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# Exploring the biofilm of *Streptococcus agalactiae* to identify virulence factors

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# Attività di ricerca

Durante il Dottorato di Ricerca mi sono occupata dello studio del biofilm di *Streptococcus agalactiae*. In particolare ho valutato l'influenza che ha il pH acido sulla capacità di formazione di biofilm *in vitro* di un ampio numero di isolati clinici. Tale analsi mi ha permesso di identificare una correlazione tra capacità di formazione di biofilm e sierotipo ed in particolare ha evidenziato che la maggior parte dei ceppi formanti biofilm appartiene all'ipervirulento *Sequence Type -17*. Ho valutato il coinvolgimento della capsula, del DNA e delle proteine nella formazione di biofilm in GBS e ho identificato, mediante spettrometria di massa, proteine espresse specificamente a pH acido che potrebbero svolgere un ruolo determinante nella prima fase di adesione del biofilm. I risultati sono descritti nella presente tesi di dottorato ed il corrispondente manoscritto è stato pubblicato recentemente (D'Urzo et al., 2014).

Parallelamente mi sono occupata della valutazione delle *performances* di espressione, intra- ed extracellulare, di proteine ricombinanti da parte di un batterio gram-positivo, *Brevibacillus choshinensis*, utilizzando due proteine modello, la GFP e l'α-amylasi. Ho inoltre implementato tale sistema di espressione utilizzando, per la prima volta, un plasmide contenente un promotore inducibile che ha permesso di incrementare le rese fino a 10 volte (D'Urzo et al., 2013). Ho infine utilizzato *Brevibacillus* per esprimere il dominio catalitico della tossina A di *Clostridium difficile*, possibile componente di un vaccino, che ho caratterizzato dal punto di vista biochimico e strutturale (D'Urzo et al., 2012).

Nel periodo del Dottorato di Ricerca sono stata co-autrice dei seguenti lavori scientifici:

1. **D'Urzo N**, Martinelli M, Pezzicoli A, De Cesare V, Pinto V; Members of DEVANI Study Group, Margarit I, Telford JL, Maione D. "Acidic pH strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17 Streptococcus agalactiae strains." Appl Environ Microbiol. 2014 Jan 31. [Epub ahead of print]
2. Leuzzi R, Spencer J, Buckley A, Brettoni C, Martinelli M, Tulli L, Marchi S, Luzzi E, Irvine J, Candlish D, Veggi D, Pansegrau W, Fiaschi L, Savino S, Swennen E, Cakici O, Oviedo-Orta E, Giraldi M, Baudner B, **D'Urzo N**, Maione D, Soriani M, Rappuoli R, Pizza M, Douce GR, Scarselli M. "Protective efficacy induced by recombinant Clostridium difficile toxin fragments" Infect Immun. 2013 Infect Immun. 2013 Aug;81(8):2851-60. doi: 10.1128/IAI.01341-12. Epub 2013 May 28.
3. **D'Urzo N**, Martinelli M, Nenci C, Brettoni C, Telford JL, Maione D. "High-level intracellular expression of heterologous proteins in Brevibacillus choshinensis SP3 under the control of a xylose inducible promoter" Microb Cell Fact. 2013 Feb 1;12:12. doi: 10.1186/1475-2859-12-12.
4. **D'Urzo N**, Malito E, Biancucci M, Bottomley MJ, Maione D, Scarselli M, Martinelli M. "The structure of Clostridium difficile toxin A glucosyltransferase domain bound to Mn2+ and UDP provides insights into glucosyltransferase activity and product release". FEBS J. 2012 Sep;279(17):3085-97. doi: 10.1111/j.1742-4658.2012.08688.x.

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# Summary

*Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS) is the primary colonizer of the anogenital mucosa of up to 40% of healthy women and an important cause of invasive neonatal infections worldwide. Among the 10 known capsular serotypes, GBS type III accounts for 30-76% of the cases of neonatal meningitis.

Biofilms are dense aggregates of surface-adherent microorganisms embedded in an exopolysaccharide matrix. Centers for Disease Control and Prevention estimate that 65% of human bacterial infections involve biofilms (Post et al., 2004). Many species of streptococci are known to form biofilms; however, the relationship between the pathogenic state and the biofilm mode of growth has been most clearly established only for the oral streptococci (Cvitkovitch et al., 2003).

In recent years, the ability of GBS to form biofilm attracted attention for its possible role in fitness and/or virulence. Here, a new *in vitro* biofilm formation protocol was developed to guarantee more stringent conditions, to better discriminate between strong-, low- and non-biofilm forming strains and reduce ambiguous data interpretation. This protocol was applied to screen the *in vitro* biofilm formation ability of more than 350 GBS clinical isolates from pregnant women and neonatal infections belonging to different serotype, in relation to media composition and pH.

The results showed the enhancement of GBS biofilm formation in acidic condition and identified a subset of isolates belonging to serotypes III and V that forms strong biofilms in these conditions. Interestingly, the best biofilm formers belonged to the serotype III hypervirulent clone ST-17.It was also found that pH 5.0 induces down-regulation of the capsule but that this

reduction is not enough by itself to ensure biofilm formation. Moreover, the ability of proteinase K to strongly inhibit biofilm formation and to disaggregate mature biofilms suggested that proteins play an essential role in promoting GBS biofilm formation and contribute to the biofilm structural stability. Finally, a set of proteins potentially expressed during the GBS *in vitro* biofilm formation, were identified by mass spectrometry.

# 1. Introduction

## 1.1 *Streptococcus agalactiae*

*Streptococcus agalactiae* is a Gram-positive bacterium, historically associated to bovine mastitis and dairy sources under the designation of *Streptococcus mastiditis* (Lancefield, 1933). It forms small (3 to 4 mm), grey-white colonies that have a narrow zone of beta hemolysis on blood agar plate (Figure 1.1). It was first identified as group B streptococci (GBS) in 1933, when Rebecca Lancefield published her studies on serological differentiation of streptococci (Lancefield, 1933). GBS was later proposed as an occasional causative agent of puerperal infections (Lancefield and Hare, 1935), and in 1938 it was recognized as an important human pathogen responsible for multiple infections (Fry, 1938). It was not until the 1970s that GBS was acknowledged as a leading cause of neonatal invasive infections (Broughton et al., 1976) and since the 1990s it has also been increasingly associated with invasive infections in non-pregnant adults (Farley et al., 1993). Despite its unquestionable importance as a human pathogen, GBS ismainly a colonizing agent of the gastrointestinal and genitourinary tracts of a significant proportion of the human population (Schuchat, 1998). GBS strains are classified into ten serotypes according to immunogenic characteristics of the capsule polysaccharides (Ia, Ib, II, III, IV, V, VI,VII, VIII and IX). Approximately 10% of strains are non-typeable (Bisharat et al., 2005;Gherardi et al., 2007;Kong et al., 2002;Skoff et al., 2009).

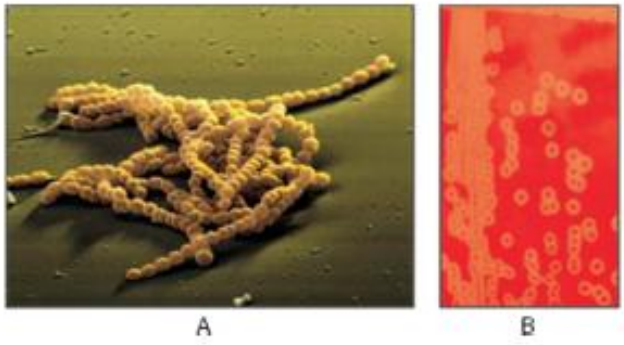



**Figure 1.1: *Streptococcus agalactiae*.**

A) Scanning Electron Microscopy (SEM) of *Streptococcus agalactiae*. B) Colonies of *Streptococcus agalactiae* on a blood agar plate. Note the zone of clear hemolysis.

## 1.2 Group B streptococcal disease

GBS colonizes the urogenital tract of more 30% of the healthy population and in particular it colonizes the vagina of 25-40% of healthy women (Dillon, Jr. et al., 1982;Hansen et al., 2004a;Schuchat, 1998). It has been found in the urethra in both men and women without causing infections and in the upper respiratory tract. Colonization also is observed in wound and soft tissue cultures in the absence of obvious infection. Determining the acquisition and transmission of *S. agalactiae* can be puzzling, as it is very invasive but produces little inflammation at the entry site.

This bacterium is an important cause of infection in three populations (Figure 1.2):

- Neonates;
- Pregnant women;
- Non pregnant adults;

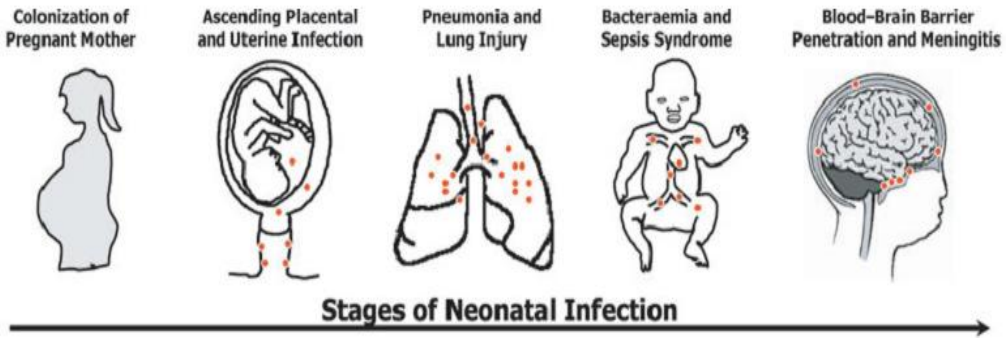



**Figure 1.2: Stages of neonatal GBS infection.** *Adapted from (Doran and Nizet, 2004).*

### 1.2.1 Neonatal infections

GBS is the leading cause of neonatal bacterial diseases in the United States of America; infection in newborns has been divided in early-onset disease (EOD) and late-onset disease (LOD) depending on the infants' age and disease manifestations. The onset of GBS infections that takes place very early in infancy, usually within the first week of life, is designated EOD, even if the majority of EOD cases occur within the first 24 hours after birth (Schuchat, 1998). LOD develops between one week and three months of age (Schuchat, 1998). Maternal carriage is a major risk factor for neonatal GBS disease, which is influenced by the degree of bacterial colonization; women with heavy colonization are more likely to have symptomatically infected infants and heavily colonized infants are more likely to develop invasive disease (Lim, DV, 1982). The onset of disease is associated with the presence of GBS in the genital tract of the mother, and transmission is thought to occur vertically due to an ascending infection during the course of pregnancy or passage through the birth canal (Schuchat, 1998). Even though perinatal transmission can occur across intact membranes, both premature and prolonged rupture of membranes increase the risk of GBS acquisition (Schuchat and Wenger, 1994). Aspiration of contaminated amniotic fluid then leads to colonization of the airways of the neonate and is

rapidly followed by the development of pneumonia (Doran and Nizet, 2004). Breaching of the pulmonary mucosal barrier leads to the entry of GBS into the bloodstream and to the development of severe sepsis in some infants (Schuchat, 1998). More than half reported cases of neonatal GBS disease now occur during the late-onset period (Centers for Disease Control and Prevention, 2005). The pathogenesis of LOD is less well understood, although some cases also suggest a maternal source, probably reflecting acquisition of the microorganism during passage through the birth canal (Schuchat, 1998). Ingestion of contaminated breast milk has also been proposed as a possible maternal source for LOD (Bingen et al., 1992). Even though nearly 50% of mothers of infants with LOD were found to carry the same GBS serotype as that causing infection in their infants, the source of infection in other infants is unclear (Schuchat, 1998). Nosocomial and horizontal transmission by hospital and community sources are probably involved in some cases of LOD, but the risk factors are not well understood (Green et al., 1994). Whereas EOD and LOD can differ in clinical presentation, mode of transmission and risk factors for disease, the most frequent clinical presentations of invasive disease in neonates are pneumonia, bacteremia and meningitis (Fernandez et al., 2001;Puopolo et al., 2005).LOD presents with meningitis and bacteremia without a focus as predominant clinical syndromes; osteoarticular infections, urinary tract infections, and pneumonia are less frequent (Schuchat, 2006). Meningitis develops when the entry of bacteria into the bloodstream is followed by the invasion of the cerebrospinal fluid.

### 1.2.2 Infections in pregnancy

GBS causes a variety of perinatal infections in pregnant women, including both symptomatic and asymptomatic bacteriuria, endometritis, amnionitis, meningitis, pyelonephritis,

and post-partum wound infections (Pass et al., 1982). It also has been suggested that GBS urinary tract infections or urinary tract, rectal, or genital colonization in pregnant woman may lead to late term abortions and preterm and low-birth-weight infants. Pregnant women are colonized at multiple sites, including rectum, vagina, cervix and throat, but many of them carry GBS in asymptomatically way (Regan et al., 1991). Approximately 10-30% of pregnant women are colonized with GBS in the vagina or rectum (Regan et al., 1991), although the colonization can be transient, chronic or intermittent (Hansen et al., 2004b). As maternal GBS carriage in the gastrointestinal and/or genital tracts is a prerequisite for EOD, the different prevalence of maternal GBS colonization could help choose preventive strategies. In most European countries, the prevalence of GBS carriage among pregnant women varies between 6.5 and 36%, with most countries reporting colonization rates of 15-20% (Barcaite et al., 2008a;Trijbels-Smeulders et al., 2004).

### 1.2.3 Infections in non-pregnant adults

In the past two decades GBS has been also increasingly associated with invasive disease in non-pregnant adults (Bergseng et al., 2008;Matsubara and Yamamoto, 2009;Phares et al., 2008;Skoff et al., 2009). Even though colonization among non-pregnant adults is less well known, vaginal and rectal colonization of healthy young and elderly adults have been reported at levels (20-34%) similar to those observed during pregnancy (Bliss et al., 2002;Edwards et al., 2005;Manning et al., 2004;Manning et al., 2002). GBS was also found to be likely transmitted between sex partners during pregnancy (Foxman et al., 2008), yet multiple transmission modes may exist (Manning et al., 2004). An increasing number of studies also suggest that limited interspecies GBS transmission is likely to occur between humans and their livestock (Manning et

al., 2010;Oliveira et al., 2006;Sukhnanand et al., 2005), further proposing a framework for GBS as a possible zoonotic infection, which can have significant public health implications (Manning et al., 2010). Such infections increase with age, occur more frequently among nursing facility residents than in the community, and are considered responsible for substantial morbidity and mortality, with case-fatality rates of nearly 25% (Farley, 2001;Henning et al., 2001). Most cases occur in individuals with significant underlying conditions; diabetes mellitus being is the most frequent co-morbidity, typically present in approximately 30% of non-pregnant adults with GBS disease (Farley et al., 1993;Jackson et al., 1995). Other risk factors have been detailed in recent years and include liver cirrhosis, heart and neurologic disease, cancer and immunosuppressive conditions (Jackson et al., 1995). The clinical spectrum of GBS disease in adults is broad, including more frequently bacteremia with or without sepsis, skin and soft tissue, osteoarticular and urinary tract infections (Farley et al., 1993). Less frequent clinical presentations include meningitis and endocarditis that are, however associated with significantly higher morbidity and mortality (Domingo et al., 1997;Sambola et al., 2002). The possible emergence of GBS as a respiratory pathogen associated with cystic fibrosis has also been proposed (Eickel et al., 2009;Sambola et al., 2002). Nosocomial disease is also raising concerns as more than 20% of patients with GBS invasive infection are thought to have acquired the bacteria from hospital settings (Jackson et al., 1995). The diversity of clinical presentations and poor outcome of invasive disease in adults are in support for the complexity of the pathogenesis of GBS infections. GBS invasive infections are more frequent in the elderly, probably reflecting the impact of risk factors that increase with age such as co-morbidities, altered integrity of anatomical barriers and immune senescence (Edwards and Baker, 2005).

## 1.3 Molecular pathogenesis of GBS and major virulence factors

Group B Streptococcus infection in human is a complex and multifactorial process which involves several virulence determinants that contribute to neonatal disease (Figure 1.3). The GBS pathogenic process can be described in four main steps: I) Colonization of mucosal surfaces II) Translocation through host cellular barriers III) Evasion of immunological clearance; IV) Activation of inflammatory response.

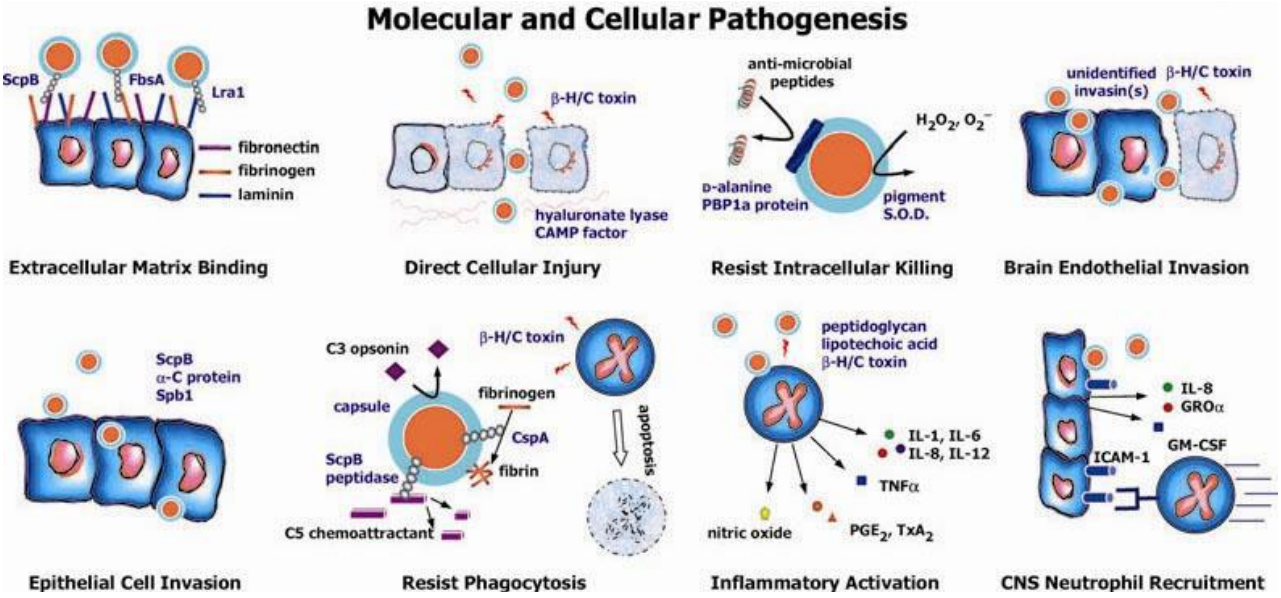



**Figure 1.3: Schematic representation of the molecular and cellular pathogenesis of GBS** (Doran and Nizet, 2004).

Although GBS usually resides as a commensal microorganism in genital and gastrointestinal tracts, it does have the ability to access several other niches such as the intrauterine compartment and multiple organs. This indicates that GBS has a survival advantage by being efficiently able to adapt to different host environments during the course of infection (Rajagopal, 2009). The development of GBS disease reflects successful bacterial colonization and the capacity to penetrate host physical barriers and requires appropriate expression and regulation of surface-

associated and secreted virulence factors that mediate host-cell interactions, including adherence to host epithelial surfaces, invasion across epithelial and endothelial barriers, and interference with innate immune clearance mechanisms (Maisey et al., 2008). Table 1 summarizes some key virulence factors of GBS, their mode of action and mechanism of regulation, detailing their proposed pathogenic mechanisms, critical for its ability to cause disease.

**Table 1.1: Regulation of virulence factor expression** (Rajagopal, 2009).

| Virulence factor | Mode of action |
|---|---|
| β-hemolysin/cytolysin ( β-H/C, CylE) | Promotes invasion of host cells and triggers host-cell lysis<br>Impairs cardiac and liver function<br>Induces inflammatory responses and apoptosis |
| CAMP factor (Cfb) | Forms pores in host-cell membrane; Binds to GPI anchored proteins |
| Sialic acid capsular polysaccharide (CPS) | Prevents recognition of GBS through molecular mimicry of host-cell surface glycoconjugates<br>Masks pro- inflammatory cell wall components |
| Superoxide dismutase (SodA) | Detoxifies singlet oxygen and superoxide |
| Pigment (rhamno-polyene) | Detoxifies singlet oxygen and superoxide |
| C5a peptidase (ScpB) | Prevents neutrophil recruitment due to cleavage of complement C5a<br>Promotes adherence by binding to ECM fibronectin and epithelial cells |
| Serine protease (CspA) | Cleaves fibrinogen and chemokines<br>Impairs neutrophil recruitment and phagocytic killing of GBS |
| Alanylation of lipotechoic acid | Decreases net negative charge on cell surface, repels AMPs |
| Penicillin-binding protein 1a (PBP1a) | Promotes resistance to AMPs through an unknown mechanism |
| Pili | Promotes resistance to AMPs through an unknown mechanism. Also promotes adherence of GBS to host cells |

**Table 1.1: Regulation of virulence factor expression (continued)** (Rajagopal, 2009).

| Virulence factor | Mode of action |
|---|---|
| Fibrinogen-binding protein A (FbsA) | Promotes adherence of GBS to host cells by biding to ECM fibrinogen |
| Fibrinogen-binding protein B (FbsB) | Promotes entry of GBS into host cells |
| Laminin-binding protein (Lmb) | Promotes adherence of GBS to host cells by binding to ECM laminin |
| Serine-rich repeat proteins (Srr) | Srr-1 promotes adherence of GBS to human keratin and epithelial (HEp2) cells<br>Srr-2 enhances virulence of GBS |
| Immunogenic bacterial adhesion (BibA) | Promotes adherence of GBS to host cells and binds complement regulatory protein C4bp |
| αC protein | Facilitates GBS adherence to epithelial cells |
| Invasion-associated gene (IagA) | Membrane anchoring of lipotechoic acid; important for blood-brain barrier invasion |

### 1.3.1 Capsule

The majority of GBS isolates recovered from human infections is encapsulated. Capsule is a major virulence determinant of GBS, being responsible for resistance to opsonophagocitic killing and phagocytosis, as well as for the inhibition of complement system clearance (Doran and Nizet, 2004).

GBS capsular polysaccharides (CPS) are predominantly composed of repeating units containing four elements: glucose, galactose, N-acetylglucosamine and sialic acid, the terminal sugar on the side chain of all serotypes. Serotypes VI and VIII are an exception to this composition by lacking the N-acetylglucosamine and serotype VIII has an additional ramnose residue (Madoff, L. C., 2006).

The biochemical and immunological properties of the GBS polysaccharide have been extensively studied. In 1987, the role of the GBS capsule in virulence was evaluated in a rat model of neonatal infection, by showing that a non-capsulated mutant of GBS presented significantly reduced virulence as compared to the encapsulated strain (Rubens et al., 1987). The importance of the capsular sialic acid for bacterial evasion of host mechanisms was also demonstrated when an encapsulated strain lost its virulence after removal of the sialic acid in a neonatal rat model of lethal GBS infection (Wessels et al., 1989). According to the chemical composition, structure, and serological properties, the GBS capsular polysaccharides are classified into ten distinct serotypes: Ia, Ib, II, III, IV, V, VI, VII, VIII and IX (Feil and Enright, 2004;Slotved et al., 2007). Moreover, a significant number of strains lack detectable capsule polysaccharide, being considered non-typeable (NT). Recently a capsular switch among very homogenous clones, as the hypervirulent CC-17, was reported. It resulted from the replacement of a type III by a type IV *cps* locus through exchange of a chromosomal segment (Bellais et al., 2012;Martins et al., 2010).

## 1.4 Characterization of GBS isolates

Bacterial epidemiologists use typing methods to study the dissemination and population dynamics of human bacterial pathogens in clinical and environmental settings, including their transmission patterns and the identification of risk-factors for the control of infectious diseases in human populations (van et al., 2007).

### 1.4.1 Phenotyping methods and Serotyping

Bacteriologists have long used phenotypic typing methods to group microorganisms according to their similarity in observable traits (phenotypes), which in turn result from the

expression of their genotypes (van et al., 2007). Conventional phenotypic methods include serotyping (based on differences in surface epitopes), phage-typing (based on resistance to infection by a standard set of bacteriophages), biotyping (according to the different metabolic capabilities of the cell), bacteriocin typing (the presence or susceptibility to a specific group of bacteriocins), and antibiotic resistance typing (susceptibility to a panel of antimicrobials). Although these methods represent a powerful tool to readily identify outbreak isolates in the short term, are in general inadequate for evolutionary studies and increasingly recognized not to afford sufficient resolution (Feil and Enright, 2004).

The serological classification of GBS is based upon the identification of capsular polysaccharides (CPS) and protein antigens (Flores and Ferrieri, 1989;Johnson and Ferrieri, 1984;Lancefield and Freimer, 1966). Many GBS capsular polysaccharide typing methods have been described (Arakere et al., 1999;Cropp et al., 1974;Holm and Hakansson, 1988;Kiely et al., 2011;Uh et al., 1997), with the most common methods based on serological tests, i.e., immunodiffusion and commercial latex agglutination methods.

### 1.4.2 Genotyping methods

Genotypic typing methods assess variation in the genomes of bacterial isolates with respect to composition (presence or absence of plasmids or mobile genetic elements), overall structure (restriction endonuclease profiles, number and position of repetitive elements), or precise nucleotide sequence (of genes or intergenic regions) (van et al., 2001). Restriction Fragment Length Polymorphism (RFLP) is based on DNA digestion with one or more endonucleases. The resulting restriction pattern of variable length fragments is obtained by separation in conventional electrophoresis and reflects the frequency and distribution of

endonuclease recognition sites (Maslow et al., 1993). Pulsed-field gel electrophoresis (PFGE) involves the exposure of chromosomal DNA to endonucleases that recognize only a few sites in the bacterial genome, generating macrorestriction fragments. However, these methods are more time-consuming and laborious. More recently, a multilocus sequence-typing (MLST) method, based on the sequence analysis of 500-bp fragments of seven housekeeping genes, has been extensively applied to investigate the clonal population structures and genetic lineages of GBS strains (Jones et al., 2003b).

### 1.4.3 PCR-based gene profiling and Multilocus sequence typing (MLST)

Polymerase chain reaction (PCR) is a nearly universal typing method, with several applications in bacterial typing systems, and exhibits an easily adjustable level of discrimination. Its major advantages include high reproducibility, technical simplicity, wide availability of equipment and reagents, and rapid turnover time (van et al., 2007). Several PCR-based typing systems have been used to genotype GBS isolates and include, among other, molecular serotyping (Kong et al., 2002;Martins et al., 2007), sub-typing within particular serotypes (Manning et al., 2005), surface protein gene profiling (Creti et al., 2004), detection of mobile genetic elements (Kong et al., 2003), and of antimicrobial resistance genes (Sutcliffe et al., 1996). MLST is a sequence-based typing method that involves sequencing of internal fragments of seven housekeeping genes (Maiden et al., 2013). The sequences are then compared with known alleles deposited at the MLST database (http://pubmlst.org/sagalactiae), and an allele number is assigned to each sequence, generating an allelic profile. Each isolate is therefore characterized by an allelic profile, a seven-integer number that can also be designated by a sequence type (ST) (Jones et al., 2003b). MLST offers a valuable tool for the characterization of bacterial strains. The

major advantage of MLST over PFGE is the precise, unambiguous and portable nature of the data obtained, so that the isolates typed in one laboratory can be rapidly compared with all previously typed strains (Feil and Enright, 2004). In recent years, MLST became increasingly used for the characterization of bacterial populations because of its ability to infer levels of relatedness between strains and the reconstruction of evolutionary events (Feil et al., 2004). These questions have been addressed based on an algorithm, eBURST, that divides an MLST data set into groups of related isolates by implementing a simple model of clonal expansion and diversification (Feil et al., 2004). This model predicts that the emergence of clonal complexes (CCs) is due to an increase in the population of the frequency of the founding genotype, as a consequence of either a fitness advantage or of random genetic drift. This genotype increases in numbers and by gradual diversification (point mutation or recombination) gives rise to a clonal complex. In terms of MLST, the descendants of the founder allelic profile will initially remain unchanged, but over time variants in one of the seven alleles will arise. These genotypes, which have allelic profiles that differ from that of the founder at only one of the seven MLST loci, are called single-locus variants (SLVs). (Feil et al., 2004).

### 1.4.4 Whole-genome sequences comparisons

The development of efficient and less expensive sequencing methods has produced a significant number of complete genome sequences of pathogenic microorganisms in recent years. The comparison of whole-genome sequences offers the possibility to assess genetic differences within a bacterial species, providing insights on how genetic variability drives the evolution of virulence mechanisms. The first complete GBS genome sequences were released in 2002 (Glaser et al., 2002;Tettelin et al., 2002). The GBS genome is nearly 2.2 Mbp long and contain over 2100

predicted coding regions. Both studies revealed substantial similarity with the genomes of the related human pathogens *Streptococcus pyogenes* and *S. pneumoniae*, representing a conserved backbone between streptococcal species. On the other hand, GBS differed from other streptococci in genome regions containing known and putative virulence genes, mostly encoding surface proteins and genes related to mobile elements, suggesting that these regions could be considered as pathogenicity islands (Glaser et al., 2002). Comparative analysis of multiple genomes reveal the concept of a "pangenome", consisting of a core genome shared by all isolates, accounting for approximately 80% of any single genome, and involved in housekeeping and regulatory functions, plus a dispensable genome consisting mostly of strain-specific genes. Again, the abundance of genes associated with mobile and extra-chromosomal elements found in the variable portion of the genome, supported the hypothesis that the acquisition of the majority of strain specific traits depends on lateral gene transfer (Tettelin et al., 2005). Furthermore, genetic heterogeneity among GBS strains, even of the same serotype, revealed that evolution within genes encoding surface and secreted proteins and those involved in the biosynthesis of the capsule is mainly due to recombination events leading to gene acquisition, duplication, and reassortment with the consequent replacement of several genes or to the allelic exchange within a particular gene. These processes allow GBS to express various combinations of virulence factors, which are likely to serve as means of adapting to host immunity (Brochet et al., 2006;Tettelin et al., 2005).

## 1.5. Molecular epidemiology of GBS

Molecular epidemiology studies has been performed to discriminate genetic lineages in order to probe for associations between specific GBS genotypes and disease.

### 1.5.1. Serotype distribution and MLST-based genetic lineages

Capsular serotyping has been the classic method used in descriptive epidemiology of *S. agalactiae*. Historically, the GBS isolates have been classified into ten different serotypes according to their capsule polysaccharides (Lindahl et al., 2005;Slotved et al., 2007). Five serotypes (Ia, Ib, II, III and V) are responsible for most human infections. There are demographic, geographic, and temporal variations with respect to the predominant serotypes present in the human population (Blumberg et al., 1996;Hickman et al., 1999;Kieran et al., 1998). Multiple surveillance studies have indicated that serotype Ia, Ib, III and V are prevalent in the vagina or perianal region of pregnant women (Barcaite et al., 2008b;Harrison et al., 1998;Phares et al., 2008;Savoia et al., 2008), whereas serotypes Ia and III are predominant isolates in neonatal invasive GBS disease, with type III, generally associated with late-onset neonatal disease (Musser et al., 1989), accounting for 30-76% of cases (Ho et al., 2007;von et al., 2008).

Among serotype III isolates, two main genetic lineages have been identified based on MLST: the ST-19 clone, frequently found among colonizing isolates (Jones et al., 2003b;Sadowy et al., 2010) and the ST-17clone, recognized as a hypervirulent clone and strongly associated with neonatal invasive infections, especially in the late-onset GBS disease (Bisharat et al., 2005;Jones et al., 2003b;Jones et al., 2006;Lin et al., 2006;Luan et al., 2005). The sequence type ST-17 more frequently cause meningitis than strains of other STs (Manning et al., 2009) Although the distinction of lineages within a particular serotype has proved useful, a complete correlation between capsular type and the genetic lineages as defined by PFGE and MLST was never found (Brochet et al., 2006;Luan et al., 2005;Manning et al., 2008). The serotype-independent clustering of strains implies that the diversification of the GBS populations is ongoing. These

observations support the hypothesis that closely and divergently related clones share the genes coding for a particular capsular type, suggesting that capsular switching probably occurs in GBS (Davies et al., 2004;Jones et al., 2003b).

## 1.6. Secreted or Surface proteins in ST-17 GBS strains

As already described in the Paragraph 1.3, surface and secreted proteins of GBS are likely to play important roles during different stages of infection, making them promising targets for vaccines development. Several virulence factors, specific of the CC-17 lineage, were already identified. These include: (I) FbsB, a fibrinogen-binding protein, (II) HvgA, encoding a cell-wall–anchored protein, (III) pili components, (IV) genetic variations in the serine-rich repeat region gene (srr), and V) the surface protein gene (spb-1).

### 1.6.1 The fibrinogen-binding protein FbsB

FbsA and FbsB are proteins with no structural homology which both bind to human fibrinogen, mediate the bacterial adhesion to or invasion of epithelial and endothelial cells, and contribute to the bacterial escape from the immune system (Gutekunst et al., 2004;Jacobsson, 2003;Samen et al., 2006). FbsB has a typical signal peptide but, differently by FbsA, lacks the LPXTG motif or other wall-anchoring signatures, suggesting that it is not a surface protein but is secreted into the extracellular medium. The *fbs* genes and the *fbs* regulator genes were not specific of either CC-17 or other CCs strains, but specific gene combinations were related to particular CCs, indicating that fibrinogen binding is a multigenic process that results from various gene combinations. Only CC-17 strains contained the *fbsA*, *fbsB*, and *rgf* genes combination. The *rogB* gene was rarely found in CC-17 strains but present in all strains of other CCs. Accordingly,

the *rogB* gene is missing in the sequenced genome of CC-17 strain COH1 (Tettelin et al., 2005), and the absence of this gene was also reported in a collection of 20 CC-17 strains (Brochet et al., 2006). Thus, each CC was characterized by a particular profile of *fbs* genes and *fbs* gene regulators that may account for differences in their fibrinogen-binding abilities. The presence of the sole *fbsA* gene was not sufficient to result in strong binding ability to fibrinogen (Rosenau et al., 2007) and mutants deleted for the *fbsA* and *fbsB* genes demonstrated that FbsB protein was the major fibrinogen binding protein of CC-17 strains (Al et al., 2011a). Indeed, the population of strains with the significantly highest ability to bind to fibrinogen had both the *fbsB* and *fbsA* genes and belonged to CC-17 phylogenetic lineage (Rosenau et al., 2007).

### 1.6.2. HvgA, a cell-wall–anchored protein

BibA is an immunogenic surface-associated antigen expressed by GBS that is involved in virulence. Four allelic variants of this protein have been identified: variant I, found in strains 2603 V/R (V) and 18RS21 (II); variant II, in strains NEM316 (III) and 515 (Ia); variant III, in strains CJB111 (V), H36B (Ib), and A909 (Ia); and variant IV, in the COH1 (III) strain. The variant IV was recently identified as a novel ST-17–specific surface-anchored protein, which is highly prevalent in cases of LOD, and was called hypervirulent GBS adhesin (HvgA, also known as gbs2018) because mediates GBS neonatal intestinal colonization and crossing of the intestinal and blood–brain barriers, leading to meningitis, which are key features of LOD (Tazi et al., 2010). Comparing the structure of the *bibA*/*hvgA* locus in GBS strains NEM316 (WT ST-23) and BM110 (WT ST-17), the nucleotide sequences of the two loci revealed that only the 5′ and 3′ ends of the two genes were highly conserved, displaying >90% sequence identity, whereas their

internal parts displayed low-level (50–60%) or no significant (<20%) sequence identity (Figure 1.4).

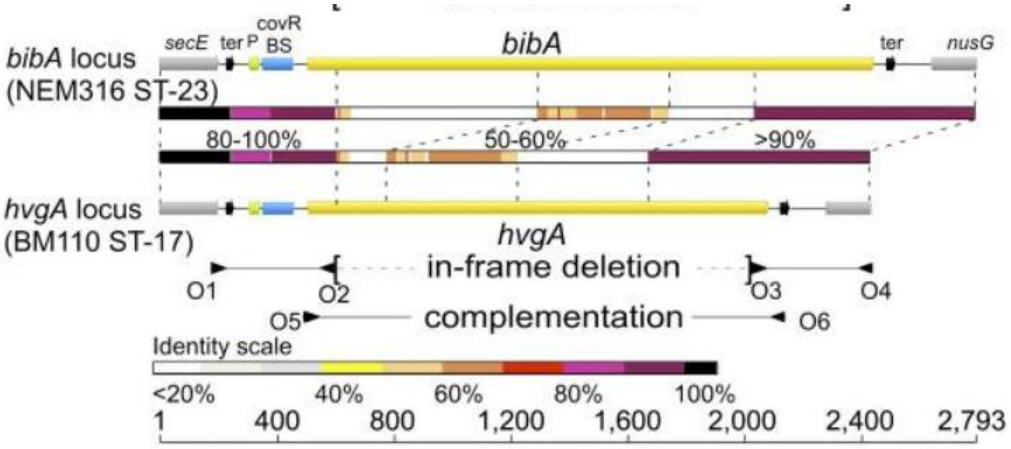



**Figure 1.4: Structure of the *bibA*/*hvgA* locus in GBS strains NEM316 and BM110** (Tazi A, 2010).

### 1.6.3 Pili components

The presence of pilus-like structures in GBS was first described in 2005 (Lauer et al., 2005). The genes encoding pili in GBS are located within two distinct loci in different regions of the genome, designated pilus-islands 1 and 2 (PI-1 and PI-2), the later presenting two distinct variants, PI-2a and PI-2b (Rosini et al., 2006a). Pili are composed of three subunits: a backbone protein (BP), the *bona fide* pilin, and two ancillary proteins, a pilus associated adhesin and a component that anchors the pili to the cell wall (Figure 1.5). Both the polymerization and attachment of the pili to the peptidoglycan cell wall occur by sortase-dependent mechanisms (Dramsi et al., 2006). Even though PI-1 is not present in all GBS strains, PI-2 is ubiquitously expressed. Serotype III ST-17 clones are characterized by the presence of PI-2b, serotype V by the presence of PI-2a. These structures have been recognized to play a role in biofilm formation, adherence, invasion and translocation of epithelial cells (Konto-Ghiorghi et al., 2009;Rosini et al., 2006a).

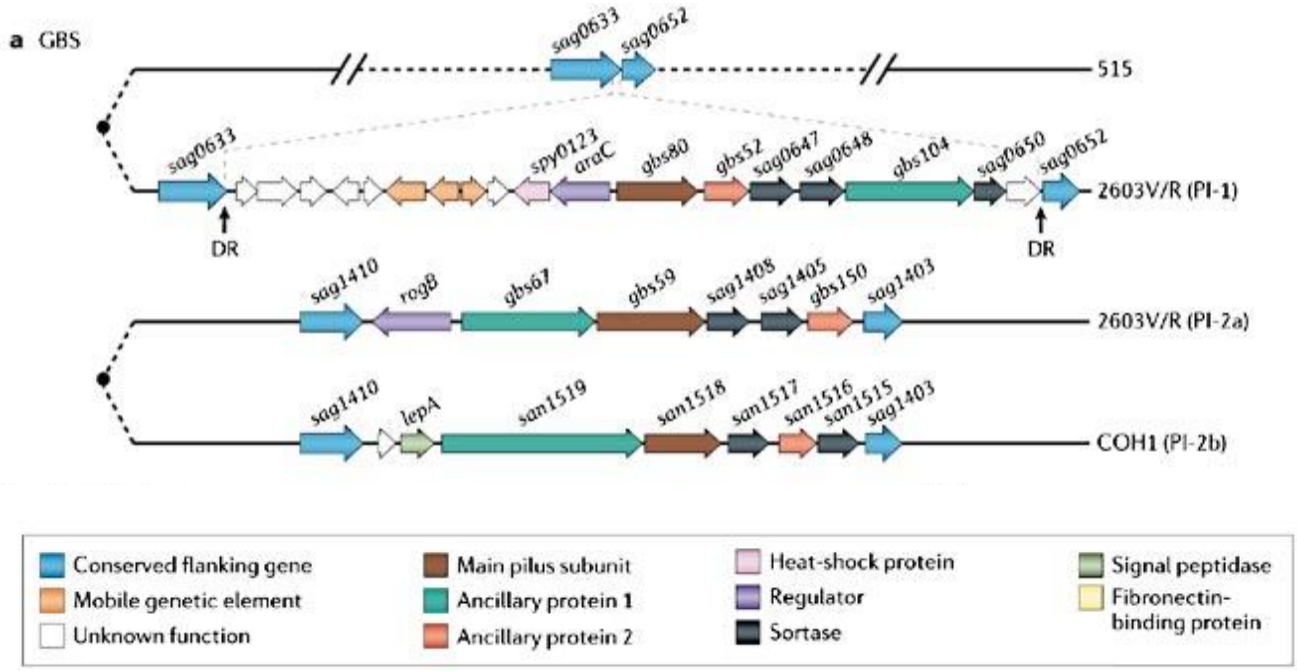



**Figure 1.5: Schematic representation of loci that encode group B *Streptococcus* pili**

In the Figure is represented Pilus island 1 (PI-1) in GBS strain 2603V/R and the same region in GBS strain 515, which is pilus negative. The operon is flanked by conserved genes *sag0633* and *sag0652* and direct repeats (DR). In the lower panel, two alleles of PI-2 flanked by conserved genes *sag1410* and *sag1403* are depicted: PI-2a from GBS strain 2603V/R, and PI-2b from *S. agalactiae* strain COH1 (Telford et al., 2006)

### 1.6.4. Srr and Spb1

Srr family proteins were first characterized in oral streptococci as serine-rich (>35%) high-molecular-mass glycosylated proteins that are transported across the membrane by a dedicated SecA2/Y2 secretion system (Chen et al., 2004;Takahashi et al., 2004). In GBS, 2 types of Srr proteins, known as Srr-1 and Srr-2, have been identified (Seifert et al., 2006). Srr-1 is surface exposed and highly conserved (>85% nucleotide identity and amino acid identity) among published genomes of GBS strains belonging to different serotypes (Samen et al., 2007). In

contrast, the expression of Srr-2 that show <20% sequence identity with Srr-1 seems to be restricted to serotype III and ST-17 strain (Seifert et al., 2006).

The *spb1* gene was identified by subtractive hybridization from a serotype III strain of the putative hypervirulent clone ET1/III-3 (Adderson et al., 2003), and the gene sequence of *spb1* indicates characteristics of a surface protein. The Spb1 protein is mainly involved in bacterial internalization into host cells as a Spb1-negative mutant was significantly reduced in the ability to invade such cells, but showed little difference in adhering to epithelial cells (Adderson et al., 2003).

## 1.7 Biofilm formation in GBS

A bacterial biofilm is composed of groups of bacteria surrounded by an extracellular polysaccharide matrix (EPS) (Hall-Stoodley and Stoodley, 2009;Kaur et al., 2009). The extracellular matrix is composed of water, polysaccharides, proteins, lipids, extracellular DNA, membrane particles and ions (Karatan and Watnick, 2009). Generally, biofilm formation is characterized by five stages (Figure 1.6): 1. adhesion of bacterial cells to the surface; 2. production of EPS resulting in more firmly adhered cells; 3. early development of biofilm architecture; 4. maturation of biofilm architecture; Stage 5, dispersion of single cells from the biofilm.

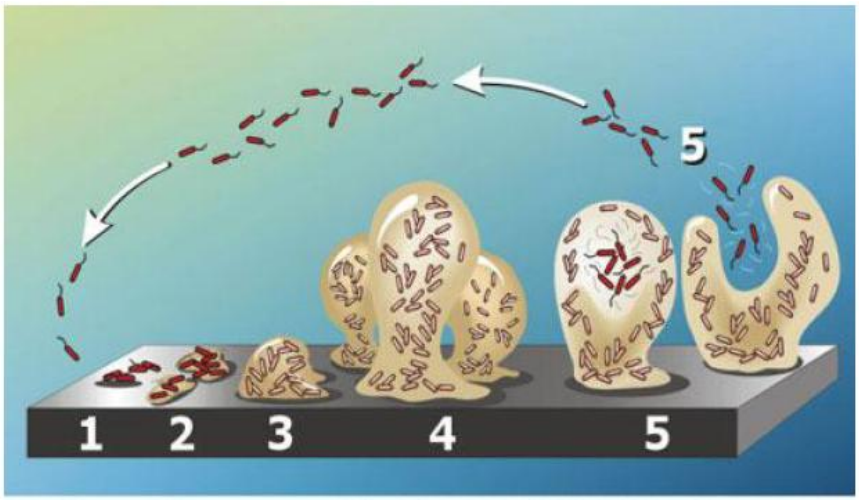



**Figure 1.6: Development of a biofilm as a five-stage process under continuous-flow conditions (e.g. flow cell system) from two dimensions to three dimensions.**

Four plausible driving forces are suggested to act behind bacterial biofilm formation (Jefferson, 2004a): (1) protection from harmful conditions in the host (defense), (2) sequestration to a nutrient rich area (colonization), (3) utilization of cooperative benefits (community), (4) bacteria normally grow as biofilms in nature (Figure 1.7). The three dimensional complex of the biofilm is a coordinated community and allows bacteria to adapt to and survive in host environments (Hall-Stoodley and Stoodley, 2009). Bacteria in biofilms detect environmental changes and respond to it in order to survive in diverse and stressful conditions (Hall-Stoodley and Stoodley, 2009). Organisms within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics better than planktonic organisms (Jefferson, 2004b). Biofilms generate resistance to antibiotics by decreasing the antibiotics penetration rate and mediating bacterial gene expression. Transmission electron micrographs reveal biofilms protect bacteria against phagocytes (Hall-Stoodley and Stoodley, 2009).

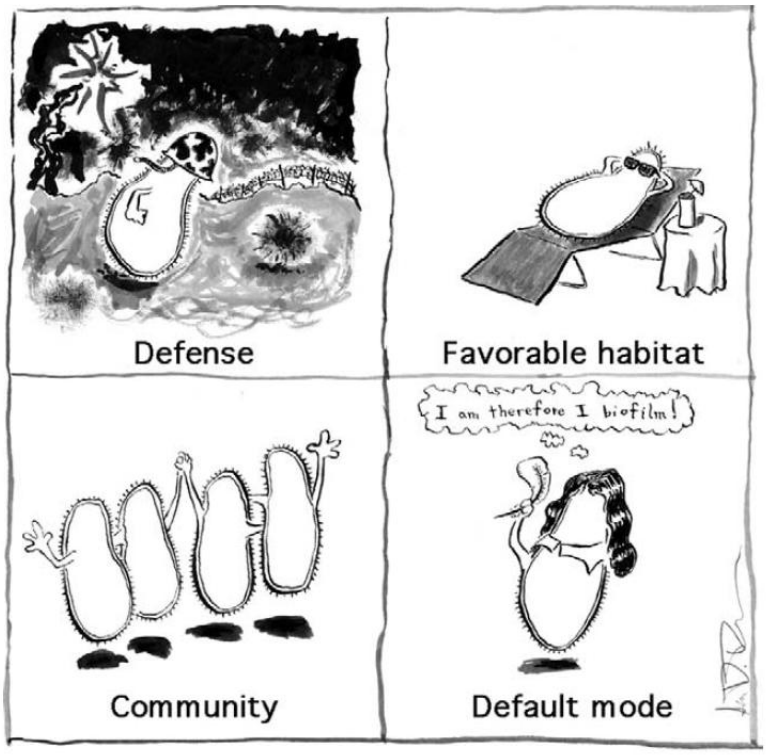



**Figure 1.7: Artistic interpretation of the four driving forces behind bacterial biofilm formation that are discussed in a review by K.K. Jefferson (Jefferson, 2004a).**

Bacterial biofilm formation is regulated by different environment signals including mechanical, nutritional, metabolic and host-derived signals, secondary messenger and protein transcriptional regulators (Karatan and Watnick, 2009). The majority of the species belonging to the *Streptococcus* family have been shown to form biofilm, while just a limited number of studies have demonstrated GBS biofilm formation in vitro (Borges et al., 2012;Kaur et al., 2009;Konto-Ghiorghi et al., 2009;Rinaudo et al., 2010a).

Glucose concentration in culture media were shown to modulate biofilm formation in GBS, although conflicting data have been reported regarding the biofilm forming capacity of isolates belonging to different serotypes, and the correlation between biofilm formation and pH (Borges et al., 2012;Kaur et al., 2009;Konto-Ghiorghi et al., 2009;Rinaudo et al., 2010a). We

hypothesized that these contradictory results could be due to absence of *in vitro* protocols that allowed clearly discriminating between strong and weak biofilm formers and unambiguously establishing the role of bacterial culture conditions.

Recent studies have demonstrated GBS biofilm formation *in vitro* (Rinaudo et al., 2010a) although the data regarding the effect of pH and media composition are controversial. In a recent study, *Yueh-Ren et al.* (Ho et al., 2012) found that the low pH condition induced biofilm fomaion in a nutrient-limited medium (M9YE) but not in THB. *Borges S. et al.* (Borges et al., 2012), *Kaur et al.* (Kaur et al., 2009) and *Yang Q. et al.* (Yang et al., 2012) found that GBS produced a greater amount of biofilm at pH 6.5 than at pH 4.2 and *Konto Ghiordi et al.* (Konto-Ghiorghi et al., 2009) reported that only LB and RPMI 1640 supplemented with 1% of glucose produced uniform biofilm and not THB. *Manetti et al.* (Manetti et al., 2010) observed that, in *S. pyogenes*, the presence of glucose resulted in auto acidification of the media and consequently biofilm formation in GBS. *Rinaudo et al.* (Rinaudo et al., 2010a) demonstrated that the presence of 1% of glucose in THB induces biofilm formation in GBS.

Recent studies also suggest that biofilm formation by some GBS strains could have an important role in host-colonization. The capability of GBS to attach to epithelial cells also increased in acidic conditions (Tamura et al., 1994). GBS adherence to A549 pulmonary epithelial cells and vaginal epithelial cells at pH 4.0 was 10 to 20 fold higher than at neutral pH. Transcription experiments showed there were 317 genes up-regulated and 61 genes down-regulated when GBS was incubated in pH 5 media compared with pH 7 medium (Santi et al., 2009). The majority of genes involved in response to environment pH change include genes regulation transport, metabolism, stress response and virulence (Santi et al., 2009).

## 1.8 Proteomics approach of vaccine candidate identification

A new approach that allows fast and consistent identification of proteins that are exposed on the bacterial surface has been recently published (Rodriguez-Ortega et al., 2006). The technique, consisting of the surface digestion of live bacteria with different proteases and analysis by mass spectrometry, identifies the so-called bacterial "surfome". This technique was already applied to analyses the proteins expressed on the surface of a GBS non-biofim forming strain, grown in standard laboratory condition (Doro et al., 2009). Here, for the first time, surfome analysis is applied to compare the expression profile of bacteria growth in both planktonic and biofilm like growth conditions.

## 1.9 Thesis overview

In the present study, the biofilm formation of more than 350 GBS clinical isolates from pregnant women and neonatal infections, using a new high-throughput *in vitro* protocol was investigated. The isolates were collected during the DEVANI project (Design of a Vaccine against Neonatal Infections) supported by the European Commission Seventh Framework, launched on 1 January 2008 (http://www.devaniproject.org).

Specifically, this study was focused on:

1. Develop a new biofilm formation protocol to guarantee more stringent conditions, reducing unambiguous data interpretation;
2. Screen 366 GBS clinical isolates from pregnant woman and from neonatal infection, belonging to different serotypes, in relation to media composition and pH;
3. Observe the correlation between biofilm formation and pH decrease in rich and limited-nutrient media;

4. Investigate the relationship between capsule amount and biofilm formation at different pH;
5. Identify the role of proteins, capsule and DNA in biofilm formation and in its structural stability.
6. Apply the surfome analysis to identify the proteins differentially expressed on the surface of GBS in growth conditions that mimic planktonic and sessile GBS life-style.

# 2. Material and methods

## 2.1 Bacterial strains and growth conditions

A total of 366 *S. agalactiae* isolates of 8 different serotypes (Ia n = 58; Ib n = 18; II n = 28; III n = 156; IV n = 10; V n = 57; VIII n = 3, IX n = 13) and non-typeable strains (n = 23) were included in the study. Among these, 357 were vaginorectal isolates obtained from pregnant women (*n* = 272) and clinical isolates from neonatal infections (*n* = 85) in Belgium, Bulgaria, the Czech Republic, Denmark, Germany, Great Britain, Italy, and Spain. These isolates were collected during the DEVANI project (Design of a Vaccine Against Neonatal Infections) supported by the European Commission Seventh Framework, launched on 1 January 2008 (http://www.devaniproject.org).

The overall aim of the DEVANI project was to assess European GBS epidemiology in order to facilitate the design of a new vaccine capable of conferring broad coverage to immunize neonates against GBS infections through a durable maternal immune response. Strains CJB111 (type V), 515 (type Ia), COH1 (serotype III) and H36B (serotype Ib), 18RS21 (serotype II), A909 (serotype Ia), D136C (serotype III) were kindly provided by Dr. Dennis Kasper (Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA, USA). 2603 V/R (serotype V) (Tettelin et al., 2002) strain was obtained from the Istituto Superiore di Sanità. The COH1 un-encapsulated mutant carries a deletion of the *cpsE* gene in the capsule locus (Cieslewicz et al., 2001) and was kindly provided by M. Cieslewicz (Channing Laboratory, Harvard Medical School Boston, MA, USA).

## 2.2 Serotype and Sequence Type identification

### 2.2.1 Total genomic DNA isolation

For genomic DNA isolation the strains were grown overnight at 37 ℃ in static conditions. 10 mL of each culture was centrifuged at 1400 rpm, washed once in PBS and suspended in 300 µL of TET (20 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100) supplemented with 5 µL Mutanolysin (10 U/µL) and 55 µL of Lysozime (12 mg/mL). The cell pellets were incubated at 37℃ for 1 hour and then processed using the Wizard DNA purification Kit (Promega). Final DNA concentration was assessed by optical density determination at 260 nm.

### 2.2.2 Serotype and ST-17 identification

GBS strains were typed by latex agglutination method (Strep-B-Latex kit;Copenhagen, Denmark), as described by *Afshar et al.*(Afshar et al., 2011). ST-17 identification was performed for all the 156 serotype III tested strains. PCR amplification and sequencing of the internal fragments of 7 housekeeping genes, namely, *adhP*, *atr*, *glcK*, *glnA*, *pheS*, *sdhA*, and *tkt* were performed as described previously (Jones et al., 2003b). Assignment to ST-17 was performed at the GBS MLST Web site (*http://pubmlst.org/sagalactiae/*). The strains showing, at least, an allele sequence non-correspondent to the ST-17 profile, were classified as non-ST-17.

## 2.3 Growth experiments

Four clinical isolates (three biofilm-forming strains and one non-biofilm-forming strain) grown overnight at 37℃ in Todd-Hewitt broth (THB) at pH 7.8 were diluted to an optical density at 600 nm (OD600) of 0.05 in optical tubes containing 10 ml of pH 7.8 THB, pH 7.8 THB

supplemented with 1% glucose, or pH 5.0 THB. The tubes were then incubated without shaking at 37°C and the OD600 was measured for 8 to 10 h. Each experiment was performed in triplicate.

## 2.4 *In vitro* biofilm formation

### 2.4.1 Standard biofilm formation protocol

The standard protocol was performed as already described (Rinaudo et al., 2010a). In brief, S*treptococcus agalactiae* strains were streaked on blood agar plates and grown at 37 °C for 18 hours. GBS strains, grown overnight in THB, were diluted 1:20 in Todd Hewitt Broth pH 7.8 (THB) or THB supplemented with 1% glucose and used to inoculate (100 µl/well) 96-weel polystirene microtiter plates (Constar; Corning Inc.; Corning ,NY).

Plates were incubated without shaking at 37°C for 18 h aerobically in 5% $CO_2$. The supernatant was removed and the wells were subjected to three cycles of washing with 200 µL of double-distilled H2O (ddH2O) to remove unattached bacteria. A Crystal Violet (CV) assay and a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay were then performed to estimate bacterial biomass and cell viability, respectively.

### 2.4.2 New biofilm formation protocol

A new protocol for *in vitro* biofilm formation was set-up. S*treptococcus agalactiae* strains were streaked on blood agar plates and grown at 37 °C for 18 hours. Bacterial suspension in Todd Hewitt Broth pH 7.8 (THB) was prepared at $OD_{600}$ 0.05 and used to inoculate (200 µL/well) a 96-well polystyrene microtiter plates (Constar; Corning Inc.; Corning ,NY). The preliminary protocol evaluation was performed with THB and THB supplemented with 1% glucose, as

already used in the standard biofilm formation protocol. The other media used to investigate the role of pH in biofilm formation were (i) RPMI GlutaMAX (Gibco-Life Technologies, Milan, Italy), (ii) RPMI GlutaMAX supplemented with 1% glucose, (iii) RPMI GlutaMAX and THB both acidified to pH 5.0, and (iv) THB supplemented with 1% glucose and buffered at pH 7.8 with the addition of HEPES (20 to 200 mM) or Tris-HCl (20 to 200 mM). The plate was sealed to limit oxygen exchange and shaken at 60 rpm at 37°C to reduce bacterial deposition. Following 8 h of adhesion at 37°C, the plates were washed to remove loosely adherent cells and the supernatant was replaced with 200 µL of fresh medium. After 15 h at 37°C, the medium was removed and the wells were subjected to three cycles of washing with 200 µL of phosphate-buffered saline (PBS) to remove unattached bacteria (Figure 2.1). CV and XTT assays were then performed to estimate bacterial biomass and cell viability, respectively.

## 2.5 Crystal Violet assay

The wells were stained for 10 min with a 0.5% (wt/vol) solution of Crystal Violet (CV) (Sigma-Aldrich, Inc., St. Louis, MO). After rinsing with ddH2O, bound dye was released from the stained cells by using 30% glacial acetic acid. Biofilm formation was quantified by measuring the OD540 of the solution with a microplate reader (Infinite M200; Tecan). Samples showing an OD540 higher than 1 were diluted 5 and 20 times in water, and the absorbance reading was repeated. The measured values were subtracted from the blank and then multiplied by the dilution factors. Each assay was performed in triplicate. Wells filled with growth medium were included as negative controls.

## 2.6 XTT viability assay.

XTT is a tetrazolium derivative XTT is a tetrazolium derivative cleaved to anorange-colored formazan product by mitochondrial dehydrogenase inviable cells (D'Urzo et al., 2014;Roehm et al., 1991). The XTT solution was prepared by dissolving 0.5 mg XTT (Sigma-Aldrich, Inc., St. Louis, MO) in 1 mL of PBS and then supplementing it with 2.5 µL of a 10 mM menadione stock solution (dissolved in acetone). 150 µL of XTT-menadione solution was added to each well. Plates were incubated in the dark for 3 h at 37°C and then centrifuged for 20 min at 4,000 rpm. 100 µL of the supernatant was transferred to the wells of a new 96-well flat-bottom plate, and the absorbance at 490 nm was measured with a microplate reader (Infinite M200; Tecan).

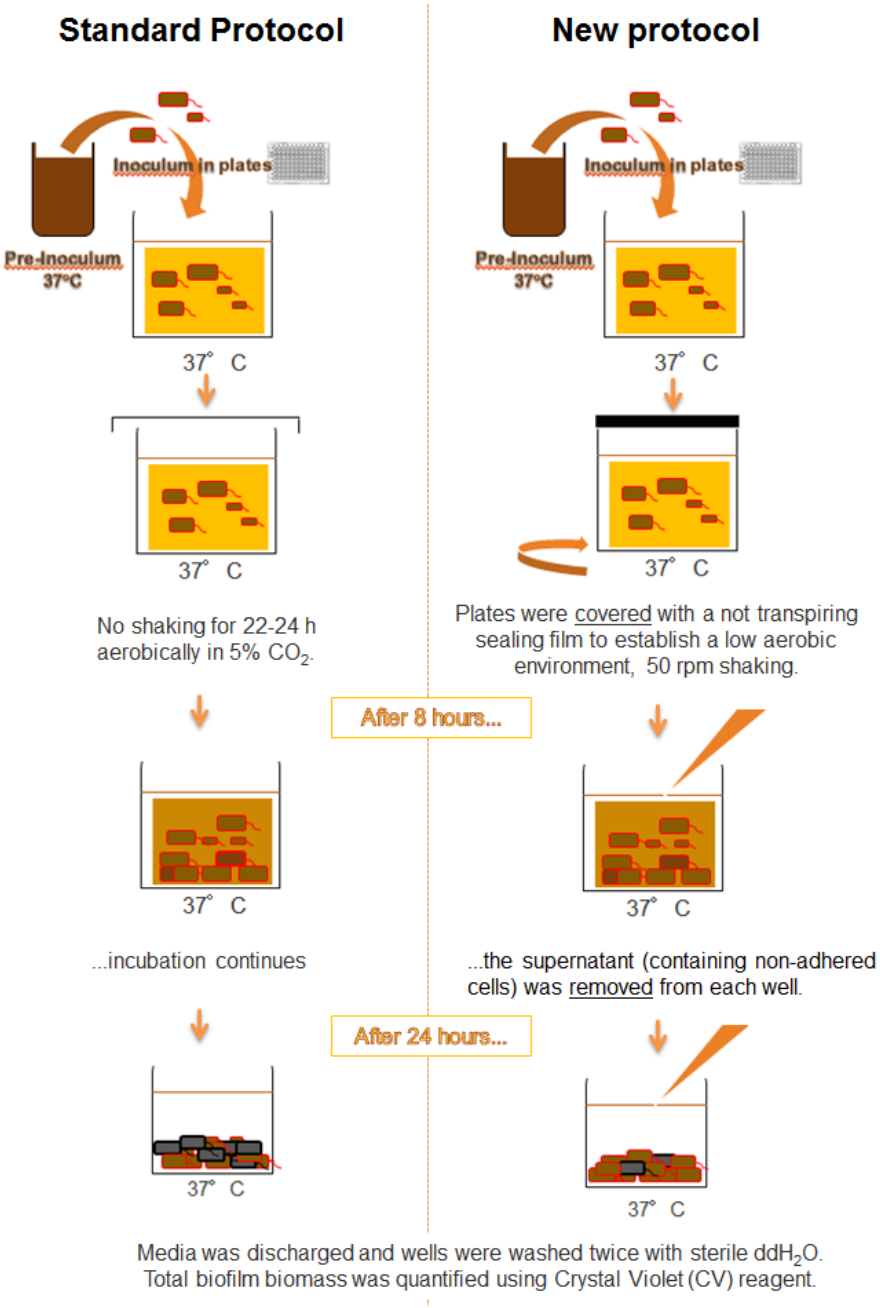



**Figure 2.1: Protocols of GBS biofilm formation.**

Comparison of the protocols used in this study. Standard protocol (on the left) and new protocol (on the right). The details regarding the two protocols are described in Material and Methods (Paragraph 2.4).

## 2.7 Confocal laser scanning microscopy

The GBS biofilms obtained by both the standard protocol (Rinaudo et al., 2010a) and the new protocol, used in this study, were visualized by Confocal Laser Scanning Microscopy (CLSM). *S. agalactiae* strains were inoculated (0.8 mL/well) in a Lab-Tek II eight-well 1.5 cover glass (VWR, Rochester, NY) containing THB or THB supplemented with 1% glucose and incubated as already described in the new biofilm formation protocol (Paragraph 2.4.2). Adherent bacteria were stained for 30 min with LIVE/DEAD BacLight fluorescent stain (Molecular Probes, Eugene, OR) and fixed with 2% formaldehyde for 30 min at room temperature.

Samples were analyzed with a Zeiss LSM710 confocal microscope by using a Plan-Apochromat 40x/1.3 objective. SYTO 9 fluorescence, corresponding to live bacteria, was acquired in the green channel (492 to 572 nm), and propidium iodide fluorescence, which does not penetrate viable bacterial cells, was acquired in the red channel (566 to 719 nm). Images were acquired by using Zen 2008 software and modified with Volocity (Improvision, Lexington, MA). ImageJ (http://rsbweb.nih.gov/ij/) and COMSTAT2 (http://www.comstat.dk) were used to evaluate the biomass and maximum and mean thicknesses of the three-dimensional biofilm images acquired by CLSM (D'Urzo et al., 2014;Heydorn et al., 2000;Roehm et al., 1991)

## 2.8 Enzymatic inhibition/eradication of biofilms

The Minimal Inhibition Concentration (MIC), Minimal Biofilm Inhibition Concentration (MBIC) and Minimal Biofilm Eradication Concentration (MBEC) were measured in 96-well polystyrene microtiter plates with 0.4-200 μg/mL proteinase K and a maximum of 200 μg/mL of DNase. For MBIC determination, the biofilm formation assays were performed as described previously using THB supplemented with 1% glucose and proteinase K (200 μg/mL) or DNase

(200 μg/mL). For MBEC determination a 24h-mature biofilm grown in the absence of proteinase K or Dnase was washed twice with PBS and further incubated in THB supplemented with 1% glucose containing proteinase K (200 μg/mL) or DNase (200 μg/mL), 3 h at 37°C. Biofilm was quantified by CV assay, as previously described.

## 2.9 Biofilm inhibition using sera from immunized rabbit

For the biofilm inhibition assay, rabbit sera for GBS80 (SAG0645) (backbone protein of pilus island-I), GBS67 (SAG1408) (ancillary protein of PI-2a), and GBS1523 (SAN 1518) (backbone protein of PI-2b) were tested for their ability to inhibit biofilm formation. Bacterial suspension in Todd Hewitt Broth pH 7.8 (THB) was prepared at $OD_{600}$ 0.05 and used to inoculate (200 μL/well) a 96-well polystyrene microtiter plates (Constar; Corning Inc.; Corning ,NY) with serial dilutions of sera. The plate was sealed to limit oxygen exchange and shaken at 60 rpm/min at 37°C to reduce bacteria deposition. Following 8 hours of adhesion at 37°C, media, including any unattached bacteria, were decanted from the wells, and any remaining planktonic cells were removed by rinsing with $ddH_2O$. Wells were air dried, and adherent bacteria were subjected to quantification by colorimetric assays. Sera from immunized New Zealand rabbits (Charles River Laboratories, Calco Italy) were kindly supply by Rinaudo CD's group and obtained as previously reported (Maione et al., 2005;Margarit et al., 2009;Rosini et al., 2006b).

## 2.10 Quantification of capsular polysaccharides

Capsular polysaccharides from COH1 strain and 4 biofilm forming strains (2 expressing type III and 2 expressing type V capsular polysaccharide) were extracted and quantified by the

resorcinol–hydrochloric acid assay as described earlier by Svennerholm (SVENNERHOLM, 1957).

Bacteria were inoculated in 15 mL of THB and THB pH 5.0 and grown to an $OD_{600}$ of 1.8 and 1.0, respectively. The cells were pelleted, washed twice with PBS, suspended in 0.8 M NaOH and incubated for 48 h at 37°C. Following neutralization with HCl, the insoluble material was removed by centrifugation; the supernatant was transferred to an Amicon Ultra-10 (Millipore, Bedford, MA), concentrated to 0.20 mL, and then perfused two times with 1 mL $dH_2O$.

A final volume of 1.5 mL of supernatant was analyzed to determine the amount of extracted polysaccharide. Briefly, 500 µL of resorcinol-HCl reagent (2% w/v aq. Resorcinol solution added to concentrated HCl and 0.1M $CuSO_4$) was added to 500 µL of extracted polysialic acids-sample which was then heated in a boiling oil bath for 20 min. The released sialic acid (N-acetylneuraminic acid [NeuNAC]) reacts with resorcinol in the presence of copper sulphate under reducing conditions to give a blue-purple color. After it cooled to room temperature, the absorbance at 564 nm was measured. NeuNAC concentrations were calculated from the standard curves, obtained using NANA standards (range: 5-25 µg/mL) and converted to total GBS saccharide (conversion factor= molecular weight of NeuNAC/ molecular weight of repeat unit GBS polysaccharide).

## 2.11 Surfome preparation of *Streptococcus agalactiae* grown in biofilm inducing and non-inducing conditions

Surfome preparation of *S. agalactiae* live cells was performed as previously described (Doro et al., 2009) with minor modifications. Briefly, *S. agalactiae* COH1 and a biofilm forming strain were inoculated in 50 mL of THB or THB pH 5.0 and incubated at 37°C until an $OD_{600}$ of 1.8 (in

THB) and 1.0 (in THB pH 5.0) was reached. Bacteria were harvested by centrifugation at 4000 rpm g for 10 min, 4°C and washed twice with phosphate buffered saline (PBS). The supernatants were used for the secretome preparation, as described below. Bacterial pellets were suspended in 700 µL of digestion buffer (33% sucrose and 50 mM Tris pH 7.0) and incubated at 37°C with 10 µg of trypsin (Promega, Madison, USA) for 30 min at 37 °C. Bacterial cells were then spin down at 4000 rpm for 10 min at 4°C, and the supernatants were filtered through 0.22-µm pore size filters (Millex, Millipore, Bedford, MA). Protease reactions were stopped with formic acid at 0.1% final concentration. Before analysis, PBS and sucrose were removed by an off-line desalting procedure using OASIS cartridges (Waters, Milford, MA) following the manufacturer's protocol. Desalted peptides were concentrated with a Centrivap Concentrator (Labconco, Kansas City, KS) and kept at −20 °C until further analysis.

Cell wall extracts of *S. agalactiae* COH1 and a biofilm forming strain, grown in THB, THB pH 5.0 and THB + 1% glucose, were compared both in protein amount both in protein profile using SDS-PAGE. GBS stains were incubated at 37°C until an $OD_{600}$ of 1.0 was reached. Bacteria were harvested by centrifugation at 4000 rpm g for 10 min, 4°C and washed twice with phosphate buffered saline (PBS). Bacterial pellets were incubated in an ice-cold protoplasting buffer containing 40% sucrose in 0.1 M $K_3PO_4$ (pH 6.2), protease inhibitors and 800U/mL of mutanolysin (Sigma-Aldrich, Inc., St. Louis, MO) for 30 min at 37°C, as previously described (Kling et al., 1999). After centrifugation the protein content of the supernatants (cell wall fractions) were quantified using the BCA assay (Pierce) and the protein profile visualized by SDS-page (Comassie staining).

## 2.12 Protein Identification by LC-MS/MS

Peptides were separated by nano-LC on a NanoAcquity UPLC system (Waters) connected to a SynaptG2 mass spectrometer equipped with a NanoLockSpray$^{TM}$ source (Waters). Samples were loaded onto a NanoAcquity 5µm Symmetry® $C_{18}$ trapping column (180µm X 20mm, Waters) using full loop injection for 2 min at a flow rate of 7.5 µL/min with mobile phase A (2% acetonitrile, 0.1% formic acid). Peptides were then separated on a NanoAcquity 1.7µm BEH130 $C_{18}$ analytical column (75µm X 250mm, Waters) using a 90 min gradient of 2–45% mobile phase B (98% acetonitrile, 0.1% formic acid) at a flow rate of 250 nL/min. The column temperature was set at 35℃. The reference, [Glu1]-fibrinopeptide was constantly infused B at 600 fmoL/µl,by the NanoAcquity auxiliary pump at a constant flow rate of 400 nL/min and acquired with an interval of 30 seconds through the reference sprayer of the NanoLockSprayTM source. The spectra of the eluted peptides were acquired in positive V-mode in a mass range of 50-2,000 m/z using a $MS^E$ program for MS/MS with 0.7-s scan times and a collision energy ramp of 20-40 eV for elevated energy scans in the transfer region of the mass spectrometer. All fragmentations were performed using argon as collision gas. Continuum $LCMS^E$ data were processed and searched against the database of *Streptococcus agalactiae* COH1 strain (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) using ProteinLynx Global Server version 2.5.2 (Waters). Methionine oxidation and glutamine and asparagine deamidation were specified as variable modifications, one trypsin missed-cleavage was allowed, and the default settings in ProteinLynx Global Server for the precursor ion and fragment ion mass tolerance were used (automatic setting). The observed mass error tolerance values were typically under 15 ppm. The search thresholds used were: minimum fragment ion matches per peptide, 3; minimum fragment ion

matches per protein, 7; minimum peptides per protein, 1; and false positive rate, 4. Only proteins with a score higher than 1000 were considered as identified.

## 2.13 Computational Analysis of Identified Proteins

A computational analysis of each identified protein sequence was performed with the PSORTb v.2.0 package (Gardy et al., 2005) to predict the subcellular localization. For the detection of lipoprotein signal sequences and cleavage sites, LipPred (Taylor et al., 2006) software was used.

## 2.14 Statistics

Statistical analysis of biofilm formation in the panel of 366 GBS strains was performed using GraphPad (GraphPad Software, La Jolla, CA). The significance of differences in the relative amount of biofilm produced by each of the *S. agalctiae* strains, under each test condition, was assessed by Univariate Analysis of Variance (ANOVA). Significance was assigned at $P \leq 0.05$.

# 3. Results

## 3.1 A new *in vitro* biofilm formation protocol minimizes false positive results

The *in vitro* protocols used to date to determine the biofilm formation abilities of different GBS isolates resulted in small differences between the strains tested and did not allow clear discrimination between strong and weak biofilm-forming GBS strains. In this study a new *in vitro* biofilm formation protocol was implemented to overcame this limitation. Two main differences from the previously described standard protocol (see Materials and Methods) were applied (Rinaudo et al., 2010a): i) replacement of culture medium with fresh medium after 8 h of incubation to remove nonadherent bacteria and (ii) incubation of the plates under shaking conditions to minimize nonspecific bacterial deposition on the bottom of the plate.

The two *in vitro* protocols (Figure 2.1) were compared by testing four GBS strains previously shown to be strong biofilm formers and four weak biofilm formers or non-biofilm formers (Rinaudo et al., 2010a). Biofilm formation was investigated by growing the bacteria in THB in the absence or presence of glucose and measuring bacterial staining with CV as described in Materials and Methods. In the absence of glucose, no biofilm formation by any of the strains tested was observed with the new protocol (Figure 3.1A), as already reported by Rinaudo et al. (Rinaudo et al., 2010a). Moreover, biofilm formation increased when glucose was added to the culture medium in both protocols. By using the new protocol in the presence of glucose, CV assay values two to four times as high as those obtained with the standard procedure were obtained in the case of strong biofilm formers (strains 1 to 4 in Figure 3.1A), while the CV assay values of weak biofilm formers were drastically reduced (strains 5 to 8 in Figure 3.1A). Confocal

microscopy analysis of biofilms produced by a representative strong biofilm-forming strain confirmed that the new protocol permitted the formation of a thicker (average thickness, 17.1 versus 2.1 μm) and more homogeneous biofilm than that obtained by the standard protocol (Figure 3.1B). The biofilms produced by the new protocol not only show a higher biovolume (8.9 versus 1.1 $\mu m^3/\mu m^2$) but also contain a higher percentage of viable cells, as suggested by the different green/red ratios in Figure 3.1B and by the XTT cell viability assay results obtained (Figure 3.2). The same analysis with a representative non-biofilm-forming strain confirmed that CV assay values of 0.3 to 0.5 do not correspond to homogeneous biofilms (Figure 3.1C). Overall, these results suggest that the newly developed protocol allows better discrimination between strong biofilm formers and non-biofilm-forming strains.

## 3.2 An acidic pH promotes biofilm formation by *Streptococcus agalactiae*

To clarify the role of pH in biofilm formation by the different GBS serotypes, biofilm assays were performed with a selection 366 *S. agalactiae* isolates of eight serotypes grown in THB (starting pH, 7.8) with or without 1% glucose and in THB at pH 5.0 without supplemented glucose. Similar to the results shown in Figure 3.1, no significant biofilm formation by any of the isolates was detected in the presence of THB at neutral pH. On the other hand, both the use of an acidified medium (Figure 3.3A) and the addition of glucose at neutral pH (Figure 3.3B) resulted in a general increase in biofilm formation, with some of the isolates reaching very high CV assay values. In the absence of glucose, a 3- to 8-fold increase in the mean CV assay value was observed for isolates of all of the serotypes grown at pH 5.0 versus those grown at neutral pH.

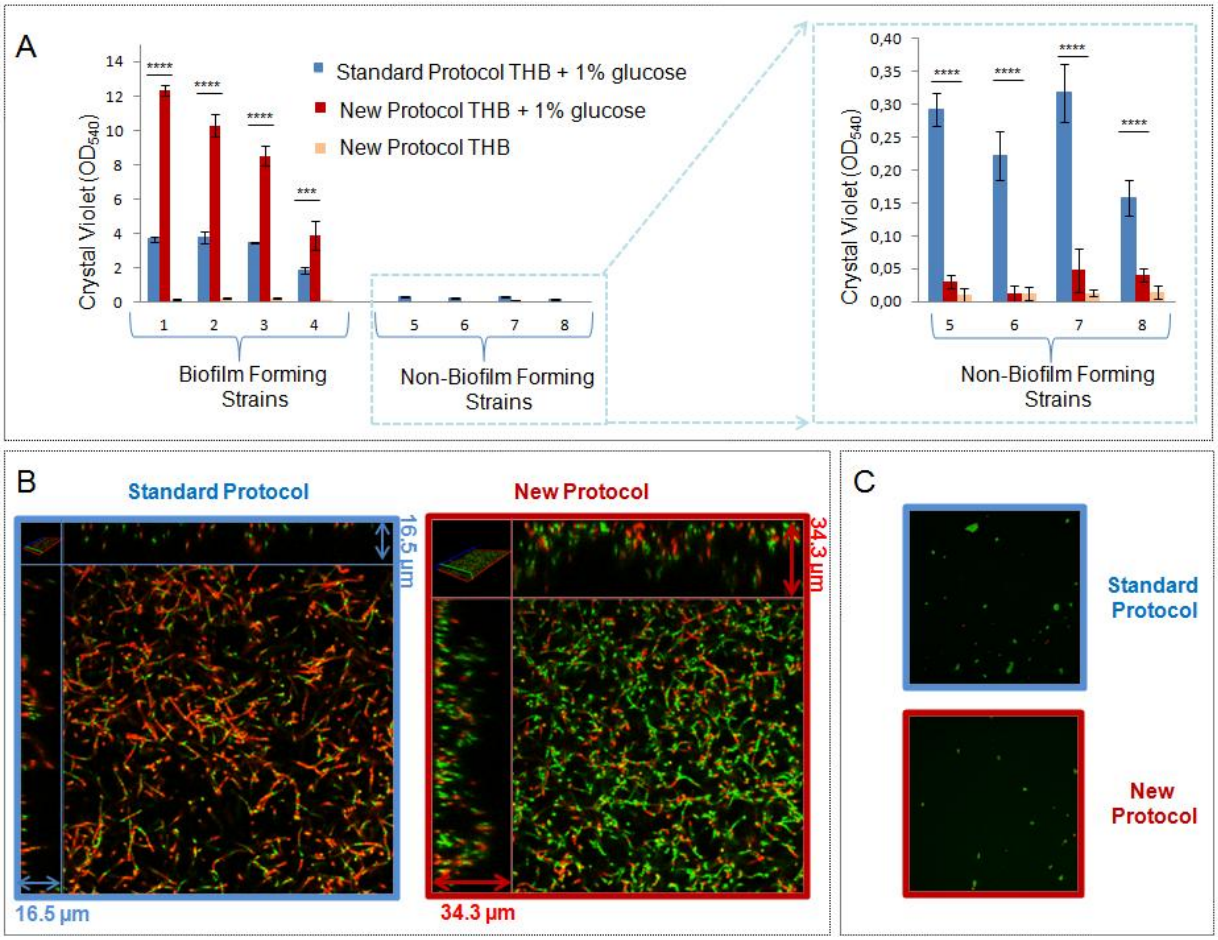



**Figure 3.1: Comparison of biofilm formation protocols.**

Comparison of biofilm formation protocols. (A) Biofilm formation abilities of four strong biofilm-forming strains and four weak biofilm formers or non-biofilm formers produced by the standard protocol and the new protocol. The mean values and standard deviations of three independent experiments are shown. Asterisks denote statistically significant difference determined by Student's *t* test (***, $P < 0.001$; ****, $P < 0.0001$). (B) CLSM of biofilms formed by a strong biofilm forming strain with the standard protocol and the new protocol in THB in the presence of 1% glucose. (C) CLSM of the biofilm produced by a non-biofilm forming strain with the standard protocol and the new protocol in THB in the presence of 1% glucose. Biofilms were stained with a LIVE/DEAD viability kit. Live and dead cells are green and red, respectively.

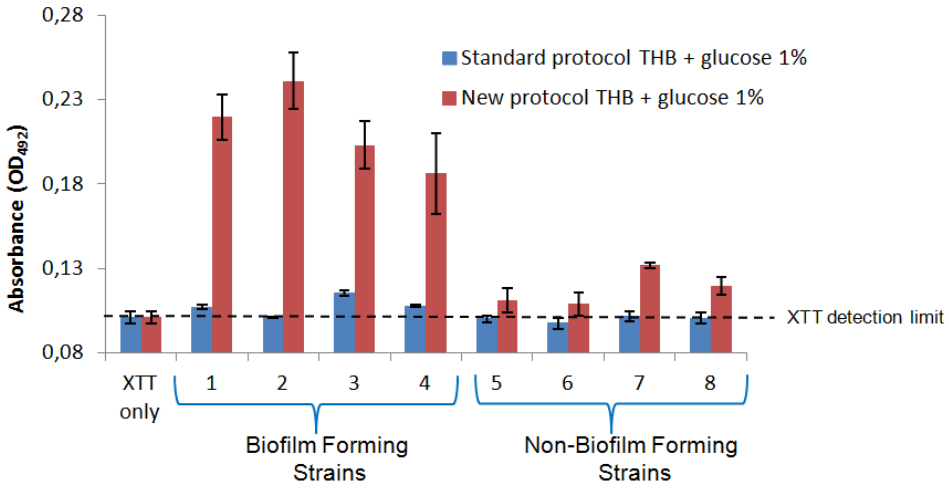



**Figure 3.2: Comparison of biofilm formation protocols by XTT**

Cell viability evaluation of four strong biofilm forming strains and four weak/non-biofilm forming strains produced by the standard protocol and the new protocol. The GBS strains were grown in THB in presence of 1% glucose (blue, standard protocol and red, new protocol). Surface-attached cells were incubated 3h at 37°C with XTT-menadione solution and cell viability was monitored by measuring the $OD_{492}$. The mean values of three independent experiments and standard deviation are shown.

The lower CV assay values observed under low-pH conditions in the absence of glucose than at pH 7.8 in the presence of glucose are most probably due to slower bacterial growth at a lower starting pH (Figure 3.4). However, no significant growth rate differences between biofilm-forming and non-biofilm-forming strains were observed in each of the media tested (Figure 3.4). As already observed in *S. pyogenes* (Manetti et al., 2010), the metabolism of the glucose, associated to organic acids production, could determine the pH decrease and could be the direct cause of the observed effect on biofilm formation. To better investigate the kinetics of biofilm formation in the presence of glucose and at low pH, we carried out time course biofilm assays by using a representative GBS that is a strong biofilm former and three different types of medium (THB pH 7.8, THB pH 7.8 supplemented with 1% glucose, and THB pH 5.0 without glucose).

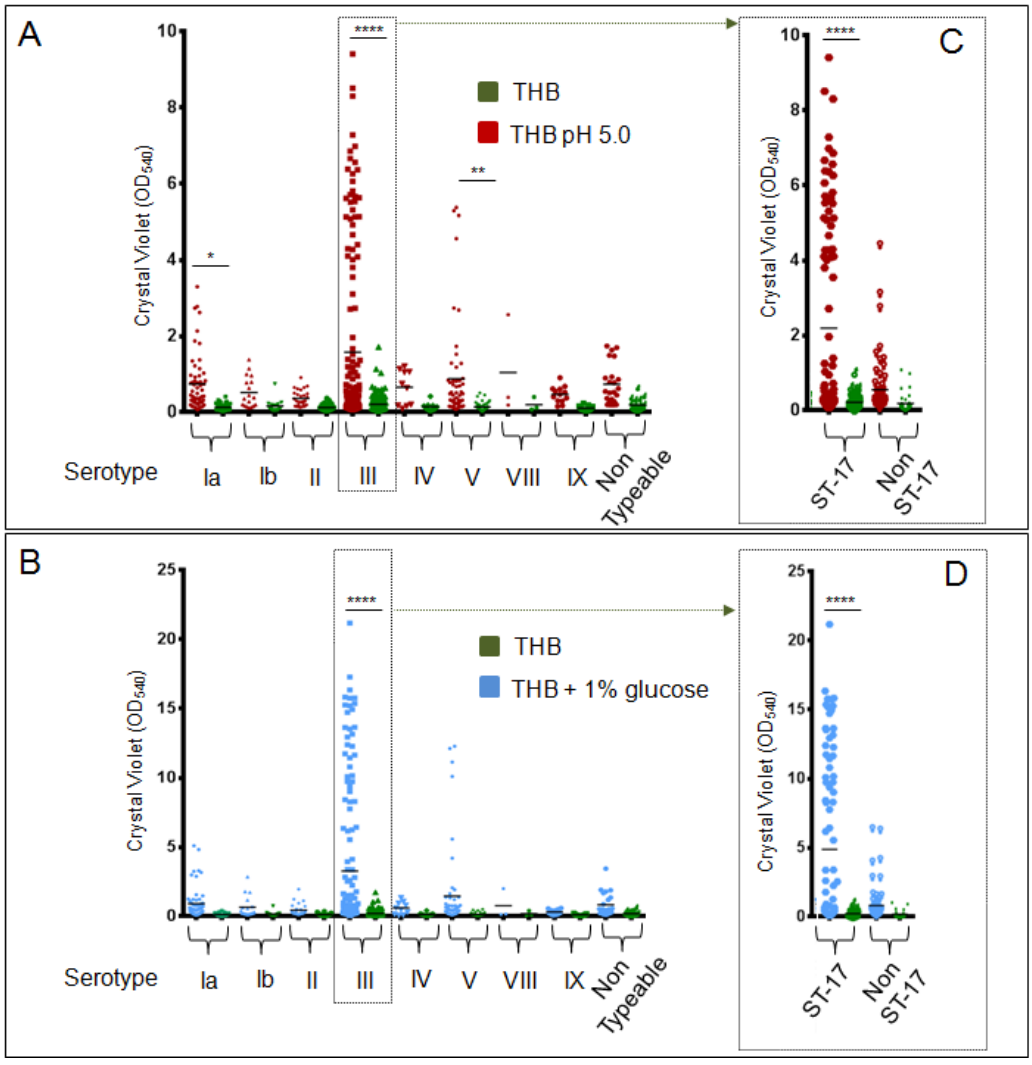



**Figure 3.3: Effect of acidic pH and glucose in GBS biofilm formation.**

Shown are the biofilm-forming abilities of 366 GBS clinical isolates of eight different serotypes and nontypeable strains grown in THB (green dots) or pH 5.0 THB (red dots) (A) or in THB (green dots) or THB supplemented with 1% glucose (red dots) (B). Panels C and D focus on serotype III strains grouped into ST-17 and non-ST-17 groups. Each dot represents the mean value of three independent experiments performed with each isolate. Asterisks denote statistically significant differences determined by ANOVA (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$).

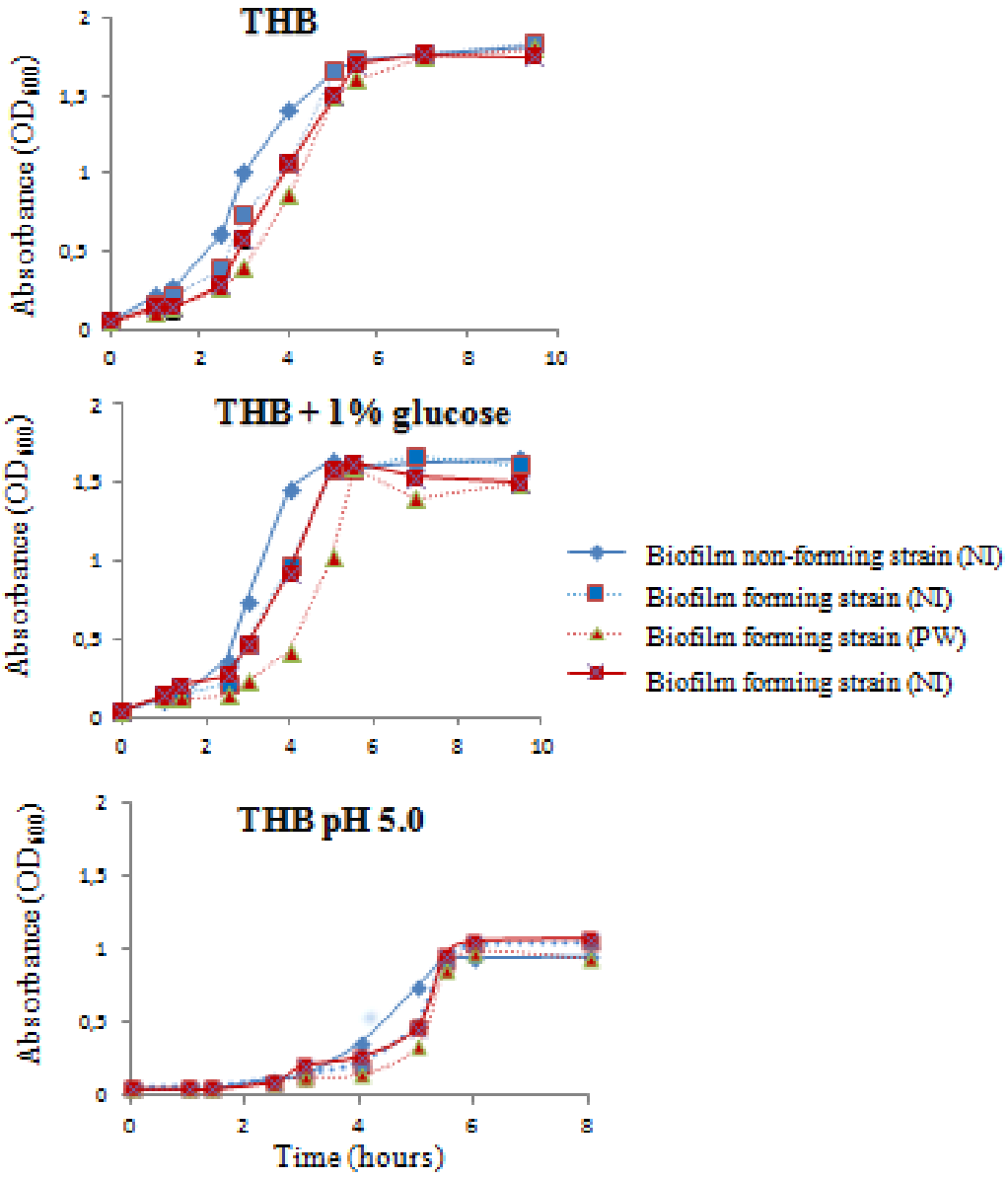



**Figure 3.4: Comparison of the growth rate of GBS biofilm forming and non-forming strains**

3 GBS biofilm forming strains (clinical isolates, serotype III, ST-17) and a GBS non-biofilm forming strain (COH1, serotype III, ST-17) were compared for their growth rate at 37°C in THB, THB supplemented with 1% glucose and THB pH 5.0. (NI) indicates the strains isolates from neonates while (PW) indicates the strain isolate from pregnant women. The $OD_{600}$ was monitored at different time point during the bacterial growth. The mean values of three independent experiments are shown.

A significant increment in the CV assay value (blue and red solid lines in Figure 3.5) was already observed after 4.5 to 5 h of incubation in THB pH 7.8 with 1% glucose, corresponding to a drop in pH to values lower than 5.0 (blue and red dotted lines in Figure 3.5), or in THB pH 5.0 without glucose. On the contrary, no biofilm formation was observed after the same strain was incubated in THB pH 7.8, where the culture pH never reached values below 5.0 (green dotted line in Figure 3.5). A similar pH profile was also detected for a representative non-biofilm-forming strain in all of the media tested (Figure 3.5). To further confirm that pH 5.0 is the signal sensed by the bacteria to start biofilm formation, a representative biofilm-forming GBS was grown in nutrition-limited RPMI GlutaMAX medium either in the presence of 1% glucose or at pH 5.0. In this case, differently from THB, the pH of the culture did not drop below 5.0 in the presence of glucose and no significant biofilm formation was observed (Figure 3.6). In contrast, significant biofilm formation (CV assay value/OD600 ratio of 3.6) was observed in the presence of RPMI GlutaMAX at pH 5.0 (Figures 3.6 and 3.7). Moreover, biofilm-forming GBS bacteria grown in THB supplemented with glucose and buffered with Tris or HEPES at concentrations that do not affect bacterial growth showed significantly reduced biofilm formation at final pH values higher than 4.5 (Figure 3.8), in agreement with data obtained with RPMI (Figure 3.6).

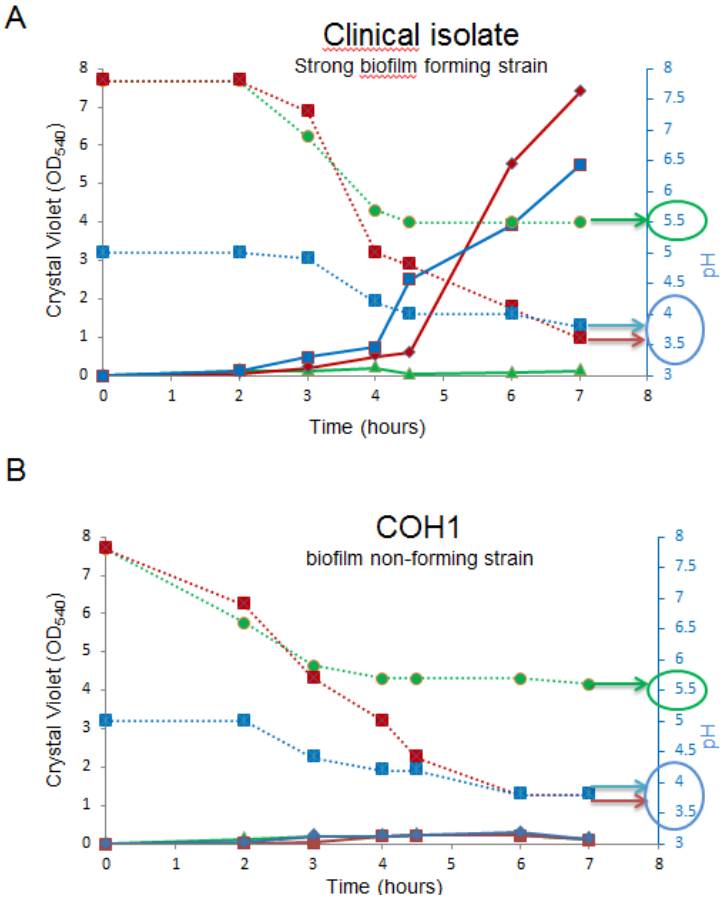
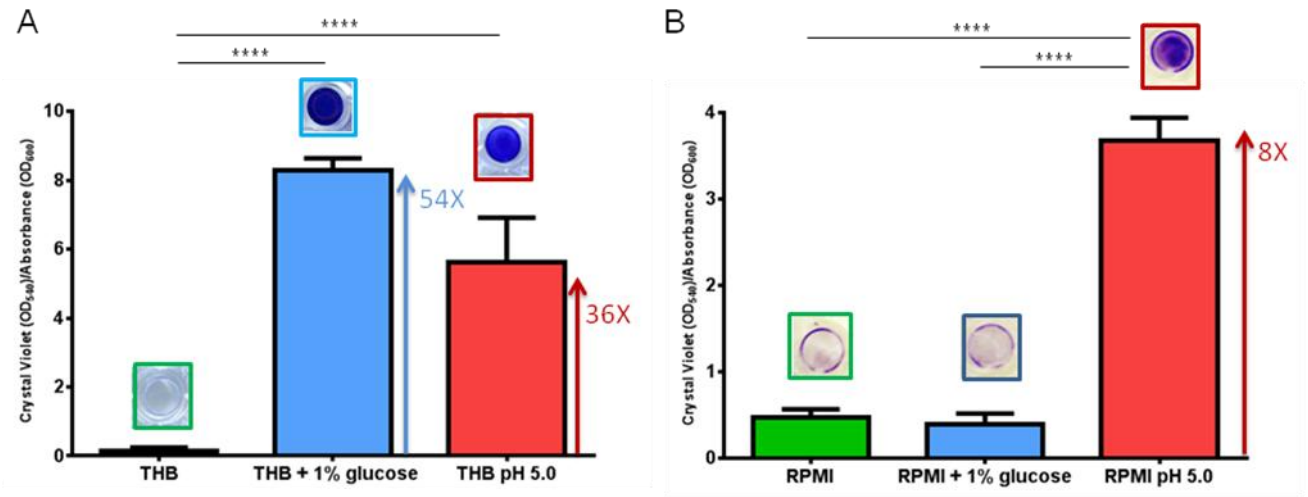



**Figure 3.5: Time-course of biofilm formation in correlation to pH.**

A) GBS biofilm forming strain (clinical isolate, serotype III, ST-17) and B) GBS non-biofilm forming strain (COH1, serotype III, ST-17) were compared for their ability to produce biofilm and induce acidification related to growth media. Three different growth media were tested: THB (green line), THB supplemented with 1% glucose (red line) and THB pH 5.0 (blue line). Biofilm formation was evaluated using Crystal Violet stain measuring the $OD_{540}$ (solid line). pH values (round dots) were measured using pH-test-strips (pH increment: 0.2). The mean values of three independent experiments, performed for both the strains in each medium, are shown.




| | THB | THB + 1% glucose | THB pH 5.0 |
|---|---|---|---|
| Final pH | 5.5 | 4.0 | 4.0 |

| | RPMI | RPMI + 1% glucose | RPMI pH 5.0 |
|---|---|---|---|
| Final pH | 6.5 | 5.5 | 4.5 |

**Figure 3.6: Acidic pH and not glucose induces GBS biofilm formation.**

Comparison of the biofilm formation ability of a strong biofilm forming strain grown in A) THB, THB supplemented with 1% of glucose and THB pH 5.0 B) RPMI, RPMI supplemented with 1% of glucose and RPMI pH 5.0. Biofilm formation was evaluated using Crystal Violet assay. Bacterial growth in planktonic form was evaluated measuring $OD_{600}$. pH values were measured using pH-test-strips (pH increment: 0.2). To normalize the biofilm formation ability with the cell growth the y-axis reports the ratio between the Crystal Violet values and $OD_{600}$. The mean values and standard deviations of three independent experiments are shown. Asterisks denote statistically significant difference, determined by Student's *t* test (****, $P < 0.0001$).The mean values of three independent experiments and standard deviation are shown.

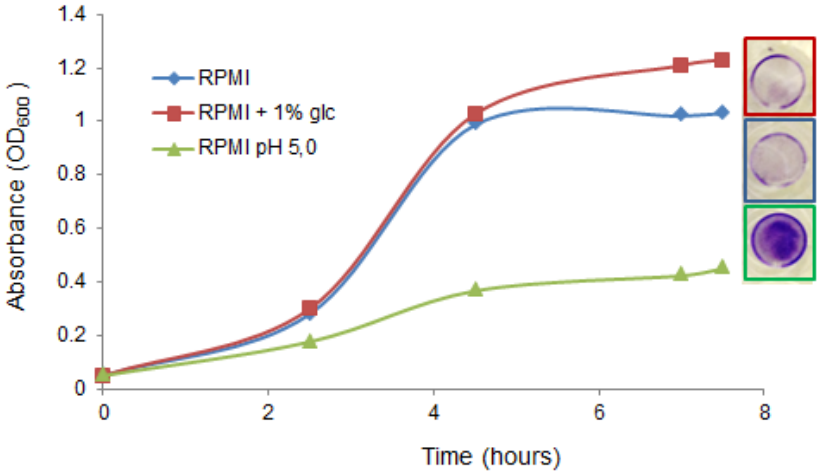
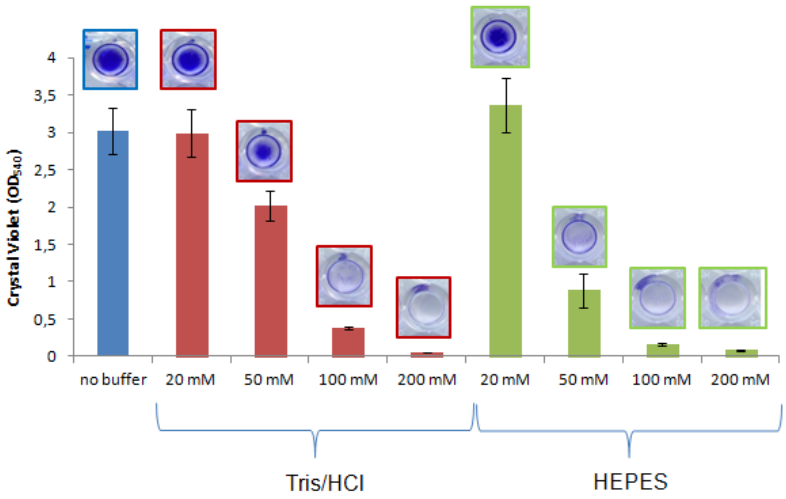



**Figure 3.7: GBS biofilm formation in a limited-nutrient medium, RPMI.**

GBS biofilm forming strain (serotype III, ST-17) was grown in three different growth media: RPMI pH 7.5 (blue line), RPMI supplemented with 1% glucose (red line) and RPMI pH 5.0 (green line) and bacterial growth was evaluated measuring $OD_{600}$. Biofilm formation was evaluated using Crystal Violet stain and the image of the well corresponding to the biofilm formation in each medium, after 7.5 hours, is reported on the right. The mean value of three independent experiments performed for both the strains and each medium.




| | No buffer | Tris-HCl | | | | Hepes | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 20 mM | 50 mM | 100 mM | 200 mM | 20 mM | 50 mM | 100 mM | 200 mM |
| Final pH | 4.3 | 4.3 | 4.5 | 4.7 | 6.5 | 4.4 | 4.6 | 4.8 | 6.0 |

**Figure 3.8: Biofilm formation in buffered THB media**

GBS strong biofilm forming strain (serotype III, ST-17) was compared for its ability to form biofilm in a THB medium supplemented with 1% of glucose and buffered with different concentration of Tris-HCl and HEPES to limit the pH-drop during the bacterial growth. Biofilm formation was evaluated 6h of incubation (37°C, 60 rpm) using Crystal Violet assay measuring the absorbance at 540 nm after. pH values (shown in the table) were measured using pH-test-strips (pH increment: 0.2). The mean values of three independent experiments are shown.

## 3.3 Most strong biofilm-forming GBS bacteria belong to the hypervirulent ST-17 lineage

Looking for a relationship between the different GBS serotypes and biofilm forming capacity, the data presented in Figure 3.3 were clustered on the basis of their capsular serotypes. The highest mean CV assay value increase and the highest number of strong biofilm-forming strains (CV values > 3) were found to belong to serotype III (Figure 3.3; Tables 3.1 and 3.2), although not all type III strains appeared to be good biofilm formers. In fact, none of the two GBS type III strains tested whose genome sequences were already available, COH1 and D136C, showed a significant ability to produce biofilm, either at an acidic pH or in the presence of glucose (Figure 3.9). Among the 156 type III isolates tested, 41 (26.4%) and 35 (22.4%) formed biofilms with CV values >3 in the presence of glucose and at low pH, respectively.

We subsequently investigated whether the subset of type III strong biofilm-forming strains belonged to the hypervirulent ST-17 clone (Figure 3.3). More than 36% of the 91 ST-17 strains showed CV values >3 in the presence of glucose (Table 3.2) or at low pH (Table 3.1), while less than 6.1% of the 65 non-ST-17 strains showed CV values >3 under the same conditions. Also, the mean CV assay value and the mean increment of the ST-17 strains were higher than those of the serotype III strains, while the values of the non-ST-17 group were comparable to those of the other serotypes (Tables 3.1 and 3.2), confirming a strong correlation between the hypervirulent ST-17 clone and GBS biofilm formation.

The possible relationship between strong biofilm formation and GBS virulence was further investigated by clustering the data presented in Figure 3.3 on the basis of GBS strain origins. A higher frequency of strong biofilm formers was observed in the 85 neonatal isolates than in the 272 in the colonizing group (18 versus 8%, $P = 0.12$). This difference was even larger when the

colonizing group was compared with the subset of infective neonatal isolates obtained from a sterile site (27 versus 8%, $P$ = 0.017). These results are clearly associated with the higher prevalence of ST-17 in the neonatal groups (40 out of 64 infective neonatal strains versus 50 out of 272 in the colonizing group). All four non-type-III strong biofilm formers belong to serotype V, and of these, two were isolated from infected neonates.

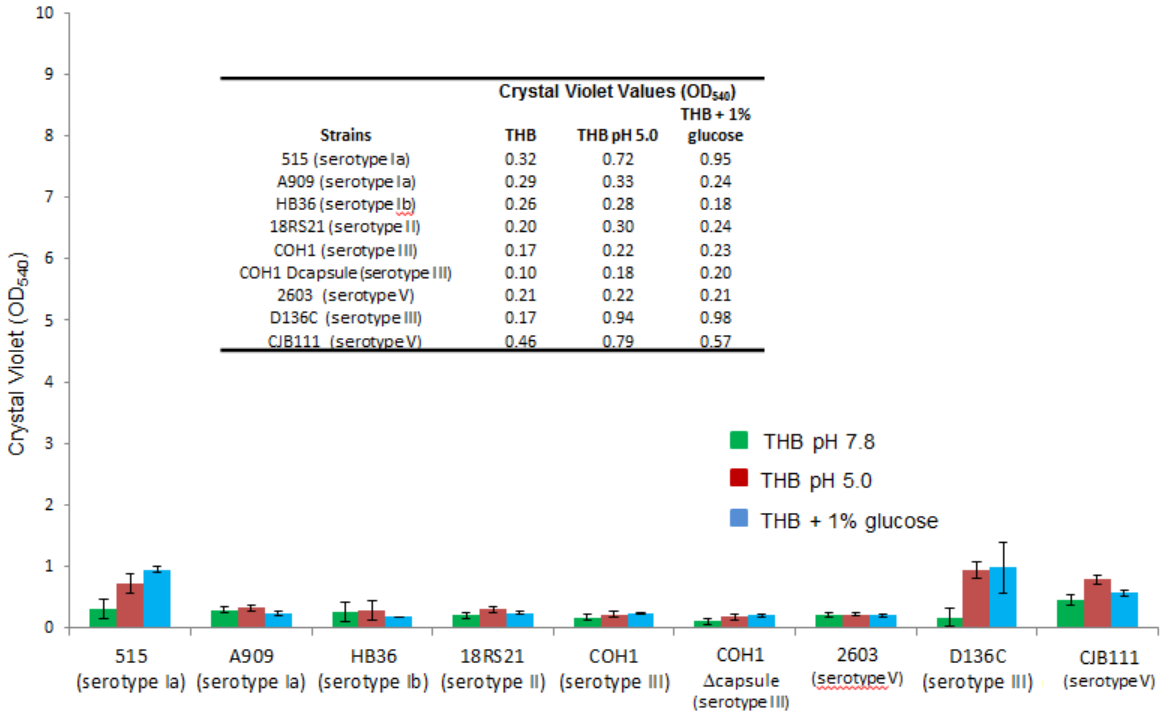



**Figure 3.9: Biofilm formation of sequence annotated GBS strains.**

Biofilm formation ability of 9 sequenced GBS strains grown in THB pH 7.8 (green bars) and THB supplemented with 1% of glucose (blue bars) and THB pH 5.0 (red bars) on 96-well polystyrene plates. Biofilm formation was evaluated using Crystal Violet stain by measuring the absorbance at 540 nm. The mean values of three independent experiments and standard deviation are shown.

## Table 3.1: Effects of THB pH 5.0 versus THB on biofilm formation by different GBS serotypes

| Serotype | Mean CV assay value | | No. (%) of strains with CV assay values of >3 | $Mean_{pH\ 5.0\ THB}/mean_{THB}$ | Mean difference | Adjusted *P* value |
|---|---|---|---|---|---|---|
| | pH 5.0 THB | THB | | | | |
| Ia | 0.74 | 0.13 | 1 (8.6) | 5.69 | 0.61 | 0.0481 |
| Ib | 0.52 | 0.17 | 0 (0) | 3.06 | 0.35 | 0.9851 |
| II | 0.37 | 0.13 | 0 (0) | 2.85 | 0.24 | 0.9952 |
| III | **1.58** | **0.21** | **35 (22.4)** | **7.52** | **1.37** | <0.0001 |
| IV | 0.66 | 0.14 | 0 (0) | 4.71 | 0.52 | 0.9740 |
| V | 0.88 | 0.14 | 4 (7.0) | 6.29 | 0.74 | 0.0083 |
| VIII | 1.05 | 0.20 | 0 (0) | 5.25 | 0.85 | 0.9864 |
| IX | 0.47 | 0.11 | 0 (0) | 4.27 | 0.36 | 0.9938 |
| Nontypeable | 0.74 | 0.21 | 0 (0) | 3.52 | 0.53 | 0.6693 |
| III (ST-17) | **2.20** | **0.22** | **33 (36.3)** | **10.00** | **1.98** | <0.0001 |
| III (non-ST-17) | 0.55 | 0.18 | 2 (3.1) | 3.06 | 0.37 | 0.3184 |

GBS biofilm formation was quantified by CV staining. Note that the percentage of biofilm formers with CV assay values of >3 is referred to the number of strains of each serotype (e.g., 156 for serotype III). Significant increments of mean CV assay values are in bold.

## Table 3.2: Effects of THB supplemented with 1% of glucose versus THB on biofilm formation by different GBS serotypes

| Serotype | Mean CV assay value | | No. (%) of strains with CV assay values of >3 | $Mean_{THB\ +\ 1\%\ glucose}/mean_{THB}$ | Mean difference | Adjusted *P* value |
|---|---|---|---|---|---|---|
| | THB + 1% glucose | THB | | | | |
| Ia | 0.91 | 0.13 | 5 (8.6) | 7.00 | 0.78 | 0.6254 |
| Ib | 0.66 | 0.17 | 0 (0) | 3.88 | 0.49 | 0.9995 |
| II | 0.45 | 0.13 | 0 (0) | 3.46 | 0.32 | >0.9999 |
| III | **3.26** | **0.21** | **41 (26.3)** | **15.52** | **3.05** | <0.0001 |
| IV | 0.61 | 0.14 | 0 (0) | 4.36 | 0.47 | >0.9999 |
| V | 1.46 | 0.14 | 6 (10.5) | 10.43 | 1.32 | 0.0568 |
| VIII | 0.76 | 0.20 | 0 (0) | 3.80 | 0.56 | >0.9999 |
| IX | 0.31 | 0.11 | 0 (0) | 2.82 | 0.20 | >0.9999 |
| Nontypeable | 0.83 | 0.21 | 1 (4.3) | 3.95 | 0.62 | 0.9900 |
| III (ST-17) | **4.878** | **0.22** | **37 (40.6)** | **22.27** | **4.68** | <0.0001 |
| III (non-ST-17) | 0.8087 | 0.18 | 4 (6.1) | 4.37 | 0.62 | 0.9187 |

GBS biofilm formation was quantified by CV staining. Note that the percentage of biofilm formers with CV assay values of >3 is referred to the number of strains of each serotype (e.g., 156 for serotype III). Significant increments of mean CV assay values are in bold.

## 3.4 Role of the capsule expression in GBS biofilm formation

To verify the possible involvement of differential capsule expression during biofilm formation at low pH, the sialic acid contents of four biofilm-forming strains (two type III ST-17 and two type V isolates) were estimated by Svennerholm's method (SVENNERHOLM, 1957) under conditions activating a planktonic or sessile GBS lifestyle. The amount of sialic acid produced by GBS grown at pH 5.0 was 50 to 60% lower than that produced by bacteria grown at pH 7.8 (Figure 3.10A), in agreement with results already reported in the literature (Kallman et al., 1998). In the biofilm-forming strains, capsule reduction corresponded to increased CV assay values (Figure 3.10B), suggesting an inverse relationship between capsule expression and biofilm formation. However, a comparable reduction in the amount of sialic acid was also observed at pH 5.0 for COH1, a non-biofilm-forming ST-17 strain (Figure 3.10), suggesting that capsule reduction per se is not sufficient to induce biofilm formation in the entire ST-17 clone.

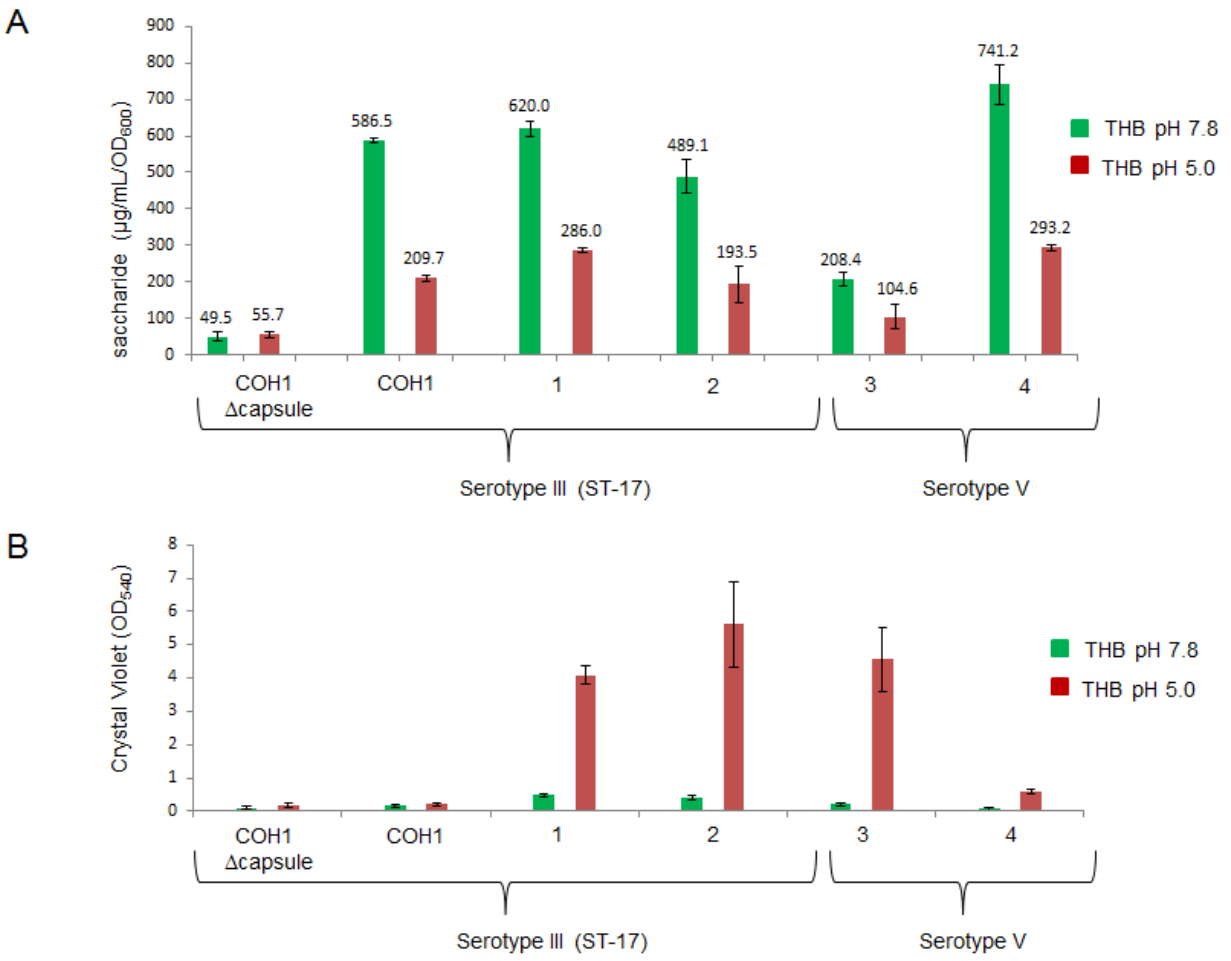



**Figure 3.10: Correlation between capsule expression and pH.**

A) Evaluation of capsule amount and B) Biofilm formation of 4 serotype III and 2 serotype V biofilm forming and non-forming strains, at pH 7.8 (green) and pH 5.0 (red). Capsular polysaccharides were isolated and quantified by the resorcinol–hydrochloric acid assay using Svennerholm's method. Surface-attached cells were Crystal Violet stained by measuring the absorbance at 540 nm. The mean values of three independent experiments and standard deviation are shown.

## 3.5 Proteins played a significant role in the GBS biofilm formation and maintenance.

Looking for the factors that, induced by acidic pH, could determine GBS biofilm formation and structural stability, biofilm inhibition and eradication by proteinase K and DNase I were evaluated for 6 strong biofilm forming strains (3 expressing type III (ST-17) and 3 expressing type V capsular polysaccharide). Concentration up to 200 µg/mL of proteinase K and DNase do not affect the planktonic bacterial growth of any tested strains (MIC ≥ 200 µg/mL). On the contrary 3 µg/mL of proteinase K were enough to induce almost total inhibition and eradication of an existing 24h-mature biofilm (Figure 3.11).

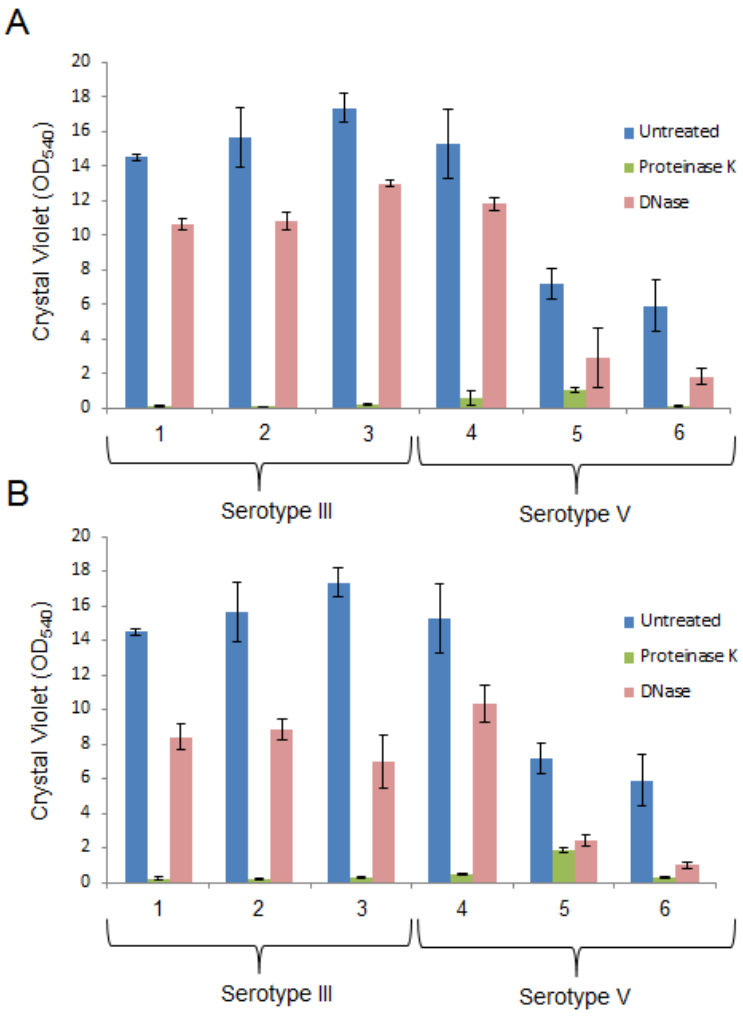



**Figure 3.11: Enzymatic inhibition or eradication of GBS biofilm.**
A) Biofilm inhibition and B) Biofilm eradication of three serotype III and three serotype V biofilm forming strains by proteinase K or DNase. Surface-attached cells were quantified by Crystal Violet measung the absorbance at 540 nm. The mean values of three independent experiments and standard deviation are shown.

The same effect was observed also decreasing the concentration of proteinase K up to 0,4 µg/mL (Figure 3.12). Addiction of 200 µg/mL of DNase determined low inhibition and partial disruption of the biofilm but the effect was weaker than that observed using proteinase K (Figure 3.11).

These results suggest that acidic pH could be important, specifically in the ST-17 strains, to regulate the expression and/or to promote the exposure of surface associated proteins, promoting bacterial adhesion and contributing to the biofilm structural stability.

Wondering if the expression the surface exposed or secreted proteins is modulated by glucose or pH specifically in the first phase of adhesion or in the second phase of maturation of the biofilm, the biofilm formation of three BFS was evaluated, in presence or in absence of glucose. Figure 3.13 shows that the presence of glucose "activates" biofilm formation already in the first 6 hours of growth and the final amount of biofilm is not affected by the absence of glucose after the replacement of media (Figure 3.13). This suggest as glucose and low pH are able to induce the expression of secreted or surface exposed proteins important to promote adhesion of bacterial cells to the surface.

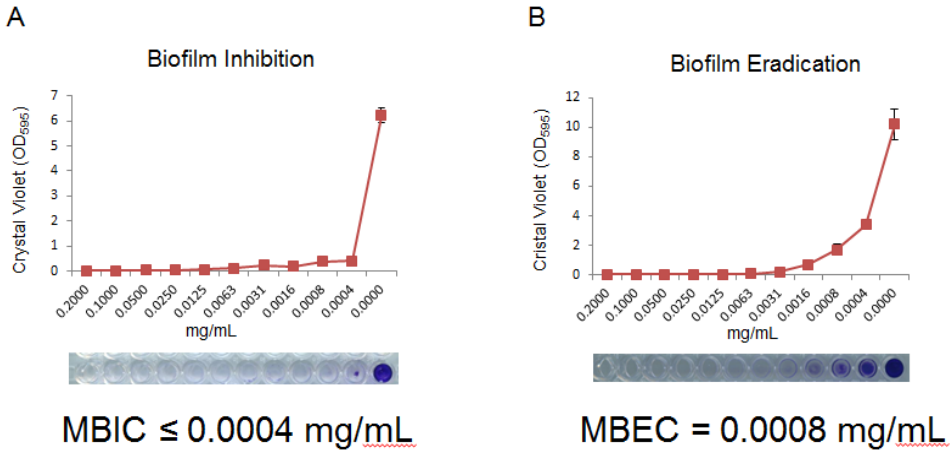



**Figure 3.12: Enzymatic biofilm inhibition and eradication using proteinase K.**

A) Minimal Biofilm Inhibition Concentration (MBIC) and B) Minimal Biofilm Eradication Concentration (MBEC) using proteinase K (range of concentration 0.2-0.0004 mg/mL). GBS biofilm forming strain (clinical isolate, serotype III, ST-17) was grown in THB pH 5.0. Biofilm formation was evaluated using Crystal Violet stain measuring the absorbance at 540 nm. The mean values of three independent experiments and standard deviation are shown. The plate stained with Crystal Violet is shown below the graph.

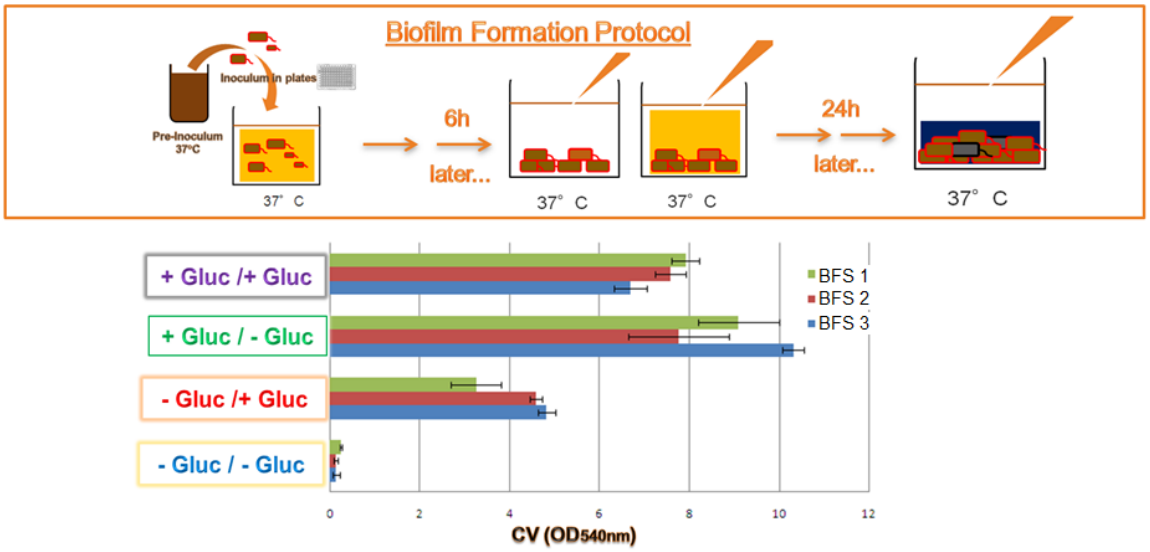



**Figure 3.13: Exposed or secreted proteins are primarily involved in the first phase of adhesion.**

The scheme of the biofilm formation-protocol is reported. 3 Biofilm Forming Strains (BFS) (serotype III, ST-17) were grown in two different growth media (THB supplemented with 1% glucose and THB) in four different combination: glucose always present (violet), glucose present only during the first 6 hours of bacterial growth (green), glucose present after the first 6 hours of growth (red) and glucose absent (blue). Bacterial growth was evaluated measuring $OD_{600}$. Biofilm formation was evaluated using Crystal Violet stain. The mean values of three independent experiments and standard deviation are shown.

## 3.6 Sulfome analysis to identify differentially expressed proteins on the surface of GBS grown in biofilm inducing and non-inducing conditions

Most of the strong biofilm formers belong to the hypervirulent clones ST-17 (Figure 3.3). Because of the differences in biofilm formation dictate by the growth-medium and the analyzed strain, we looked for the GBS surface-associated proteins differentially expressed in condition that favor sessile (THB pH 5.0) and planktonic (THB pH 7.8) life-style, using a proteomics approach. We compared the proteins differentially expressed in COH1 (a non-biofilm forming strain) and in a Biofilm Forming Strain (BFS). Mass spectrometry based peptide sequencing identified the GBS proteins expressed when cells were held at a steady state of growth at each of the two different growth conditions described in Material and Methods (Paragraph 2.11). Sodium dodecyl sulfate-polyacrilamide gel electrophoresis analysis of GBS cell wall-associated and membrane proteins did not reveal significant differences in expression depending on GBS strains (BFS or not) in biofilm formation activating condition (Figure 3.14).

Among the 32-39 cell wall-associated and membrane proteins detected in THB pH 5.0 (Table 3.3), 15 (38%) were detected exclusively under biofilm formation conditions both in COH1 both in a clinical BFS (Table 3.4). Only 3 proteins (9%) were detected exclusively under biofilm formation conditions (THB pH 5.0) in a clinical biofilm forming strain (Table 3.5), similarly 8 proteins were detected under biofilm formation conditions (THB pH 5.0) in the non-biofilm forming strain COH1 (Table 3.6).

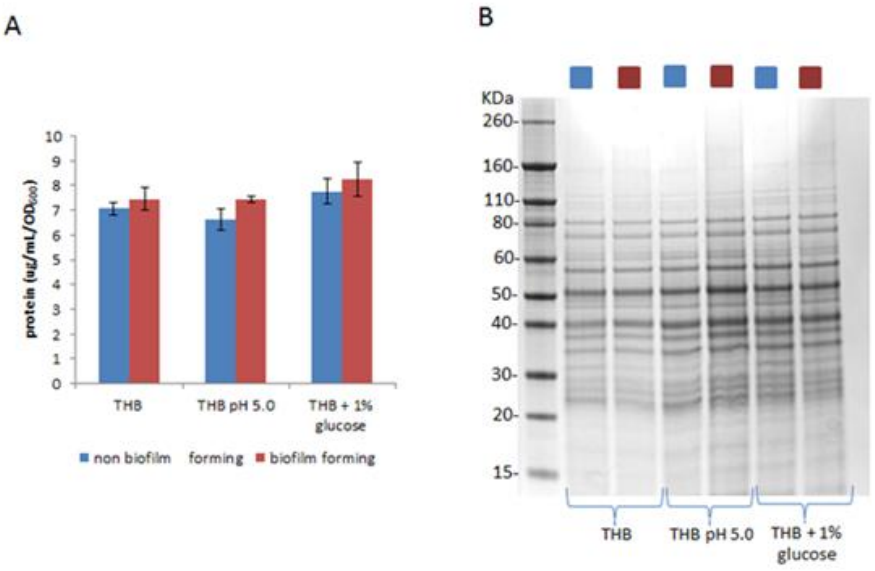



**Figure 3.14: Protein cell wall extraction and quantification of surface associated proteins**
Protein cell wall extracts of a biofilm forming and a biofilm non-forming strain, grown in THB, THB pH 5.0 and THB supplemented with 1% glucose. A) Protein content in cell wall fractions, quantified using BCA assay; B) Protein profile visualized by SDS-PAGE (Comassie staining).

Future studies, as gene expression analysis and/or knock-out mutants production, will be necessary to understand if some of these proteins, identified by surfome analysis, are really important to promote biofilm formation by ST-17 GBS strains.

**Table 3.3:** *Group B Streptococcus* ST-17 cell wall-associated and membrane proteins under biofilm and non-biofilm growth condition (respectively THB pH 5.0 and THB) in COH1 and in a BFS.

| Samples | Total no. of proteins detected |
|---|---|
| **COH1 grown in THB** | 16 |
| **BFS grown in THB** | 10 |
| **COH1 grown in THB pH 5.0** | 39 |
| **BFS grown in THB pH 5.0** | 32 |

BFS: Biofilm Forming Strain

**Table 3.4:** Predicted group B Streptococcus surface-associated proteins (n=15) detected exclusively under biofilm formation conditions (THB pH 5.0), both in COH1 both in a clinical biofilm forming strain.

| Locus | Protein Annotation |
|---|---|
| **SAN_1556** | ABC transporter maltose maltodextrin binding protein |
| **SAN_1559** | Maltose-maltodextrin ABC transporter |
| **SAN_0785** | Hypothetical protein |
| **SAN_0509** | Reticulocyte binding protein |
| **SAN_1313** | Zn-dependent protease |
| **SAN_1534** | Hypothetical protein |
| **SAN_0118** | N-acetylmuramoyl-L-alanine amidase_ family 4 protein |
| **SAN_0480** | Serine protease (htrA) |
| **SAN_0545** | Hypothetical protein |
| **SAN_1491** | Sulfatase |
| **SAN_0909** | Rotamase family protein |
| **SAN_0570** | Cell division protein FtsZ |
| **SAN_0325** | Penicillin-binding protein 1A |
| **SAN_0197** | Penicillin-binding protein 1B |
| **SAN_2288** | Membrane protein_ putative |

**Table 3.5**: Predicted group B Streptococcus surface-associated proteins (n=3) detected exclusively under biofilm formation conditions (THB pH 5.0) in a clinical biofilm forming strain.

| Locus | Protein Annotation |
|---|---|
| **SAN_1360** | Protein of unknown function |
| **SAN_1756** | YaeC family protein |
| **SAN_0524** | Hypothetical protein |

**Table 3.6:** Predicted group B Streptococcus surface-associated proteins (n=8) detected under biofilm formation conditions (THB pH 5.0) in the non-biofilm forming strain COH1.

| Locus | Protein Annotation |
|---|---|
| **SAN_1460** | efflux transporter_ RND family_ MFP subunit subfamily |
| **SAN_1830*** | conserved hypothetical protein |
| **SAN_0198*** | penicillin-binding protein 1B_ putative |
| **SAN_0072** | MORN motif family protein |
| **SAN_0698** | cell wall surface anchor family protein |
| **SAN_1040*** | lipoprotein (tmbC) |
| **SAN_1457** | ABC transporter_ ATP-binding protein |
| **SAN_2207*** | pathogenicity protein_ putative (HvgA) |

* Surface associated proteins detected also in the BFS in biofilm formation conditions (THB pH 5.0) but only in 1 out of 3 experiments.

## 3.7 Antibodies against pilus 2a, but not pilus 2b, inhibit biofilm formation

*Rinaudo et al.* demonstrated that pili proteins demonstrate that polyclonal antibodies against backbone protein (BP) and ancillary proteins of the pilus 2a inhibited the ability of GBS wild type strain 515 to form biofilms. Here also, it was investigated whether polyclonal antibodies against each of the three structural pilus proteins (BP, AP1 and AP2) inhibited the ability of type III ST-17 and type V strong-biofilm forming strains.

As reported in Figure 3.15A, the presence of anti-BP antibodies significantly inhibited biofilm formation in a dose-dependent manner of the type V BFS. On the contrary, antibodies specific for the backbone protein of pilus type 1 and 2b did not inhibit biofilm formation of type III ST-17 (Figure 3.15B). These data further confirm specific involvement of pilus type 2a, but not pilus types 1 and 2b in biofilm development, as already demonstrated by Rinaudo et al., 2010 (Rinaudo et al., 2010b).

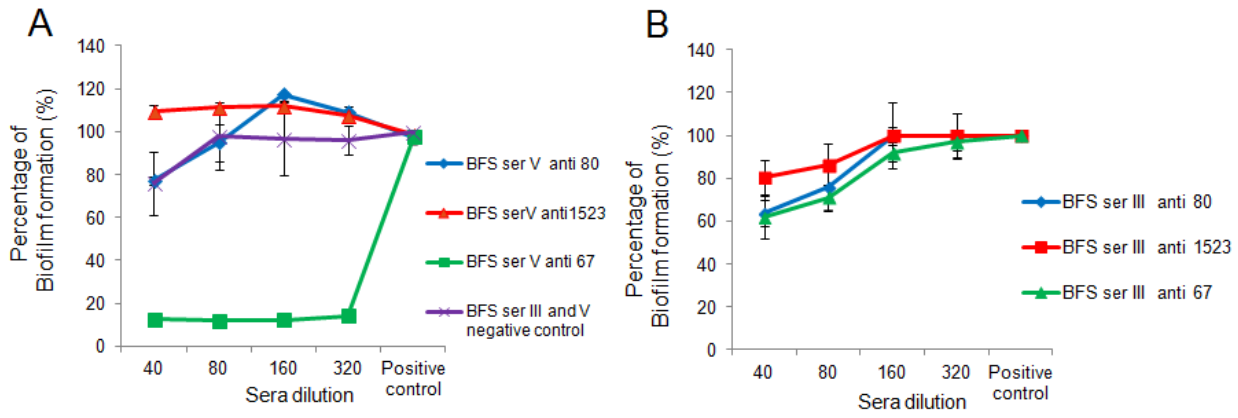



**Figure 3.15: Inhibition of biofilm formation by antibodies against pilus 2a proteins.**

A)Type V GBS BFS and B) Type III GBS BFS were grown in 96-well polystyrene plates at 37 °C for 8 hours in the presence of serial dilutions of sera containing polyclonal antibodies directed against each of the pilus 2a structural protein (anti-BP-2a) and against the structural proteins of pilus 1 and 2b. Sera from pre-immunized mice were used as a control. Biofilm formation was measured by CV assay, determining the absorbance at 540 nm. The mean values of three independent experiments and standard deviation are shown.

# 4. Discussion

Recent studies have demonstrated GBS biofilm formation *in vitro* (Borges et al., 2012;Kaur et al., 2009;Konto-Ghiorghi et al., 2009;Rinaudo et al., 2010a) although the data regarding the effect of pH and media composition are controversial. In a recent study, *Ho et al.* (Ho et al., 2012) found that the pH and nutrient-limited medium (M9YE) together, induce biofilm formation in GBS strains, but contradictorily, they found that in THB medium the capacity for GBS to produce biofilm increased with higher pH values. *Borges S. et al.* (2011), *Kaur et al.* (2009) and *Yang Q. et al.* (2012) found that GBS produced a greater amount of biofilm at pH 6.5 than at pH 4.2, and *Konto Ghiordi et al.* (2009) reported that only on LB and RPMI 1640 supplemented with 1% of glucose produced uniform biofilm and not on THB medium. Manetti et al. (Manetti et al., 2010) observed that in *S. pyogenes*, the presence of glucose resulted in auto acidification of the media and consequently biofilm formation. *Rinaudo et al.* (2010) demonstrated that the presence of 1% of glucose in THB induces biofilm formation by GBS. .

A major limitation of the static protocol used to screen the biofilm formation used in that studies is the absence of the fluid circulation encountered in the host. *Konto Ghiordi et al.* (2009) (Konto-Ghiorghi et al., 2009) showed that a protocol under low flow conditions, was preferable respect to a static-condition protocol for GBS adherence to epithelial cells. To approach the conditions of the laminar-flow chamber system while maintaining the throughput of multiwell-based protocols, in this study we developed an in-batch *in vitro* protocol including a medium replacement step.

The biofilm, produced by the strong biofilm forming strains, using the new protocol, was thicker and more homogeneous than that obtained with the standard protocol (Rinaudo et al., 2010a)

(Figure 3.1). In contrast weak biofilm forming strains or non biofilm forming strains produced reduced biofilms with this protocol (Figure 3.1). These data suggest that our biofilm formation protocol, based on more stringent conditions, reduces false positive results and unambiguous data interpretation.

Appling the new protocol to a large number of GBS clinical isolates (about 360), we obtained for the first time unequivocal evidence that an acidic pH induces GBS biofilm formation in both a nutrition-rich (THB) and nutrition-limited environment (RPMI) (Figure 3.6). As already reported for other pathogens, like GAS (Manetti et al., 2010) and *Staphylococcus aureus* (Boles and Horswill, 2008;Regassa et al., 1992) also for GBS the addiction of 1% glucose induces biofilm formation only if results in the acidification of the media with a pH below 5 (Figures 3.5 and 3.6), as observed in THB but not in RPMI. These data not only clarify previous observations regarding the role of pH during biofilm formation by GBS but also revealed, for the first time, a significant divergence between different GBS serotypes.

Interestingly the majority of the strong biofilm-forming GBS bacteria are serotype III ST-17 strains, suggesting an ST-biofilm correlation (Figure 3.3). The ST-17 lineage of GBS strains causes significantly more meningitis in neonates than strains of other GBS lineages and hence is considered as a highly virulent clone (Hery-Arnaud et al., 2005;Manning et al., 2009). A higher frequency of strong biofilm formers was also observed among the infective neonatal isolates than in the colonizing group included in our strain collection. This result is clearly associated with the higher prevalence of ST-17 in our neonatal group (Fig. 3.3). In fact, the ST-17 GBS lineage is more prevalent in infected neonates than in other lineages, particularly during late-onset disease (Bellais et al., 2012;Lin et al., 2006;Tazi et al., 2010).

A high heterogeneity in the ability to produce biofilm was observed not only between serotypes but also within the very homogeneous hypervirulent ST-17 clone (40, 41) (Fig. 3.3). A general reduction of the capsule amount was observed in biofilm-forming serotype III and V strains in response to a pH decrease (Figure 3.10). In fact, for all the selected tested strains (biofilm and non-biofilm formers), the amount of sialic acid, produced growing GBS at pH 5.0, was reduced by 50-60% respect to the amount of sialic acid produced at pH 7.8, as previously observed (Kallman et al., 1998). Although this capsule reduction correlated with increased biofilm formation by some, but not all, of the ST-17 isolates tested, such as COH1 (Figure 3.10), capsule down regulation at pH 5.0 is not sufficient per se to ensure biofilm formation by all ST-17 strains. If the presence of the capsule favors bacterial escape of complement-mediated killing (Areschoug et al., 2008) in genital tracts with a low pH, where capsule formation is down regulated, the biofilm lifestyle of ST-17 strains may represent an alternative strategy to guarantee their persistence.

Proteinase K, at a concentration that does not affect the cell growth, inhibited biofilm formation and induced biofilm detachment (Figure 3.11), suggesting that surface exposed or secreted proteins, specifically modulated by pH, play a major role in promoting bacterial adhesion and biofilm structural stability (Figure 3.11). These results suggest that an acidic pH may be important in unmasking surface-associated proteins that promote adhesion by biofilm-forming bacterial strains. Alternatively, the acidic pH could up-regulate the expression of some surface-associated proteins specifically in biofilm-forming GBS bacteria. It has already been shown that an acidic pH can modulate the expression of a large number of proteins in GBS, including the proteins involved in surface adhesion (Park et al., 2012;Santi et al., 2009).

Several proteins were already reported to be important, in ST-17 strains, for adhesion to solid surfaces or attachment to host cells or extracellular matrix. These include: (I) *fbsB*, a fibrinogen-binding gene (Al et al., 2011b;Rosenau et al., 2007), (II) HvgA, encoding a cell-wall–anchored protein (Tazi et al., 2010), (III) pili components (Sharma et al., 2013) and (IV) genetic variations in the serine-rich repeat region gene (srr), the surface protein gene (spb-1) and the alpha C protein gene (Brochet et al., 2006;Seifert et al., 2006). Surfome analysis enabled us the identification of several GBS-surface proteins under both activating and non-activating biofilm conditions. 26 proteins were exclusively detected at acidic pH. 15 out of the 26 proteins were detected in both BFS and non-BFS and 3 of them typically in BFS: I) SAN1360, a cell surface-exposed lipoprotein; II) SAN1756, an O-sialoglycoprotein endopeptidase and III) SAN0524, a cell surface protein. These three proteins are interesting because they seem to be specific of the BFS. Little is known about their role in GBS and further studies are necessary to verify if these proteins promote GBS biofilm formation and in *vivo* colonization. We cannot exclude that the high number of proteins detected at pH 5.0 is due to the reduction of capsular polysaccharide that could unmask some cell wall-associated proteins and make them more available to the enzymatic proteolysis. In *S. aureus*, evidences of the ability of the capsule to mask critical determinants of virulence in endocarditis were reported (Kuypers and Proctor, 1989;Moreillon et al., 1995). It was also observed that capsule hinders the in vitro interactions between *S. aureus* and mammalian cells (Pohlmann-Dietze et al., 2000;Tuchscherr et al., 2005) ). Moreover our surface proteome analysis provides qualitative but not quantitative results in terms of protein expression in GBS surface. Therefore, it is also possible that changes in the expression level of some of the 15 proteins, detected specifically at pH 5.0 in both BFS and non-BFS, may contribute to induce biofilm formation. This group includes a Serine protease HtrA (SAN0480), a Reticulocyte

binding protein (SAN0509), a Zn-dependent protease (SAN1313), ABC transporters (SAN1556, SAN1559) and a family of PBPs (SAN0325, SAN0197). HtrA, in *S. mutans*, a causative agent of dental caries, plays a role in the regulation of genetic competence, biofilm formation, and the biogenesis of cell wall-associated and secreted proteins (Ahn et al., 2005;Biswas and Biswas, 2005). Also *S. aureus* expresses two surface-exposed HtrA homologues, involved in the secretion of virulence factors essential for bacterial dissemination (Rigoulay et al., 2005). 3 PBPs (SAN0325, SAN0197, SAN0198) were detected in at least one of the GBS tested strains under the biofilm-activating growth condition. Other 2 members PBPs (SAN2210, SAN0314) were detected in COH1 at pH 5.0 but only 1 out of 3 replicates and therefore excluded from Table 3.6. PBPs have been proposed as virulence factors in several gram-positive bacteria (Graham and Clark-Curtiss, 1999;Mei et al., 1997). Pbp1a has been shown to be critical for GBS pathogenesis in a neonatal rat model of infection (Jones et al., 2003a) and GBS Pbp2b has been reported to bind ME-180 cervix epithelial cells (Johri et al., 2007).

Whether the newly identified GBS proteins described herein do indeed play a role in biofilm formation awaits further experimentation. The difference in terms of protein expression levels can be assessed performing qPCR experiments and, moreover the role of the identified proteins in GBS biofilm formation could be confirmed testing the biofilm formation, invasion, or virulence of a set of knock-out mutants in comparison with the parent strains.

In conclusion applying a new *in vitro* biofilm formation protocol to screen a large number of clinical isolates, we were able to clearly demonstrate that acidic pH is able to induce biofilm formation in GBS in a serotype dependent manner. Further efforts are necessary to: i) understand if the effects of pH on biofilm formation by different GBS serotypes correlate with the ability to adhere to vaginal cells; ii) confirm the result obtained by mass spectrometry: a) identifying the

genes differently regulated by low pH in strong biofilm formers and non-biofilm formers (e.g. COH1) belonging to ST-17 lineage and b) constructing isogenic mutants to verify the altered phenotype compared with wild-type stains. The identification of those proteins that promote cell adhesion in biofilm-forming GBS strains will lead to a better understanding of the mechanism of biofilm formation by GBS and allow the design of new therapeutic approaches against this pathogen.

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