foletta *et al*., 1998). Genes involved in these activities was identified in cluster 1 (DUSP1), cluster 2 (DUSP5, CXCR4, EDN1), and cluster 3 (RGS2, RCAN1, DST, and NFKBIZ). Yet, no genes for B cell activation and regulation of immunoglobulin production could be detected in any of the 5 clusters.

Based on gene annotation from InnateDB, JUN and FOS are involved in IL-mediated signalling (IL-1, 2, 6, 12 for JUN and IL-2, 3, 6, 12 for FOS). No other gene involved in interleukin signalling was found in any of the 5 clusters. Yet, the JUN and FOS mediated pro-inflammatory response and activation of immune system could help the HEp-2 cell to cope with a *S. pyogenes* infection.

The AP-1 complex also has a role in apoptosis induction. Genes important for this pathway were found in cluster 1 (DDIT4 and IER3), cluster 2 (EGLN3, HTATIP2, PIM1, CXCR4, and RHOB), and in cluster 3 (ACTN4). This finding does not correlate with the improved survival rates especially in the presence of the *S. oralis* strains.

Reduced *S. pyogenes* adherence to or internalization into HEp-2 cell could also be related to *S. salivarius* or *S. oralis* - induced alterations in eukaryotic cytoskeleton factors and surface

proteins such as integrin, laminin and fibronectin. *S. pyogenes* binds to the latter two matrix proteins, which in turn bind to the cell membrane protein integrin. Therefore, the three proteins act as bridging molecules between the *S. pyogenes* adhesins and the host cell. Once this bridge is formed, a signalling cascade is implemented leading to *S. pyogenes* uptake into the eukaryotic cells by a zipper-like mechanism (Cue *et al*., 1998; Ozeri *et al*., 1998; Dombek *et al*., 1999; Molinari *et al.*, 2000; Yaoi *et al*., 2000; Terao *et al*., 2002; Wang *et al*., 2006).

Altered integrin-related mRNA quantities in response to contact with *S. oralis* are RhoB and CTGF (cluster 2) as well as ACTN4 and DST (cluster 3). Differentially expressed genes which are involved in cytoskeleton formation and cell adhesion were mainly found in response to *S. oralis*: FOS (cluster 1), JUN, CTGF, CYR61, RhoB, VEGFA, PBEF1, EDN1 (cluster 2), RASEF, DST, MKLN1 and ACTN4 (cluster 3). The Wnt signalling pathway, which is affected by JUN (cluster 2), has been linked to endocytosis events (Hynes *et al*., 2000; Marsden & DeSimone, 2003; Ulrich *et al*., 2005). Endocytosis represents a major uptake process which involves integrin recycling. The recycling process had been associated with the amount of extracellular matrix proteins such as fibronectin (Pellinen & Ivaska, 2006; LaFlamme *et al*., 2008). The factor VEGFA has been related with laminin and integrin turnover (Sudhakaran *et al*., 2008), while PLAUR was found to affect extracellular fibronectin levels (Monaghan *et al.,* 2004).

Up-regulated genes involved in proteolysis were found in response to *S. oralis* (PLAUR and YOD) and to *S. salivarius* (CTSZ). Such factors could protect HEp-2 cells from *S. pyogenes* adherence or cytotoxicity by degrading GAS surface proteins or toxins, respectively. On the other hand, the chaperone GRPEL1 (cluster 2) could interfere with the *S. pyogenes* proteases and thus, could contribute to improved HEp-2 cell homeostasis.

Taken together, the study highlighted several avenues by which *S. oralis* could induce protection of the eukaryotic cells even without binding to the cells or covering them by a biofilm for subsequent exposure to the *S. pyogenes* pathogen. The somewhat less effective protection of HEp-2 cells by *S. salivarius* involved expression changes only in a very restricted panel of genes and in stead, could predominantly be exerted by building an almost impermeable, potentially bacteriocin-producing wall of *S. salivarius* biofilm in front of the host cell target.

V. Conclusion

The data of the present study demonstrate that *S. pyogenes* can establish itself as a member of mixed species biofilms with typical species, i.e., *S. oralis*, of the resident oro-pharyngeal microflora. However, the species which most efficiently supports *S. pyogenes* growth in mixed species biofilms and simultaneously, does not affect virulence factor production in the β-hemolytic streptococci, also most efficiently protects underlying epithelial host cells from *S. pyogenes*-inflicted damages. The study also demonstrates that viable bacteria of the resident microflora such as *S. salivarius* can act as probiotics by suppressing *S. pyogenes* growth and growing as protective biofilms on top of the eukaryotic target cells. Finally, interference with *S. pyogenes* virulence factor production as exerted by *E. faecalis* as part of the transient microflora does not necessarily predict a protective function of such bacteria in more complex but also more realistic assays.

Part of this thesis work has been presented in:

Short talk:

Riani C*, Podbielski A, Kreikemeyer B, "*Streptococcus pyogenes* biofilm development and virulence functions in presence of physiologic oral or probiotic bacteria". The 58. Tagung der DGHM (Deutschen Gesellschaft für Hygiene und Mikrobiologie e.V.). Würzburg, Germany. Oktober 1 – 4, 2006

Kreikemeyer B*, Riani C, Lembke C, Standar K, Podbielski A, "Mixed species biofilms of Streptococcus pyogenes and oral streptococci – molecular and structural details of bacterial interactions and consequences for exposed human cells", The Fourth ASM Conference on Biofilms. Quebec City, Quebec, Canada. March 25-29, 2007

Lembke C*, Riani C, Podbielski A, Kreikemeyer B (2006) Identification and characterization of biofilm formation phenotypes of several clinically relevant *Streptococcus pyogenes* serotype strains. Biofilms II, Leipzig, Germany

Poster:

Kreikemeyer B*, Lembke C, Riani C, Köller T, Podbielski A (2007) Structures and components of *Streptococcus pyogenes* biofilms. The Fourth ASM Conference on Biofilms. Quebec City, Quebec, Canada. March 25-29, 2007

Riani C, Podbielski A, Kreikemeyer B*, "Mixed species biofilm interactions of the human pathogen *Streptococcus pyogenes* with resident and benign oral bacteria" as a poster at the International Biofilms III Conference. Munich, Germany. October 6−8, 2008

*) The presenter of the short talk or poster.

Reference

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

Abbott JD, Shannon R (1958) A Method for Typing *Shigella sonnei*, using Colicine Production as a Marker. *J Clin Pathol* **11**:71-77

Akiyama H, Morizane S, Yamasaki O, Oono T, Iwatsuki K (2003) Assessment of *Streptococcus pyogenes* microcolony formation in infected skin by confocal laser scanning microscopy. *J Dermatol Sci* **32**:193-199

Amerongen AN, Veerman E (2002) Saliva: the defender of the oral cavity. *Oral Dis* **8**:12-22

Aoki SK, Pamma R, Hernday AD, Bickham JE, Braaten BA, Low DA (2005) Contact-Dependent Inhibition of Growth in *Escherichia coli*. *Science* **309**:1245-1248

Autieri SM, Lins JJ, Leatham MP, Laux DC, Conway T, Cohen PS (2007) L-Fucose Stimulates Utilization of D-Ribose by *Escherichia coli* MG1655 Δ*fuc*AO and *E. coli* Nissle 1917 Δ*fuc*AO Mutants in the Mouse Intestine and in M9 Minimal Medium. *Infect Immun* **75**:5465-5475

Baldassarri L, Creti R, Recchia S, Imperi M, Facinelli B, Giovanetti E, Pataracchia M, Alfarone G, Orefici G (2006) Therapeutic Failures of Antibiotics Used To Treat Macrolide-Susceptible *Streptococcus pyogenes* Infections May Be Due to Biofilm Formation. *J Clin Microbiol* **44**:2721-2727

Beall B, Facklam R, Thompson T (1996) Sequencing *emm*-specific PCR products for routine and accurate typing of group A streptococci. *J Clin Microbiol* **34**:953-958

Bernstein, Faden HS, Scannapieco F, Belmont M, Dryja D, Wolf J (2002) Interference of nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis* by *Streptococcus oralis* in adenoid organ culture: a possible strategy for the treatment of the otitis-prone child. *Ann Otol Rhinol Laryngol* **111**:696-700

Bottone E, Allerhand J, Pisano MA (1971) Characteristics of a Bacteriocin Derived from *Streptococcus faecalis* var. *zymogenes* Antagonistic to *Diplococcus peumoniae*. *Appl Environ Microbiol* **22**:200-204

Brook I, Gilmore JD (1993) Evaluation of bacterial interference and beta-lactamase production in management of experimental infection with group A beta-hemolytic streptococci. *Antimicrob Agents Chemother* **37**:1452-1455

Brook I (2005) The Role of Bacterial Interference in Otitis, Sinusitis and Tonsillitis. *Otolaryngol Head Neck Surg* **133**:139-146

Brook I, Gober AE (2006) Recovery of interfering and β-lactamase-producing bacteria from group A β-haemolytic streptococci carriers and non-carriers. *J Med Microbiol* **55**:1741-1744

Burton JP, Chilcott CN, Moore CJ, Speiser G, Tagg JR (2006a) A preliminary study of the effect of probiotic *Streptococcus salivarius* K12 on oral malodour parameters. *J Appl Microbiol* **100**:754-764

Burton JP, Wescombe PA, Moore CJ, Chilcott CN, Tagg JR (2006) Safety Assessment of the Oral Cavity Probiotic *Streptococcus salivarius* K12. *Appl Environ Microbiol* **72**:3050-3053

Byers HL, Homer KA, Beighton D (1996) Utilization of sialic acid by viridans streptococci. *J Dent Res* **75**:1564-1571

Caparon, MG, Scott JR (1991) Genetic manipulation of pathogenic streptococci. *Methods Enzymol*. **204**:556–586.

Carlsson J (1967) Presence of various types of non-haemolytic streptococci in dental plaque and in other sites of the oral cavity in man. *Odontol Revy* **18**:55-74

Carlsson J, Grahnen H, Jonsson G, Wikner S (1970) Early Establishment of *Streptococcus salivarius* in the Mouths of Infants. *J Dent Res* **49**:415-418

Casadevall A, Pirofski LA, Catalano MT (1990) Primary group A streptococcal peritonitis in adults. *Am J Med* **88**:63N-64N

Challacombe S, Russell MJH, Hawkes J (1978) Passage of intact IgG from plasma to the oral cavity via crevicular fluid. *Clin Exp Immunol* **34**:417-422

Chan KG, Mayer M, Davis EM, Halperin SA, Lin TJ, Lee SF (2007) Role of D-Alanylation of *Streptococcus gordonii* Lipoteichoic Acid in Innate and Adaptive Immunity. *Infect Immun* **75**:3033-3042

Chapple ILC, Brock G, Eftimiadi C, Matthews JB (2002) Glutathione in gingival crevicular fluid and its relation to local antioxidant capacity in periodontal health and disease. *Mol Pathol* **55**:367-373

Chen YM, Weaver CA, Burne RA (2000) Dual Functions of *Streptococcus salivarius* Urease. *J Bacteriol* **182**:4667-4669

Chinenov Y, Kerppola TK (2001) Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene* **20**:2438-2452

Cho KH, Caparon MG (2005) Patterns of virulence gene expression differ between biofilm and tissue communities of *Streptococcus pyogenes*. *Mol Microbiol* **57**:1545-1556

Chole RA, Faddis BT (2003) Anatomical Evidence of Microbial Biofilms in Tonsillar Tissues: A Possible Mechanism to Explain Chronicity. *Arch Otolaryngol Head Neck Surg* **129**:634-636

Cisar JO, Kolenbrander PE, McIntire FC (1979) Specificity of coaggregation reactions between human oral streptococci and strains of *Actinomyces viscosus* or *Actinomyces naeslundii*. *Infect Immun* **24**:742-752

Cisar JO, Sandberg AL, Reddy GP, Abeygunawardana C, Bush CA (1997) Structural and antigenic types of cell wall polysaccharides from viridans group streptococci with receptors for oral actinomyces and streptococcal lectins. *Infect Immun* **65**:5035-5041

Clemans DL, Kolenbrander PE, Debabov DV, Zhang Q, Lunsford RD, Sakone H, Whittaker CJ, Heaton MP, Neuhaus FC (1999) Insertional Inactivation of Genes Responsible for the D-Alanylation of Lipoteichoic Acid in *Streptococcus gordonii* DL1 (Challis) Affects Intrageneric Coaggregations. *Infect Immun* **67**:2464-2474

Cohen SN, Chang ACY, Hsu L (1972) Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of *Escherichia coli* by R-Factor DNA. *Proc Natl Acad Sci USA***69**:2110-2114

Cole MF, Hsu SD, Baum BJ, Bowen WH, Sierra LI, Aquirre M, Gillespie G (1981) Specific and nonspecific immune factors in dental plaque fluid and saliva from young and old populations. *Infect Immun* **31**:998-1002

Conley J, Olson ME, Cook LS, Ceri H, Phan V, Davies HD (2003) Biofilm Formation by Group A Streptococci: Is There a Relationship with Treatment Failure? *J Clin Microbiol* **41**:4043-4048

Cook G, Costerton J, Lamont R (1998) Biofilm formation by *Porphyromonas gingivalis* and *Streptococcus gordonii*. *J Periodontal Res* **33**:323-327

Cosseau C, Devine DA, Dullaghan E, Gardy JL, Chikatamarla A, Gellatly S, Yu LL, Pistolic J, Falsafi R, Tagg J, Hancock REW (2008) The Commensal *Streptococcus salivarius* K12 Downregulates the Innate Immune Responses of Human Epithelial Cells and Promotes Host-Microbe Homeostasis. *Infect Immun* **76**:4163-4175

Costerton JW (1999) Introduction to biofilm. *Int J Antimicrob Agents* **11**:217-221

Courtney HS, Hasty DL (1991) Aggregation of group A streptococci by human saliva and effect of saliva on streptococcal adherence to host cells. *Infect Immun* **59**:1661-1666

Courtney HS, Podbielski A (2004) Group A streptococcal invasion of host cells. In *Bacterial Invasion of Host Cells*. Lamont, RJ (ed) Cambridge University Press, 239

Coykendall AL (1989) Classification and identification of the viridans streptococci. *Clin Microbiol Rev* **2**:315-328

Cue D, Dombek PE, Lam H, Cleary PP (1998) *Streptococcus pyogenes* Serotype M1 Encodes Multiple Pathways for Entry into Human Epithelial Cells. *Infect Immun* **66**:4593-4601

Cunningham MW (2000) Pathogenesis of Group A Streptococcal Infections. *Clin Microbiol Rev* **13**:470-511

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm. *Science* **280**:295-298

Delima AJ, Van Dyke TE (2003) Origin and function of the cellular components in gingival crevice fluid. *Periodontol 2000* **31**:55-76

Dempster RP, Tagg JR (1982) The production of bacteriocin-link substances by the oral bacterium *Streptococcus salivarius*. *Arch Oral Biol* **27**:151-157

Dierksen KP, Moore CJ, Inglis M, Wescombe PA, Tagg JR (2007) The effect of ingestion of milk supplemented with salivaricin A-producing *Streptococcus salivarius* on the bacteriocin-like inhibitory activity of streptococcal populations on the tongue. *FEMS Microbiol Ecol* **59**:584-591

Doern CD, Roberts AL, Hong W, Nelson J, Lukomski S, Swords WE,Reid SD (2009) Biofilm formation by group A Streptococcus: a role for the streptococcal regulator of virulence (Srv) and streptococcal cysteine protease (SpeB). *Microbiology* **155**:46-52

Dombek PE, Cue D, Sedgewick J, Lam H, Ruschkowski S, Finlay BB, Cleary PP (1999) High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements. *Mol Microbiol* **31**:859-870

Dunny GM, Lee LN, LeBlanc DJ (1991) Improved electroporation and cloning vector system for gramm-positive bacteria. *Appl Environ Microbiol* **57:**1194-1201

Ebersole JL (2003) Humoral immune responses in gingival crevice fluid: local and systemic implications. *Periodontol 2000* **31**:135-166

Egland PG, Palmer RJ, Kolenbrander PE (2004) Interspecies communication in *Streptococcus gordonii-Veillonella atypica* biofilms: Signaling in flow conditions requires juxtaposition. *Proc Natl Acad Sci USA* **101**:16917-16922

Elley BM, Cox SW (2003) Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice fluid. *Periodontol 2000* **31**:105-124

Facklam R, Beall B, Efstratiou A, Fischetti V, Johnson D, Kaplan E, Kriz P, Lovgren M, Martin D, Schwartz B, Totolian A, Bessen D, Hollingshead S, Rubin F, Scott J, Tyrrell G (1999) *emm* Typing and Validation of Provisional M Types for Group A Streptococci. *Emerg Infect Dis* **5**:247-253

Facklam R (2002) What Happened to the Streptococci: Overview of Taxonomic and Nomenclature Changes. *Clin Microbiol Rev* **15**:613-630

Falck G, Grahn-Hãkansson E, Holm SE, Roos K, Lagergren L (1999) Tolerance and Efficacy of Interfering Alpha-streptococci in Recurrence of Streptococcal Pharyngotonsillitis: a Placebo-controlled Study. *Acta Otolaryngol* **119**:944-948

Federle MJ, Bassler BL (2003) Interspecies communication in bacteria. *J Clin Invest* **112**:1291-1299

Fernie-King BA, Seilly DJ, Davies A, Lachmann PJ (2002) Streptococcal Inhibitor of Complement Inhibits Two Additional Components of the Mucosal Innate Immune System: Secretory Leukocyte Proteinase Inhibitor and Lysozyme. *Infect Immun* **70**:4908-4916

Fernie-King BA, Seilly DJ, Lachmann PJ (2004) The interaction of streptococcal inhibitor of complement (SIC) and its proteolytic fragments with the human beta defensins. *Immunology* **111**:444-452

Fiorentino T, Beall B, Mshar P, Bessen D (1997) A Genetic-Based Evaluation of the Principal Tissue Reservoir for Group A Streptococci Isolated from Normally Sterile Sites. *J Infect Dis* **176**:177-182

Foletta VC, Segal DH, Cohen DR (1998) Transcriptional regulation in the immune system: all roads lead to AP-1. *J Leukoc Biol* **63**:139-152

Fox JW, Marcon MJ, Bonsu BK (2006) Diagnosis of Streptococcal Pharyngitis by Detection of *Streptococcus pyogenes* in Posterior Pharyngeal versus Oral Cavity Specimens. *J Clin Microbiol* **44**:2593-2594

Frick IM, Mörgelin M, Björck L (2000) Virulent aggregates of *Streptococcus pyogenes* are generated by homophilic protein-protein interactions. *Mol Microbiol* **37**:1232-1247

Frick IM, Akesson P, Rasmussen M, Schmidtchen A, Bjorck L (2003) SIC, a Secreted Protein of *Streptococcus pyogenes* That Inactivates Antibacterial Peptides. *J Biol Chem* **278**:16561-16566

Friedemann TE (1938) Metabolism of Pathogenic Bacteria I.: Bacteriological and Chemical Methods. *J Bacteriol* **35**:527-546

Fuller R (1989) Probiotics in man and animals. *J Appl Microbiol* **66**:365-378

Gálvez A, Valdivia E, Abriouel H, Camafeita E, Mendez E, Martínez-Bueno M, Maqueda M (1998) Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. *Arch Microbiol* **171**:59-65

Ghosh J, Caparon MG (2006) Specificity of *Streptococcus pyogenes* NAD^+ glycohydrolase in cytolysin-mediated translocation. *Mol Microbiol* **62**:1203-1214

Glück U, Gebbers JO (2003) Ingested probiotics reduce nasal colonization with pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, and β-hemolytic streptococci). *Am J Clin Nutr* **77**:517-520

Gough H, Luke GA, Beeley JA, Geddes DAM (1996) Human salivary glucose analysis by high-performance ion-exchange chromatography and pulsed amperometric detection. *Arch oral biol* **41**:141-145

Griffiths GS (2003) Formation, collection and significance of gingival crevice fluid. *Periodontol 2000* **31**:32-42

Habermann W, Zimmermann K, Skarabis H, Kunze R, Rusch V (2001) Einfluss eines bakteriellen Immunstimulans (humane *Enterococcus faecalis*-Bakterien) auf die Rezidivhäufigkeit bei Patienten mit chronischer Bronchitis. *ArzneimForsch* **11**:931-937

Habermann W, Zimmermann K, Skarabis H, Kunze R, Rusch V (2002) Verminderung der Rezidivhäufigkeit bei Patienten mit chronisch rezidivierender hypertrophischer Sinusitis unter Behandlung mit einem bakteriellen Immunstimulans (*Enterococcus faecalis*-Bakterien humaner Herkunft). *ArzneimForsch* **8**:622-627

Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**:95-108

Hasegawa Y, Mans JJ, Mao S, Lopez MC, Baker HV, Handfield M, Lamont RJ (2007) Gingival Epithelial Cell Transcriptional Responses to Commensal and Opportunistic Oral Microbial Species. *Infect Immun* **75**:2540-2547

Hatakka K, Saxelin M (2008) Probiotics in Intestinal and Non-Intestinal Infectious Diseases - Clinical Evidence. *Curr Pharm Des* **14**:1351-1367

Henderson B, Poole S, Wilson M (1996) Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol Mol Biol Rev* **60**:316-341

Hirota K, Murakami K, Nemoto K, Ono T, Matsuo T, Kumon H, Miyake Y (1998) Fosfomycin Reduces CD15s-Related Antigen Expression of *Streptococcus pyogenes*. *Antimicrob Agents Chemother* **42**:1083-1087

Hollingshead SK, Fischetti VA, Scott JR (1986) Complete nucleotide sequence of type 6 M protein of the group A *Streptococcus*. Repetitive structure and membrane anchor. *J Biol Chem* **261**:1677-1686

Horstmann RD, Sievertsen HJ, Knobloch J, Fischetti VA (1988) Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc Natl Acad Sci USA***85**:1657-1661

Horz AM, Houben B, Conrads G (2007) Distribution and persistence of probiotic *Streptococcus salivarius* K12 in the human oral cavity as determined by real-time quantitative polymerase chain reaction. *Oral Microbiol Immunol* **22**:126-130

Hyink O, Wescombe PA, Upton M, Ragland N, Burton JP, Tagg JR (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**:1107-1113

Hynes W (2004) Virulence factors of the group A streptococci and genes that regulate their expression. *Front biosci* **9**:3399-3433

Jakubovics NS, Gill SR, Iobst SE, Vickerman MM, Kolenbrander PE (2008) Regulation of Gene Expression in a Mixed-Genus Community: Stabilized Arginine Biosynthesis in *Streptococcus gordonii* by Coaggregation with *Actinomyces naeslundii*. *J Bacteriol* **190**:3646-3657

Kolenbrander PE (2000) Oral Microbial Communities: Biofilms, Interactions, and Genetic Systems. *Annu Rev Microbiol* **54**:413-437

Kreikemeyer B, Boyle MDP, Buttaro BAL, Heinemann M, Podbielski A (2001) Group A streptococcal growth phase-associated virulence factor regulation by a novel operon (Fas)

with homologies to two-component-type regulators requires a small RNA molecule. *Mol Microbiol* **39**:392-406

Kreikemeyer B, McIver KS, Podbielski A (2003) Virulence factor regulation and regulatory networks in *Streptococcus pyogenes* and their impact on pathogen-host interactions. *Trends Microbiol* **11**:224-232

Kreikemeyer B, Klenk M, Podbielski A (2004) The intracellular status of *Streptococcus pyogenes*: role of extracellular matrix-binding proteins and their regulation. *Int J Med Microbiol* **294**:177-188

Kreikemeyer B, Nakata M, Koller T, Hildisch H, Kourakos V, Standar K, Kawabata S, Glocker MO, Podbielski A (2007) The *Streptococcus pyogenes* Serotype M49 Nra-Ralp3 Transcriptional Regulatory Network and Its Control of Virulence Factor Expression from the Novel *eno ralp3 epf sagA* Pathogenicity Region. *Infect Immun* **75**:5698-5710

Kreth J, Zhang Y, Herzberg MC (2008) Streptococcal Antagonism in Oral Biofilms: *Streptococcus sanguinis* and *Streptococcus gordonii* Interference with *Streptococcus mutans*. *J Bacteriol* **190**:4632-4640

Kuboniwa M, Tribble GD, James CE, Kilic AO, Tao L, Herzberg MC, Shizukuishi S,Lamont RJ (2006) *Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Mol Microbiol* **60**:121-139

LaFlamme SE, Nieves B, Colello D, Reverte CG (2008) Integrins as regulators of the mitotic machinery. *Current Opinion in Cell Biology*, **20:** 576-582.

Lafontaine ER, Wall D, Vanlerberg SL, Donabedian H, Sledjeski DD (2004) *Moraxella catarrhalis* Coaggregates with *Streptococcus pyogenes* and Modulates Interactions of *S. pyogenes* with Human Epithelial Cells. *Infect Immun* **72**:6689-6693

Lancefield RC (1928) The antigenic complex of *Streptococcus haemoliticus*: I. demonstration of a type-specific substance in extract of *Streptococcus haemoliticus*. *J Exp Med* **47**:91-103

Lancefield RC (1933) A serological differentiation of human and other groups of hemolytic streptococci. *J Exp Med* **57**:571-595

Lasaro MA, Salinger N, Zhang J, Wang Y, Zhong Z, Goulian M, Zhu J (2008) F1C fimbriae play an important role in biofilm formation and intestinal colonization of the *Escherichia coli* commensal Nissle 1917. *Appl Environ Microbiol* **75**:246-51

Lembke C, Podbielski A, Hidalgo-Grass C, Jonas L, Hanski E, Kreikemeyer B (2006) Characterization of Biofilm Formation by Clinically Relevant Serotypes of Group A Streptococci. *Appl Environ Microbiol* **72**:2864-2875

Li J, Helmerhorst EJ, Leone CW, Troxler RF, Yaskell T, Haffajee AD, Socransky SS, Oppenheim FG (2004) Identification of early microbial colonizers in human dental biofilm. *J Appl Microbiol* **97**:1311-1318

Li Y-H, Chen YYM, Burne RA (2000) Regulation of urease gene expression by *Streptococcus salivarius* growing in biofilms. *Environ Microbiol* **2**:169-177

Li Z, Sledjeski DD, Kreikemeyer B, Podbielski A, Boyle MDP (1999) Identification of *pel*, a *Streptococcus pyogenes* Locus That Affects both Surface and Secreted Proteins. *J Bacteriol* **181**:6019-6027

Liljemark WF, Bloomquist C (1996) Human Oral Microbial Ecology and Dental Caries and Periodontal Diseases. *Crit Rev Oral Biol Med* **7**:180-198

Luke NR, Jurcisek JA, Bakaletz LO, Campagnari AA (2007) Contribution of *Moraxella catarrhalis* Type IV Pili to Nasopharyngeal Colonization and Biofilm Formation. *Infect Immun* **75**:5559-5564

Madden JC, Ruiz N, Caparon M (2001) Cytolysin-Mediated Translocation (CMT): A Functional Equivalent of Type III Secretion in Gram-Positive Bacteria. *Cell* **104**:143-152

Manetti AG, Zingaretti C, Falugi F, Capo S, Bombaci M, Bagnoli F, Gambellini G, Bensi G, Mora M, Edwards AM, Musser JM, Graviss EA, Telford JL, Grandi G, Margarit I. (2007) *Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation. *Mol Microbiol* **64**:968-983

Marsden M, DeSimone DW (2003) Integrin-ECM Interactions Regulate Cadherin-Dependent Cell Adhesion and Are Required for Convergent Extension in Xenopus. *Curr Biol* **13**:1182-1191

Martin NJ, Kaplan EL, Gerber MA, Menegus MA, Randolph M, Bell K,Cleary PP (1990) Comparison of epidemic and endemic group G streptococci by restriction enzyme analysis. *J Clin Microbiol* **28**:1881-1886

Meehl MA, Caparon MG (2004) Specificity of streptolysin O in cytolysin-mediated translocation. *Mol Microbiol* **52**:1665-1676

Meehl MA, Pinkner JS, Anderson PJ, Hultgren SJ, Caparon MG (2005) A Novel Endogenous Inhibitor of the Secreted Streptococcal NAD-Glycohydrolase. *PLoS Pathog* **1**:e35

Molinari G, Talay SR, Valentin-Weigand P, Rohde M, Chhatwal GS (1997) The fibronectin-binding protein of *Streptococcus pyogenes*, SfbI, is involved in the internalization of group A streptococci by epithelial cells. *Infect Immun* **65** :1357-1363

Molinari G, Rohde M, Guzman CA, Chhatwal GS (2000) Two distinct pathways for the invasion of *Streptococcus pyogenes* in non-phagocytic cells. *Cell Microbiol* **2**:145-154

Mombelli B, Gismondo MR (2000) The use of probiotics in medical practice. *Int J Antimicrob Agents* **16**:531-536

Monaghan E, Gueorguiev V, Wilkins-Port C,McKeown-Longo PJ (2004) The Receptor for Urokinase-type Plasminogen Activator Regulates Fibronectin Matrix Assembly in Human Skin Fibroblasts. *J Biol Chem* **279**:1400-1407

Moore WEC, Moore LVH (1994) The bacteria of periodontal diseases. *Periodontol 2000* **5**:66-77

Mullis K, Paloona F, Scharf S, Saiki R, Horn G, Erlich H, (1986) Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction, *Cold Spring Harb Symp Quant Biol* 51:263-273

Murray PA, Levine MJ, Tabak LA, Reddy MS (1982) Specificity of salivary-bacterial interactions: II. Evidence for a lectin on *Streptococcus sanguis* with specificity for a NeuAcα2,3Ga1β1,3Ga1NAc sequence. *Biochem Biophys Res Comm* **106**:390-396

Nakata M, Podbielski A, Kreikemeyer B (2005) MsmR, a specific positive regulator of the *Streptococcus pyogenes* FCT pathogenicity region and cytolysin-mediated translocation system genes. *Mol Microbiol* **57**:786-803

Oliveros, JC (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. *http://bioinfogp.cnb.csic.es/tools/venny/index.html.*

Olsen RJ, Shelburne III SA, Musser JM (2008) Molecular mechanisms underlying group A streptococcal pathogenesis. *Cell Microbiol* **11**:1-12

Ozeri V, Rosenshine I, Mosher DF, Hanski E, Fässler R (1998) Roles of integrins and fibronectin in the entry of *Streptococcus pyogenes* into cells via protein F1. *Mol Microbiol* **30**:625-637

Österlund A, Engstrand L (1997) An Intracellular Sanctuary for *Streptococcus pyogenes* in Human Tonsillar Epithelium-Studies of Asymptomatic Carriers and In Vitro Cultured Biopsies. *Acta Otolaryngol* **117**:883-888

Palmer RJ. Jr., Gordon SM, Cisar JO, Kolenbrander PE (2003) Coaggregation-Mediated Interactions of Streptococci and Actinomyces Detected in Initial Human Dental Plaque. *J Bacteriol* **185**:3400-3409

Park Y, Simionato MR, Sekiya K, Murakami Y, James D, Chen W, Hackett M, Yoshimura F, Demuth DR, Lamont RJ (2005) Short Fimbriae of *Porphyromonas gingivalis* and Their Role in Coadhesion with *Streptococcus gordonii*. *Infect Immun* **73**:3983-3989

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE (2001) Bacterial Diversity in Human Subgingival Plaque. *J Bacteriol* **183**:3770-3783

Pellinen T, Ivaska J (2006) Integrin traffic. *J Cell Sci* **119**:3723-3731

Podbielski A, Melzer B, Lütticken R (1991) Application of the polymerase chain reaction to study the M protein(-like) gene family in beta-hemolytic streptococci. *Medical Microbiology and Immunology* **180**:213-227

Podbielski A, Spellerberg B, Woischnik M, Pohl B, Lütticken R (1996) Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* **177**:137-147

Podbielski A, Woischnik M, Leonard BAB, Schmidt KH (1999) Characterization of *nra*, a global negative regulator gene in group A streptococci. *Mol Microbiol* **31**:1051-1064

Pollanen MT, Salonen JI, Uitto VJ (2003) Structure and function of the tooth-epithelial interface in health and disease. *Periodontol 2000* **31**:12-31

Power DA, Burton JP, Chilcott CN, Dawes PJ, Tagg JR (2008) Preliminary investigations of the colonisation of upper respiratory tract tissues of infants using a paediatric formulation of the oral probiotic *Streptococcus salivarius* K12. *Eur J Clin Microbiol Infect Dis* **27**:1261-1263

Rafay AM, Homer KA, Beighton D (1996) Effect of mucin and glucose on proteolytic and glycosidic activities of *Streptococcus oralis*. *J Med Microbiol* **44**:409-417

Riani C, Standar K, Srimuang S, Lembke C, Kreikemeyer B, Podbielski A (2007) Transcriptome analyses extend understanding of *Streptococcus pyogenes* regulatory mechanisms and behavior toward immunomodulatory substances. *Int J Med Microbiol* **297**:513-523

Ribble JC (1967) A Mechanism of Bacterial Interference in vitro. *J Immunol* **98**:716-723

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* **11**:94-100

Rods K, Holm SE, Grahn-Hãkansson E, Lagergren L (1996) Recolonization with Selected α-Streptococci for Prophylaxis of Recurrent Streptococcal Pharyngotonsillitis - a Randomized Placebo-controlled Multicentre Study. *Scand J Infect Dis* **28**:459-462

Roos K, Grahn E, Holm SE, Johansson H,Lind L (1993) Interfering α-streptococci as a protection against recurrent streptococcal tonsillitis in children. *Int J Pediatr Otorhinolaryngol* **25**:141-148

Roos K, Hãkansson EG, Holm S (2001) Effect of recolonisation with "interfering" α streptococci on recurrences of acute and secretory otitis media in children: randomised placebo controlled trial. *BMJ* **322**:210-212

Roos K, Holm SE, Grahn-Hãkansson E, Lind L (2003) Alpha-streptococci as supplementary treatment of recurrent streptococcal tonsillitis: A randomized placebo-controlled study. *Scand J Infect Dis* **25**:31-35

Rosenbach FJ (1884) Mikro-Organismen bei den Wund-Infections-Krankheiten des Menschen. Wiesbaden: J.F. Bergmann Verlag, 22-29

Rosenkranz W, Grundmann E (1994) Immunomodulator action of living, nonpathogenic *Enterococcus faecalis* bacteria from humans. *ArzneimForsch* **44**:691-695

Ruiz N, Wang B, Pentland A, Caparon M (1998) Streptolysin O and adherence synergistically modulate proinflammatory responses of keratinocytes to group A streptococci. *Mol Microbiol* **27**:337-346

Russel C, Tagg JR (1981) Role of Bacteriocin during Plaque Formation by *Streptococcus salivarius* and *Streptococcus sanguis* on a Tooth in an Artificial Mouth. *J Appl Microbiol* **50**:305-313

Ryan PA, Pancholi V, Fischetti VA (2001) Group A Streptococci Bind to Mucin and Human Pharyngeal Cells through Sialic Acid-Containing Receptors. *Infect Immun* **69**:7402-7412

Salim KY, de Azavedo JC, Bast DJ, Cvitkovitch DG (2007) Role for *sag*A and *sia*A in Quorum Sensing and Iron Regulation in *Streptococcus pyogenes*. *Infect Immun* **75**:5011-5017

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 3rd ed.

Shelburne III SA, Sumby P, Sitkiewicz I, Granville C, DeLeo FR, Musser JM (2005) Central role of a bacterial two-component gene regulatory system of previously unknown function in pathogen persistence in human saliva. *Proc Natl Acad Sci USA* **102**:16037-16042

Shelburne III SA, Keith DB, Davenport MT, Horstmann N, Brennan RG, Musser JM (2008) Molecular characterization of group A *Streptococcus* maltodextrin catabolism and its role in pharyngitis. *Mol Microbiol* **69**:436-452

Simionato MR, Tucker CM, Kuboniwa M, Lamont G, Demuth DR, Tribble GD, Lamont RJ (2006) *Porphyromonas gingivalis* Genes Involved in Community Development with *Streptococcus gordonii*. *Infect Immun* **74**:6419-6428

Smith T, Brown JH (1915) A Study of Streptococci isolated from certain presumably milk-borne Epidemics of Tonsillitis occurring in Massachusetts in 1913 and 1914. *J Med Res* **31**:455-502

Stanley NR, Lazazzera BA (2004) Environmental signals and regulatory pathways that influence biofilm formation. *Mol Microbiol* **52**:917-924

Stenfors LE, Räisänen S (1991) Attachment of bacteria to tonsillar epithelium during acute tonsillitis. *J Laryngol Otol* **105**:29-32

Stinson MW, Alder S, Kumar S (2003) Invasion and Killing of Human Endothelial Cells by Viridans Group Streptococci. *Infect Immun* **71**:2365-2372

Stoodley P, Sauer K, Davies DG, Costerton JW (2002) Biofilms as complex differentiated communities. *Annu rev microbiol* **56**:187-209

Sun J, Gunzer F, Westendorf AM, Buer J, Scharfe M, Jarek M, Gößling F, Blöcker H, Zeng AP (2005) Genomic peculiarity of coding sequences and metabolic potential of probiotic *Escherichia coli* strain Nissle 1917 inferred from raw genome data. *J Biotechnol* **117**:147-161

Sudhakaran P, Viji R, Kiran M, Kumar SV (2008) Endothelial cell-laminin interaction: modulation of LDH expression involves α6β4 integrin-FAK-p38MAPK pathway. *Glycoconj J* **25** (online first)

Suntharalingam P, Cvitkovitch DG (2005) Quorum sensing in streptococcal biofilm formation. *Trends Microbiol* **13**:3-6

Swidsinski A, Goktas O, Bessler C, Loening-Baucke V, Hale LP, Andree H, Weizenegger M, Holzl M, Scherer H, Lochs H (2007) Spatial organisation of microbiota in quiescent adenoiditis and tonsillitis. *J Clin Pathol* **60**:253-260

Tagg JR, Dierksen KP (2003) Bacterial replacement therapy: adapting `germ warfare' to infection prevention. *Trends Biotechnol* **21**:217-223

Takemura N, Noiri Y, Ehara A, Kawahara T, Noguchi N, Ebisu S (2004) Single species biofilm-forming ability of root canal isolates on gutta-percha points. *Eur J Oral Sci* **112**:523-529

Tano K, Grahn-Hãkansson E, Holm SE, Hellström S (2000) Inhibition of OM pathogens by α-hemolytic streptococci from healthy children, children with SOM and children with rAOM. *Int J Pediatr Otorhinolaryngol* **56**:185-190

Tano K, Grahn Hãkansson E, Holm SE, Hellström S (2002) A nasal spray with alpha-haemolytic streptococci as long term prophylaxis against recurrent otitis media. *Int J Pediatr Otorhinolaryngol* **62**:17-23

Terao Y, Kawabata S, Nakata M, Nakagawa I, Hamada S (2002) Molecular Characterization of a Novel Fibronectin-binding Protein of *Streptococcus pyogenes* Strains Isolated from Toxic Shock-like Syndrome Patients. *J Biol Chem* **277**:47428-47435

Thomas M, Del Mar C, Glasziou P (2000) How effective are treatments other than antibiotics for acute sore throat. *Br J Gen Pract* **50**:817-820

Uitto VJ (2003) Gingival crevice fluid - an introduction. *Periodontol 2000* **31**:9-11

Uitto VJ, Overall CM, McCulloh C (2003) Proteolytic host cell enzymes in gingival crevice fluid. *Periodontol 2000* **31**:77-104

Ulrich F, Krieg M, Schötz EM, Link V, Castanon I, Schnabel V, Taubenberger A, Mueller D, Puech PH, Heisenberg CP (2005) Wnt11 Functions in Gastrulation by Controlling Cell Cohesion through Rab5c and E-Cadherin. *Dev Cell* **9**:555-564

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. *J Bacteriol* **183**:3931-3938

Van der Hoeven JS, Van den Kieboom CWA, Camp PJM (1990) Utilization of mucin by oral *Streptococcus* species. *Antonie van Leeuwenhoek* **57**:165-172

Van der Hoeven JS, Camp PJ (1991) Synergistic degradation of mucin by *Streptococcus oralis* and *Streptococcus sanguis* in mixed chemostat cultures. *J Dent Res* **70**:1041-1044

Virtaneva K, Porcella SF, Graham MR, Ireland RM, Johnson CA, Ricklefs SM, Babar I, Parkins LD, Romero RA, Corn GJ, Gardner DJ, Bailey JR, Parnell MJ, Musser JM (2005) Longitudinal analysis of the group A Streptococcus transcriptome in experimental pharyngitis in cynomolgus macaques. *Proc Natl Acad Sci USA* **102**:9014-9019

Vitkova A, Votava M (2005) Inhibition of hemolytic activity of Staphylococcus aureus 3-hemolysin by an exosubstance produced by some Enterococcus faecalis strains. *Epidemiol Mikrobiol Imunol.* **54**:11-15

Wade W (2002) Unculturable bacteria-the uncharacterized organisms that cause oral infections. *J R Soc Med* **95**:81-83

Walls T, Power D, Tagg J (2003) Bacteriocin-like inhibitory substance (BLIS) production by the normal flora of the nasopharynx: potential to protect against otitis media? *J Med Microbiol* **52**:829-833

Wang B, Cue D, Cleary P (2006) Intracelluar invasion by *Streptococcus pyogenes*: invasins, host receptors, and relevance to human disease. In *Gram-positive pathogens*. Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI(eds). Washington, DC: ASM Press, 29-36

Wang BY, Kuramitsu HK (2005) Interactions between Oral Bacteria: Inhibition of *Streptococcus mutans* Bacteriocin Production by *Streptococcus gordonii*. *Appl Environ Microbiol* **71**:354-362

Watnick P, Kolter R (2000) Biofilm, City of Microbes. *J Bacteriol* **182**:2675-2679

Weaver CA, Chen YY, Burne RA (2000) Inactivation of the ptsI gene encoding enzyme I of the sugar phosphotransferase system of *Streptococcus salivarius*: effects on growth and urease expression. *Microbiology* **146**:1179-1185

Weerkamp AH, McBride BC (1980) Adherence of *Streptococcus salivarius* HB and HB-7 to oral surfaces and saliva-coated hydroxyapatite. *Infect Immun* **30**:150-158

Weerkamp AH, Handley PS, Baars A, Slot JW (1986) Negative staining and immunoelectron microscopy of adhesion-deficient mutants of *Streptococcus salivarius* reveal that the adhesive protein antigens are separate classes of cell surface fibril. *J Bacteriol* **165**:746-755

Weerkamp AH, Jacobs T (1982) Cell Wall-Associated Protein Antigens of *Streptococcus salivarius*: Purification, Properties, and Function in Adherence. *Infect Immun* **38**:233-242

Wescombe PA, Upton M, Dierksen KP, Ragland NL, Sivabalan S, Wirawan RE, Inglis MA, Moore CJ, Walker GV, Chilcott CN, Jenkinson HF, 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. *Appl Environ Microbiol* **72**:1459-1466

Wilkins JC, Beighton D, Homer KA (2003) Effect of Acidic pH on Expression of Surface-Associated Proteins of *Streptococcus oralis*. *Appl Environ Microbiol* **69**:5290-5296

Wilson M (2005) Microbial Inhabitants of Humans, Their Ecology and Role in Health and Disease. London: Cambridge University Press

Yaoi T, Laksanalamai P, Jiemjit A, Kagawa HK, Alton T, Trent JD (2000) Cloning and Characterization of *fts*Z and *pyr*F from the Archaeon *Thermoplasma acidophilum*. *Biochem Biophys Res Comm* **275**:936-945

Yoshida Y, Yang J, Peaker PE, Kato H, Bush CA, Cisar JO (2008) Molecular and Antigenic Characterization of a *Streptococcus oralis* Coaggregation Receptor Polysaccharide by Carbohydrate Engineering in *Streptococcus gordonii*. *J Biol Chem* **283**:12654-12664

Websites:

NetAffx™ analysis center (http://www.affymetrix.com/analysis/index.affx)

PANTHER (http://www.pantherdb.org)

InnateDB (http://www.innatedb.com)

VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html)

VII. Appendix

Table 8: List of genes symbol and genes name

Gene symbol	Gene Name
ACTN4	actinin alpha 4
ADM	adrenomedullin
ANKRD37	ankyrin repeat domain 37
ARRDC3	arrestin domain containing 3
BHLHB2	basic helix-loop-helix domain containing, class b, 2
C5orf28	chromosome 5 open reading frame 28
C5orf5	chromosome 5 open reading frame 5
C7orf44	chromosome 7 open reading frame 44
CCNG2	cyclin G2
CDKN1B	cyclin-dependent kinase inhibitor 1b (p27, kip1)
CHD2	chromodomain helicase DNA binding protein 2
CLINT1	clathrin interactor 1
CLK1	cdc (cell division cycle)-like kinase 1
CSTF3	cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kDa
CTGF	connective tissue growth factor
CTSZ	cathepsin Z
CXCR4	chemokine (c-x-c motif) receptor 4
CYP39A1	cytochrome p450, family 39, subfamily A, polypeptide 1
CYR61	cysteine-rich, angiogenic inducer, 61
DDIT4	DNA-damage-inducible transcript 4
DKFZp547E087	hypothetical gene LOC283846
DST	dystonin
DTWD1	DTW domain containing 1
DUSP1	dual specificity phosphatase 1
DUSP5	dual specificity phosphatase 5
EDN1	endothelin 1
EEF1A1	eukaryotic translation elongation factor 1 alpha 1
EGLN3	EGL 9 homolog 3 (*Caenorhabditis elegans*)
ERGIC1	endoplasmic reticulum-golgi intermediate compartment 1
FLJ11236	hypothetical protein FLJ11236
FLJ38717	hypothetical protein FLJ38717
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
GRPEL1	GrpE-like 1, mitochondrial (*E. coli*)
GUSBP1	glucuronidase beta pseudogene 1
H19	H19, imprinted maternally expressed transcript (non-protein coding)
HIG2	hypoxia-inducible protein 2
HINT3	histidine triad nucleotide binding protein 3

Gene symbol	Gene Name
HTATIP2	HIV-1 tat interactive protein 2, 30 kDa
IER3	immediate early response 3
JUN	jun oncogene
JUNB	jun B proto-oncogene
KIAA0100	KIAA0100
LOC100134282	hypothetical protein loc100134282
LOC284454	hypothetical protein loc284454
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog f (avian)
MIRN21	microRNA 21
MKLN1	muskelin 1, intracellular mediator containing Kelch motifs
NAMPT	nicotinamide phosphoribosyltransferase
ND6	mitochondrially encoded NADH dehydrogenase 6
NFIL3	nuclear factor interleukin 3 regulated
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NKTR	natural killer-tumor recognition sequence
PIM1	pim-1 oncogene
PLAUR	plasminogen activator, urokinase receptor
POLR2J2	polymerase (RNA) II (DNA directed) polypeptide J2
PPP1R15B	protein phosphatase 1, regulatory (inhibitor) subunit 15b
PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3c
PSORS1C3	psoriasis susceptibility 1 candidate 3
RABGGTB	Rab geranylgeranyltransferase beta subunit
RASEF	Ras and EF-hand domain containing
RCAN1	regulator of calcineurin 1
RGS2	regulator of G-protein signalling 2, 24kda
RHOB	ras homolog gene family, member B
RP4-621O15.2	hypothetical protein FLJ31401
SERTAD2	SERTA domain containing 2
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3
SNF1LK	Snf1-like kinase
STC2	stanniocalcin 2
TAF9B	TAF9B RNA polymerase II, TATA box binding protein-associated factor, 31kDa
TFRC	transferrin receptor (p90, cd71)
TIPARP	TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin)-inducible poly(ADP-ribose) polymerase
TMEM49	transmembrane protein 49
TncRNA	non-protein coding RNA 84
TXNIP	thioredoxin interacting protein
VEGFA	vascular endothelial growth factor A
YOD1	YOD1 OTU deubiquinating enzyme 1 homolog (*Sacharomices cerevisiae*)
ZC3H12A	zinc finger CCCH-type containing 12A

Table 9: Overrepresented molecular functions, biological processes, and pathways of differentially expressed gene

Cluster	Molecular function	Biological process	Pathway
1	**DNA binding,** hydrolase activity, **MAP kinase tyrosine/serine/threonine phosphatase activity,** non-membrane spanning, phosphoprotein phosphatase activity, phosphoric monoester hydrolase activity, protein binding, protein dimerization activity, protein heterodimerization activity, **protein tyrosine/ serine/threonine phosphatase activity,** specific RNA polymerase II transcription factor activity, **transcription factor activity**	**anatomical structure morphogenesis**, anti-apoptosis, **apoptosis**, **cell cycle, dephosphorylation**, DNA methylation, inflammatory response, **intracellular signaling cascade**, **negative regulation of signal transduction**, nervous system development, protein amino acid dephosphorylation, **regulation of transcription**, regulation of transcription from RNA polymerase II promoter, regulation of transcription, response to oxidative stress, **response to stress**	Smooth muscle contraction, **TGF Beta Signaling Pathway**
2	24-hydroxycholesterol 7alpha-hydroxylase activity, actin binding, adenyl-nucleotide exchange factor activity, ATP binding, ATP-dependent DNA helicase activity, catalytic activity, C-C chemokine receptor activity, cell surface binding, **chaperone binding**, chemokine receptor activity, chromatin binding, CoA hydrolase activity, coreceptor activity, C-X-C chemokine receptor activity, cyclin-dependent protein kinase inhibitor activity, cysteine-type peptidase activity, **cytokine activity**, DNA binding, electron carrier activity, endothelin A & B receptor binding, enzyme binding, **extracellular matrix binding**, **glucose transmembrane transporter activity**, **G-protein coupled receptor activity**, **growth factor activity**, **GTP binding**, **GTPase activity**, **helicase activity**, heme binding, heparin binding, hormone activity, hydrolase activity, insulin-like growth factor binding, integrin binding, iron ion binding, **kinase activity**, L-ascorbic acid binding, magnesium ion binding, MAP kinase tyrosine/serine/ threonine phosphatase activity, monooxygenase activity, myosin light chain binding, NAD+ ADP-ribosyl-transferase activity, nicotinamide phosphoribosyl-transferase activity, non-membrane spanning protein tyrosine kinase & phosphatase activity, **nucleic acid binding**, nucleotide binding, **oxidoreductase activity**, oxysterol 7-alpha-hydroxylase activity, peptidase activity, phosphoprotein phosphatase activity, phosphoric monoester hydrolase activity, **PDGF receptor binding**, prenyltransferase activity, protein binding, protein dimerization activity, protein homodimerization activity, **protein kinase activity**, protein kinase inhibitor	**activation of MAPK activity**, activation of protein kinase C activity, ameboidal cell migration, amino acid and derivative metabolic process, anatomical structure morphogenesis, **angiogenesis**, **apoptosis**, autophagic cell death, bile acid catabolic & biosynthetic process, blood circulation, **blood coagulation**, blood vessel morphogenesis, body fluid secretion, **calcium-mediated signaling**, cAMP biosynthetic process, **carbohydrate metabolic process**, carbohydrate transport, cartilage development, cell adhesion, **cell cycle**, **cell cycle checkpoint, cell differentiation**, cell division, cell migration, **cell motility, cell proliferation, cell surface receptor linked signal transduction, cell-cell signaling**, cell-matrix adhesion, cellular process, chemotaxis, cholesterol catabolic process, chromatin assembly or disassembly, dephosphorylation, digestion, diuresis, DNA replication, dorsal/ventral pattern formation, elevation of cytosolic calcium ion concentration, endosome to lysosome transport, entrainment of circadian clock, epidermis development, ER to Golgi vesicle-mediated transport, excretion, fibroblast growth factor receptor signaling pathway, G1/S transition of mitotic cell cycle, germ cell development, germ cell migration, glucose transport, glycogen biosynthetic process, **glycogen metabolic process**, G-protein coupled receptor protein signaling pathway, G-protein signaling phospholipase D activating pathway, **immune response**, induction of apoptosis, induction of positive chemotaxis, inflammatory response, integrin-mediated signaling pathway, **intracellular signaling cascade**, leading edge cell differentiation, leukocyte activation, **lipid metabolic process**, membrane depolarization, metabolic process, **mitosis**, motor axon guidance,	**Apoptosis**, Apoptosis GenMAPP, Circadian Exercise, **G1 to S cell cycle**, GPCRDB Class A Rhodopsin-like, Hypertrophy model, **MAPK Cascade**, mRNA processing, **Peptide GPCRs**, Prostaglandin synthesis regulation, Smooth muscle contraction, **TGF Beta Signaling Pathway, Wnt signaling pathway**

Cluster	Molecular function	Biological process	Pathway
	activity, Rab-protein geranyl-geranyltransferase activity,receptor activity, rhodopsin-like receptor activity, **RNA binding**, **RNA polymerase II transcription factor activity**, sequence-specific DNA binding, **signal transducer activity**, steroid 7-alpha-hydroxylase activity, sugar:hydrogen symporter activity, transcription coactivator activity, transcription corepressor activity, **transcription factor activity**, transcription regulator activity, **transferase activity**, transferring glycosyl groups, transforming growth factor beta receptor, cytoplasmic mediator activity, **transporter activity**, unfolded protein binding, U-plasminogen activator receptor activity, vascular endothelial growth factor receptor binding, zinc ion binding	multicellular organismal development, **mRNA processing**, natriuresis, nervous system development, neural crest cell development, neuron migration, nitric oxide transport, nuclear import, ossification, oxidation reduction, parturition, peptide hormone secretion, phosphoinositide 3-kinase cascade, PDGF receptor signaling pathway, potassium ion transport, protein amino acid ADP-ribosylation, protein amino acid dephosphorylation, **protein amino acid phosphorylation**, **protein folding**, protein import into mitochondrial matrix, protein kinase C deactivation, **protein kinase cascade, protein metabolic process, protein modification process**, protein transport, pyridine nucleotide biosynthetic process, regulation of pH, **regulation of proteolysis**, regulation of translation, **regulation of vasoconstriction**, respiratory gaseous exchange, response to hypoxia, response to nutrient, **response to stress**, response to virus, response to wounding, Rho protein signal transduction, rhythmic excitation, rhythmic process, **signal transduction, small GTPase mediated signal transduction, steroid metabolic process**, T cell proliferation, **transcription,translation**, ubiquitin cycle, vasculogenesis, vesicle-mediated transport	
3	**actin binding**, actin filament binding, ATP binding, calcium ion binding, calmodulin binding, cyclosporin A binding, **DNA binding**, **DNA-directed RNA polymerase activity**, GTP binding, GTPase activator activity,**GTPase activity**, integrin binding, **isomerase activity**, malate dehydrogenase (acceptor) activity, **mismatched DNA binding**, nucleoside binding, peptide binding, peptidyl-prolyl cis-trans isomerase activity, protein binding, protein C-terminus binding, protein dimerization activity, protein homodimerization activity, protein N-terminus binding, **receptor activity, receptor binding, signal transducer activity**, specific RNA polymerase II transcription factor activity, structural constituent of cytoskeleton, structural molecule activity, transcription coactivator activity, transcription factor activity, transferrin receptor activity, **translation elongation factor activity**, zinc ion binding	actin cytoskeleton organization and biogenesis, **actin filament bundle formation**, blood circulation, calcium-mediated signaling, **cell adhesion, cell cycle**, **cell motility**, cell-matrix adhesion, cellular iron ion homeostasis, central nervous system development, cytoskeleton organization and biogenesis, **endocytosis**, integrin-mediated signaling pathway, intermediate filament cytoskeleton organization and biogenesis, **iron ion transport**, keratinocyte differentiation, **mismatch repair**, negative regulation of cell motility, negative regulation of signal transduction, positive regulation of cell motility, positive regulation of sodium: hydrogen antiporter activity, **protein folding**, protein transport, regulation of apoptosis, **regulation of G-protein coupled receptor protein signaling pathway**, regulation of transcription, response to hypoxia, **signal transduction**, small GTPase mediated signal transduction, transcription, transcription from RNA polymerase II promoter, **translation**, translational elongation, transmembrane receptor protein tyrosine kinase signaling pathway, tricarboxylic acid cycle	**RNA transcription, Smooth muscle contraction,** Striated muscle contraction

Cluster	Molecular function	Biological process	Pathway
4	catalytic activity, cysteine-type endopeptidase activity, hydrolase activity, peptidase activity	**proteolysis**	
5	GTPase activator activity, lipid binding, NADH dehydrogenase (ubiquinone) activity, oxidoreductase activity, protein binding	endocytosis,mitochondrial electron transport (NADH to ubiquinone), oxidation reduction, **signal transduction**, transport	Electron Transport Chain

Bold printed pathways were retrieved from NetAff™ Analysis Center, PANTHER and InnateDB; underlined pathways and molecular functions were retrieved from PANTHER and InnateDB; bold printed molecular functions and biological processes were retrieved from NetAff™ Analysis Center and PANTHER.

Acknowledgement

I would like to thank Prof. Dr. Dr Andreas Podbielski, for his guidance and patience in finishing my PhD study.

I would like to thank PD Dr. Bernd Kreikemeyer, for his helpful suggestions and support during my thesis work and writing.

I would like to thank DAAD for the scholarship.

I would like to thank Prof. Ludwig Jonas and all technical assistant in Electron Microscope Center, Dept. Pathology, Rostock University for help and suggestions during work with electron microscope; to Dr. Peter Lorenz (Molecular Cell Biology, Institute of Immunology, Rostock University) for his help with scanning confocal laser microscope, Dr. Dirk Koczan (Molecular Immunology, Institute of Immunology, Rostock University) for his help and explanation in transcriptome work of HEp-2 cell.

I would like to thank all technicians (especially Yvonne and Jana) and all members of research group in Prof. Dr Andreas Podbielski lab for their help, warm friendship and patience with my poor German during my work and stay in Rostock. Hopefully, we still keep in touch.

I would like to thank all my Indonesian friends in Germany and especially in Rostock for their support, help and losing my home sick also all friends and people who made my stay in Rostock feels like at home.

Special thanks to my father, Katiman, and my mother, Rohana, for their motivation and endless praying for my happiness and success. And also to my brothers and sister and for my nieces and nephews and all my big family and friends in Indonesia who always made me happy with our telephone during my stay in Rostock.

Curriculum Vitae

Name	Catur Riani
Date/ Place of birth	13.08.1976, Pulau Sambu, Indonesia

Education

2004 (October) - 2009	Ph.D degree in Rostock University (Sponsored by DAAD)
1999 - 2002	Master degree in Microbiology, Dept. Pharmacy, Fact. Mathematics and Natural Sciences, Bandung Institute of Technology, Indonesia
1997 - 1998	Pharmacist, Dept. Pharmacy, Fact. Mathematics and Natural Sciences, Bandung Institute of Technology, Indonesia
1993 - 1997	Bachelor degree, Dept. Pharmacy, Fact. Mathematics and Natural Sciences, Bandung Institute of Technology, Indonesia
1990 - 1993	Senior High School, SMAN 1 Tanjungpinang, Indonesia
1987 - 1990	Junior High School, SMPN 1 Belakangpadang, Indonesia
1981 - 1987	Elementary School, SDN 1 Pertamina Pulau Sambu, Indonesia

Work Experience

Participate in several projects during Master degree and working as research assistant in Inter University Research Center, Bandung Institute of Technology, Indonesia

- KMNRT-LIPI (Ministry of Research and Technology), 2001-2003, RUT VIII, "Biological functions of *Streptococcus pyogenes* M12-human albumin interaction on signal transduction mechanism", as a member.
- KMNRT-LIPI (Ministry of Research and Technology), 2002-2003, RUT IX, "The role of *Streptococcus pyogenes* HTH2 Mga protein in *mga* gene autoregulation and its ability in activating virulence factor *emm* & *scp* gene *in vivo*", as a principle investigator.
- DIKTI, (Ministry of High Education), 2003-2004, Hibah Bersaing XII, “Laminin Binding Protein as Vaccine Candidate for *Steptococcus pyogenes*”, as a principle investigator.

List of publication, short talk and poster:

Riani C, Standar K, Srimuang S, Lembke C, Kreikemeyer B, Podbielski A (2007) Transcriptome analyses extend understanding of *Streptococcus pyogenes* regulatory mechanisms and behavior toward immunomodulatory substances. *Int J Med Microbiol* **297**:513-523

Sugareva V, Arlt R, Fiedler T, Riani C, Podbielski A, Kreikemeyer B* (2009) Serotype- and strain- dependent contribution of the CovRS two-component system to *Streptococcus pyogenes* pathogenesis. Submitted in *Int J Med Microbiol*

Short talk:

Riani C*, Podbielski A, Kreikemeyer B, "*Streptococcus pyogenes* biofilm development and virulence functions in presence of physiologic oral or probiotic bacteria". The 58. Tagung der DGHM (Deutschen Gesellschaft für Hygiene und Mikrobiologie e.V.). Würzburg, Germany. Oktober 1 – 4, 2006

Kreikemeyer B*, Riani C, Lembke C, Standar K, Podbielski A, "Mixed species biofilms of Streptococcus pyogenes and oral streptococci – molecular and structural details of bacterial interactions and consequences for exposed human cells", The Fourth ASM Conference on Biofilms. Quebec City, Quebec, Canada. March 25-29, 2007

Lembke C*, Riani C, Podbielski A, Kreikemeyer B (2006) Identification and characterization of biofilm formation phenotypes of several clinically relevant *Streptococcus pyogenes* serotype strains. Biofilms II, Leipzig, Germany

Köller T*, Nakata M, Lembke C, Standar K, Riani C, Redanz S, Glocker MO, Kreikemeyer B (2008) Role of the cell wall anchoring proteins SortaseA and SortaseC2 in *Streptococcus pyogenes* pathogenesis. VAAM-/GBM-Jahrestagung 2008, Frankfurt

Poster:

Kreikemeyer B*, Lembke C, Riani C, Köller T, Podbielski A (2007) Structures and components of *Streptococcus pyogenes* biofilms. The Fourth ASM Conference on Biofilms. Quebec City, Quebec, Canada. March 25-29, 2007

Riani C, Podbielski A, Kreikemeyer B*, "Mixed species biofilm interactions of the human pathogen *Streptococcus pyogenes* with resident and benign oral bacteria" as a poster at the International Biofilms III Conference. Munich, Germany. October 6−8, 2008

Sugareva V*, Riani C, Arlt R, Podbielski A, Kreikemeyer B (2008) Serotype-dependent characterization of two-component signal transduction systems in *Streptococcus pyogenes*. The XVII Lancefield Symposium on Streptococci and Streptococcal Diseases, Porto Heli, Greece

Rostock, 28.01.2009 Catur Riani

*) The presenter of the short talk or poster

Selbständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmitteln verwendet habe.

Rostock, 28.01.2009 Catur Riani