Acidic pH strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17 *Streptococcus agalactiae* strains


Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

*To whom correspondence should be address. E-mail: manuele.martinelli@novartis.com

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**Running Title:** ST-17 GBS strains produce biofilm at low pH
Abstract

*Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS) is a 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.

In recent years, the ability of GBS to form biofilm attracted attention for its possible role in fitness and 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 biofilm formation ability of 366 GBS clinical isolates from pregnant women and from neonatal infections belonging to different serotypes, in relation to media composition and pH.

The results identified a subset isolates belonging to serotypes III and V that formed strong biofilms under acidic conditions. Importantly, the best biofilm formers belonged to the serotype III hypervirulent clone ST-17. 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 initiation and contribute to the biofilm structural stability.
Streptococcus agalactiae, also known as Group B Streptococcus (GBS) is a leading cause of invasive neonatal infections worldwide. It is a common colonizer of the gastro-intestinal and urogenital tracts of up to 40% of healthy individuals (1). However, in certain circumstances, GBS can become a life-threatening pathogen causing invasive infections in human neonates (2;3). Early-onset group B streptococcal disease (EOD) occurs in infants younger than 7 days and late-onset disease (LOD) occurs in infants between 7 and 89 days old. GBS is usually transmitted from mothers to newborns during childbirth (4), but it can also penetrate the human placenta (5), and in the case of LOD it can be nosocomially acquired.

Historically, GBS isolates have been classified into 10 different serotypes according to their capsule polysaccharide composition (6;7). Multiple surveillance studies have indicated that all serotypes are able to colonize the vagina and perianal region of pregnant women, but five serotypes (Ia, Ib, II, III and V) are predominant, and are also the most frequent in human infections (8-12). In particular, serotype III accounts for 30-76% of neonatal disease cases (13;14). The application of Multilocus Sequence Typing (MLST) allowed classifying GBS isolates independently from their capsular serotype and identifying the bacterial genogroups more often associated with invasive infections in newborns (15). Serotype III isolates belonging to a particular genotype cluster, the Sequence Type 17 (ST-17), disproportionately cause late-onset GBS disease (15-19), and more frequently cause meningitis in comparison with other STs (20). The precise mechanism by which ST-17 causes LOD more frequently than other ST types is not well understood, although recent evidence indicated the ST-17 displays a conserved specific combination of the secreted and surface exposed proteins (21;22).

Biofilm is known to facilitate colonization and persistence of a large variety of bacterial and fungal species and to support dissemination of virulent clones (23;24). Organisms within biofilms can withstand nutrient deprivation, pH changes, oxygen radicals, disinfectants, and antibiotics better than planktonic organisms (25). 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 (26-29). 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 (26;27;29). 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.

In the present work, a new in vitro biofilm formation protocol was applied to evaluate the ability of a large collection of GBS isolates to produce biofilm in different growth conditions. The protocol permitted to clearly demonstrate that GBS biofilm formation is enhanced at acid pH and to identify a subset of serotype III strains belonging to the sequence type ST-17 as strong biofilm formers. The contribution of DNA, capsule and proteins in inducing bacterial adherence was also investigated.
Material and Methods

Strains

A total of 366 S. agalactiae isolates belonging to 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 isolates from neonates (n = 85, of which 64 obtained from a sterile site) 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. 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 (Harvard Medical School, Boston, MA, USA). 2603 V/R (serotype V) (30) 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 (31) and was kindly provided by M. Cieslewicz (Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, USA).

Serotype and ST-17 identification

GBS strains were typed by latex agglutination method (Strep-B-Latex kit; SSI, Denmark), following the procedure described by Afshar et al. (32). 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, gleK, glnA, pheS, sdhA, and tkt were performed as described previously (15). 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.

Growth experiments
Four clinical isolates (3 biofilm forming and 1 non-biofilm forming strains), grown overnight at 37°C in Todd Hewitt Broth pH 7.8 (THB), were diluted to OD₆₀₀ 0.05 in optical tubes containing 10 mL of THB pH 7.8, THB pH 7.8 supplemented with 1% glucose or THB pH 5.0. The tubes were then incubated without shaking at 37°C and the optical density at 600nm was measured for 8-10 h. Each experiment was performed in triplicate.

**Standard biofilm formation protocol**

The standard protocol was performed as already described (26). In brief, *Streptococcus agalactiae* strains were streaked on blood agar plates and grown at 37 °C for 18 hours at 37 °C.

GBS strains, grown overnight in THB pH 7.8 were diluted 1:20 in THB and THB supplemented with 1% glucose; and used to inoculate (100 μL/well) a 96-well polystyrene microtiter plates (Costar, Corning Inc. USA). Plates were incubated without shaking at 37°C for 18 h aerobically in 5% CO₂. The supernatant was removed and the wells were subjected to 3 cycles of washes with 200 μL of ddH₂O to remove unattached bacteria. Crystal Violet (CV) assay and XTT assay were then performed to estimate bacterial biomass and cell viability respectively.

**New biofilm formation protocol**

A new protocol for *in vitro* biofilm formation was set-up. *Streptococcus agalactiae* strains were streaked on blood agar plates and grown at 37 °C for 18 h. Bacterial suspension in THB was diluted to OD₆₀₀ 0.05 and used to inoculate (200 μL/well) a 96-well polystyrene microtiter plates (Costar, Corning Inc. USA). The preliminary protocol evaluation was performed using THB and THB supplemented with 1% of glucose, as already used in the *Standard biofilm formation protocol*. Other media were also used to investigate the role of pH in biofilm formation a)RPMI GlutaMAX (Gibco-Life Technologies, Milan, Italy); b) RPMI GlutaMAX supplemented with 1% glucose; c) RPMI GlutaMAX and THB both acidified to pH 5.0; d)THB supplemented with 1% glucose and buffered at pH 7.8 with addiction of Hepes (20-200mM range) or Tris-HCl (20-200mM range).
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 the plates were washed to remove loosely adherent cells and the supernatant was replaced by 200 μL of fresh medium. After 15 h at 37°C, the medium was removed and the wells were subjected to 3 cycles of washes with 200 μL of PBS to remove unattached bacteria. CV assay and XTT assay were then performed to estimate bacterial biomass and cell viability respectively.

**Crystal Violet assay**

The wells were stained for 10 min with 200 μL of 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 absorbance of the solution at 540 nm using a microplate reader (Tecan, Infinite M200). 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. Plate wells filled with growth medium were included as negative controls.

**XTT viability assay**

The 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) is a tetrazolium derivate cleaved to an orange-colored formazan product by mitochondrial dehydrogenase in viable cells (33). The XTT solution was prepared by dissolving 0.5 mg XTT (Sigma-Aldrich, Inc., St. Louis, MO) in 1 ml of PBS and then supplemented with 2.5 μL of 10 mM menadione stock solution, (dissolved in acetone). 150 μL of XTT–menadione solution were added to the wells. Plates were incubated in the dark for 3 h at 37°C and then centrifuged 20 min at 4000 rpm. 100 μl of the supernatant was transferred to a new 96-well flat bottom plate and the absorbance was measured at 490 nm using a microplate reader (Tecan, Infinite M200).
Confocal laser scanning microscopy

The GBS biofilms obtained by both the standard protocol (26) 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 Labtek II 8-well 1.5 Coverglass (Labtek II, VWR, Rochester, USA) containing THB or THB supplemented with 1% of glucose and incubated as already described in the New biofilm formation protocol paragraph. Adherent bacteria were stained for 30 min with the fluorescent BacLight Live/Dead stain (Molecular Probes, Eugene, OR, USA) and fixed with 2% formaldehyde for 30 min at RT. 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” (492-572 nm) and Propidium Iodide fluorescence, which not penetrates viable bacterial cells, were acquired in the “red channel” (566-719 nm). Images were acquired by using Zen 2008 software and modified with Volocity (Improvision, Lexington, MA). Image J (http://rsbweb.nih.gov/ij/) and COMSTAT2 (http://www.comstat.dk) were used to evaluate the biomass, maximum thickness and mean thickness from the three-dimensional biofilm images acquired by CLSM (34).

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 (Costar, Corning Inc. USA) using 0.4-200 μg/mL of proteinase K and maximum 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 or DNase. For MBEC determination a 24h-mature biofilm growth in absence of proteinase K or DNase, was washed twice with PBS and further incubated in THB supplemented with 1% glucose containing...
proteinase K or DNase for 3 h at 37°C. Biofilm was quantified by CV assay, as previously described.

**Quantification of capsular polysaccharides**

Capsular polysaccharides from COH1 strain and 4 strong/weak biofilm forming strains (3 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 (35).

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$_2$O. 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 (NeuNAC) reacts with resorcinol in the presence of copper sulphate under reducing conditions to give a blue-purple color. After cooling 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 GBS total saccharide (conversion factor= MW NeuNAC / MW Repeat Unit GBS polysaccharide).

**Statistics**

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

A novel *in vitro* biofilm formation protocol

*In vitro* protocols applied to date to determine the biofilm formation ability of different GBS isolates resulted in small differences between the tested strains and did not allow clearly discriminating among strong and weak GBS biofilm formers. Here, a novel experimental protocol was implemented to overcome this limitation. The main differences respect to the previously described standard protocol (see *Methods section*) (26) were: i) replacement of culture medium with fresh medium after 8 hours of incubation to remove non-adherent bacteria and ii) incubation of the plates under shaking conditions to minimize non-specific bacterial deposition on the bottom of the plate.

The two *in vitro* protocols were compared by testing 4 GBS strains previously shown as strong biofilm formers and 4 weak or non-biofilm formers (26). Biofilm formation was investigated by growing the bacteria in Todd Hewitt Broth (THB) medium in the absence or in the presence of glucose, and measured by bacterial staining with Crystal Violet (CV) as reported in the *Methods section*. No biofilm was observed for any of the tested strains in the absence of glucose with the new protocol (Figure 1A) as already reported by Rinaudo (26) using the standard protocol. Moreover biofilm formation increased when glucose was added to the culture medium in both protocols. By applying the new protocol in the presence of glucose, CV values 2-4 times higher compared to the standard procedure were obtained in the case of strong biofilm formers (strains 1, 2, 3 and 4 in Figure 1A), while the CV values of weak biofilm formers were drastically reduced (strains 5, 6, 7 and 8 in Figure 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 μm vs 2.1 μm) and more homogenous biofilm compared to that obtained using the standard protocol (Figure 1B) The biofilms produced using the new protocol show not only an higher biovolume (8.9 μm³/μm² vs 1.1 μm³/μm²) but also contain an higher percentage of viable cells, as
suggested by the different green/red ratio in Figure 1B and by the XTT cell viability assay (Figure S1). The same analysis using a representative non-biofilm forming strain, confirmed that CV values of 0.3-0.5 do not correspond to homogenous biofilms (Figure 1C). Overall, those results suggest that the newly developed protocol allows better discrimination between strong biofilm formers and non-biofilm forming strains.

**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 using a selection 366 *S. agalactiae* isolates belonging to 8 serotypes grown in THB (starting pH 7.8) without or with 1% glucose and in THB at pH 5.0 without supplemented glucose. Similar to what observed in Figure 1, no significant biofilm was detected in presence of THB at neutral pH for any of the isolates. On the other hand, both the use of an acidified medium (Figure 2A) and the addition of glucose at neutral pH (Figure 2B) resulted in a general increase in biofilm formation, with some of the isolates reaching very high CV values. In the absence of glucose, a 3-8 fold increase in the CV mean was observed for isolates belonging to all serotypes grown at pH 5.0 versus neutral pH.

The lower CVs observed at low pH conditions in the absence of glucose compared to pH 7.8 in presence of glucose are most probably due to slower bacterial growth at lower starting pH (Figure S2). However no significant differences in terms of growth rate were observed between biofilm forming strains and non-biofilm forming strains, in each the tested media (Figure S2).

As already reported for *S. pyogenes* (34), the production of organic acids associated with the metabolism of glucose could determine a pH decrease and be the direct cause of the observed effect on GBS biofilm formation in glucose rich media. To better investigate the kinetics of biofilm formation in the presence of glucose and at low pH, we carried out time-course biofilm assays using a representative GBS strong biofilm former and three different types of media (THB pH 7.8, THB pH 7.8 supplemented with 1% glucose and THB pH 5.0 without glucose). A significant increment
in the CV value (blue and red solid lines, Figure S3A) was observed already after 4.5-5 hours of incubation in THB pH 7.8 with 1% of glucose, in correspondence to a drop of pH to values lower than 5.0 (blue and red dotted lines, Figure S3A), or in THB pH 5.0 without glucose. On the contrary, no biofilm formation was observed after incubating the same strain in THB pH 7.8, where the culture pH never reached values below 5.0 (green dotted line, Figure S3A). A similar pH profile was detected also for a representative non-biofilm forming strain, in all tested media (Figure S3B).

To confirm that pH 5.0 is the signal sensed by the bacteria to start biofilm formation, a representative GBS biofilm former was grown in a nutrition-limited RPMI GlutaMAX medium, either in presence of 1% glucose or at pH 5.0. In this case differently from THB (Figure 3A), the pH of the culture did not drop below 5.0 in presence of glucose and no significant biofilm formation was observed. In contrast, significant biofilm formation (CV/OD600 of 3.6), was observed in presence of RPMI GlutaMAX pH 5.0 (Figure 3B). Moreover, GBS biofilm formers grown in THB supplemented with glucose and buffered with Tris- or Heps- at concentrations that do not affect the bacterial growth, showed significantly reduced biofilm formation in correspondence of final pH values higher than 4.5, in agreement with data obtained using RPMI (Figure S4).

Most GBS strong biofilm formers belong to the hypervirulent ST-17 lineage

Looking for a relationship between the different serotypes and biofilm production capacity, the data on the different isolates presented in Figure 2 were clustered on the basis of capsular serotype. The highest CV mean increase and the highest number of strong biofilm forming strains (CV>3) were found to belong to serotype III (Figure 2, Table 1), although not all type III strains appeared to be good biofilm formers. In fact, none of the two tested GBS type III strains for which the genome sequence was already available, COH1 and D136C, showed a significant ability to produce biofilm, neither at acidic pH nor in presence of glucose (Figure S5). Among the 156 type III tested isolates, 41 (26.4%) and 35 (22.4%) formed biofilms with CV values higher than 3 in presence of glucose or 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 2C and Figure 2D). More than 36% of the 91 ST-17 showed a CV>3 in presence of glucose or at low pH, while less than 6.1% of the 65 non-ST-17 showed CV>3 in the same conditions (Table 1). Also the CV mean and the mean increment obtained for the ST-17 strains were higher than those obtained for the serotype III while the values obtained for the non-ST-17 group were comparable to the other serotypes (Table 1), confirming a strong correlation between the hypervirulent ST-17 clone and GBS biofilm formation.

The possible relationship between the high biofilm formation and GBS virulence was further investigated by clustering the data presented in Figure 2 on the basis of the GBS strain origin. A higher frequency of strong biofilm formers was observed in the 85 neonatal isolates in comparison to the 272 of the colonizing group (18% versus 8%, P=0.12). This difference was even higher if the colonizing group is 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 high biofilm formers belong to serotype V, and of these, two were isolated in infected neonates.

**Role of capsule expression in GBS biofilm formation**

To verify the possible involvement of differential capsule expression during biofilm formation at low pH, the sialic acid content of 4 biofilm forming strains (2 type III ST-17 and 2 type V isolates) was estimated using Svennerholm’s method (35), in conditions activating GBS planktonic or sessile life-style. The amount of sialic acid produced by GBS grown at pH 5.0 was reduced by 50-60% compared with bacteria grown at pH 7.8 (Figure 4A), in agreement to results already reported in the literature (36). In the biofilm forming strains capsule reduction corresponded to increased CV values (Figure 4B), 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 ST-17 non-biofilm forming strain, (Figure 4), suggesting that the capsule reduction is not sufficient per se to induce biofilm formation in the entire ST-17 clone.

Proteins play a significant role in GBS biofilm formation and maintenance

To understand what type of low pH-induced factors could determine GBS biofilm formation and structural stability, biofilm inhibition and eradication in 6 strong biofilm forming strains (3 ST-17 expressing type III and 3 expressing type V capsular polysaccharide) were evaluated in the presence of proteinase K and DNase. The maximum concentration of proteinase K (200 μg/mL) and DNase (200 μg/mL) used in MBIC and MBEC determination does not affect the planktonic bacterial growth (MIC ≥ 200 μg/mL). An almost total inhibition and eradication of 24h-mature biofilms was observed for all tested strains in presence of 3 μg/mL of proteinase K (Figure 5). The same effect was observed also when the concentration of proteinase K was decreased to 0.4 μg/mL (Figure S6).

Addition of 200 μg/mL of DNase resulted in low inhibition and partial disruption of the biofilm, but the effect was weaker than that observed using proteinase K (Figure 5).

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, inducing bacterial adhesion and contributing to the biofilm structural stability.
Recent studies have demonstrated GBS biofilm formation in vitro (26-29) although the data regarding the effect of pH and media composition are controversial. In a recent study, Yueh-Ren et al. (37) found that low pH condition induced biofilm formation in nutrient-limited medium (M9YE) but not in THB. Borges S. et al. (27), Kaur et al. (28) and Yang Q. et al. (38) found that GBS produced a greater amount of biofilm at pH 6.5 than at pH 4.2 and Konto Ghiordi et al. (29) reported that only LB and RPMI 1640 supplemented with 1% of glucose produced uniform biofilm and not THB. Manetti et al. (39) observed that, in S. pyogenes, the presence of glucose resulted in auto acidification of the media and consequently biofilm formation. Rinaudo et al. (26) demonstrated that the presence of 1% of glucose in THB induces biofilm formation in GBS.

A major limitation of the static protocol used to screen biofilm formation in most studies is the absence of the fluid circulation encountered in the host. Konto Ghiordi et al. (29) showed that a protocol using low flow conditions was preferable 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 multi-well based protocols, we developed an in batch-in vitro protocol, containing a medium replacement step. The biofilm produced by strong biofilm forming strains using the new protocol was thicker and more homogeneous than that obtained using the standard protocol (26) (Figure 1). In contrast, weak or non- biofilm forming strains produced much reduced biofilms using this protocol (Figure 1). Applying the new protocol to a large number of GBS clinical isolates, we provide unequivocal evidence that acidic pH induces GBS biofilm formation in both a nutritionally rich environment (THB) and a limited environment (RPMI) (Figure 3).

These data clarify previous observations regarding the role of pH during biofilm formation by GBS and revealed, for the first time, a significant divergence between different GBS serotypes. Interestingly, the majority of the GBS strong biofilm formers belong to serotype III ST-17 strains suggesting a sequence type-biofilm correlation (Figure 2). A higher frequency of strong biofilm formers was also observed in the infective neonatal isolates rather than the colonizing group.
included in our strain collection. This result is clearly associated with the higher prevalence of ST-17 in our neonatal group (Figure 2). In fact, the ST-17 GBS lineage is highly prevalent in infected neonates compared to other lineages, particularly during late onset disease and is considered as a highly virulent clone (18;20;21;40). A high heterogeneity in the ability to produce biofilm was observed not only between serotypes but also within the very homogenous hypervirulent ST-17 clone (40;41) (Figure 2).

A general reduction of the capsule amount was observed for biofilm forming strains belonging to both serotype III and serotype V, in response to pH decrease (Figure 4). Although the capsule reduction correlated with increased biofilm formation of some but not all the ST-17 tested isolates, such as COH1 (Figure 4), the down-regulation of capsule at pH 5.0 is not sufficient per se to ensure biofilm formation in all ST-17 strains. If the presence of the capsule favors bacterial escape to complement mediated killing (42), in genital tracts with low pH, where capsule is down-regulated, the biofilm-lifestyle of ST-17 strains may represent an alternative strategy to guarantee their persistence.

Proteinase K, at concentration that does not affect the cell growth, inhibited biofilm formation and induced biofilm detachment (Figure 5), suggesting that proteins play the major role to promote bacterial adhesion and biofilm structural stability (Figure 5). These results suggest that acidic pH may be important to unmask surface-associated proteins promoting bacterial adhesion in biofilm forming strain. Alternatively, acidic pH may up-regulate the expression of some surface associated proteins specifically in the GBS biofilm formers. It was already shown that acidic pH to modulate the expression of a large number of proteins in GBS, including the proteins involved in surface adhesion (43;44). 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 (21;42;45;46). Our data suggest that the contradictory results reported about the effects of pH on GBS adherence to
epithelial cells (44;47-49), as those previously reported about the effects of pH on biofilm formation, could be due to the limited number of GBS strains tested and methods used. Further efforts will be necessary to: 1) understand if the effects of pH on biofilm formation, in different GBS serotypes, correlate to their ability to adhere to vaginal cells; 2) identify those proteins unmasked or differently regulated by low pH in strong biofilm formers and non-biofilm formers (e.g. COH1) belonging to ST-17 lineage. The identification of those proteins that promote cell adhesion in GBS biofilm forming strains will lead to better understand the mechanism of biofilm formation in GBS and allow designing new therapeutic approaches against this pathogen.

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Competing Interests
The authors declare that they have no competing interests.


Figure Captions

Figure 1. Comparison of biofilm formation protocols.

A) Biofilm formation ability of 4 strong biofilm forming strains and 4 weak/non-biofilm forming strains produced by the standard protocol and the new protocol. The GBS strains were grown in THB (pink, new protocol) and THB in presence of 1% glucose (blue, standard protocol and red, new protocol). Surface-attached cells were Crystal Violet stained and quantified by measuring the absorbance at 540 nm. The mean values of three independent experiments and standard deviation are shown. B) Confocal Laser Scanning Microscopy (CLSM) of biofilms formed by a strong biofilm forming strain, using the standard protocol (blue) and the new protocol (red) in THB in presence of 1% glucose. C) Confocal Laser Scanning Microscopy (CLSM) of the biofilm produced by a non-biofilm forming strain using the standard protocol (blue) and the new protocol (red) in THB in presence of 1% glucose. Biofilms were stained with LIVE/DEAD Viability Kit. Green and red colors indicate live- and dead-cells, respectively. Asterisks denote statistically significant difference determined by Student's t test, (***) $P < 0.001$ and (****) $P < 0.0001$.

Figure 2. Effect of acidic pH and glucose in GBS biofilm formation.

Biofilm formation ability of 366 GBS clinical isolates belonging to 8 different serotypes and non-typeable strains grown in A) THB (green dots) and THB pH 5.0 (red dots) B) THB (green dots) and THB supplemented with 1% of glucose (red dots). On the right, panels C and D, focus on serotype III strains grouped in Sequence type ST-17 and non-ST-17. Biofilm formation was evaluated using Crystal Violet stain measuring the absorbance at 540 nm. Each dot represents the mean value of three independent experiments performed for each isolates. Asterisks denote statistically significant difference, determined by Univariate Analysis of Variance (ANOVA), (*) $P < 0.05$ (**) $P < 0.01$, (***) $P < 0.001$ and (****) $P < 0.0001$. 
Figure 3. **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 stain measuring the absorbance at 540 nm. 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 of three independent experiments and standard deviation are shown. Asterisks denote statistically significant difference, determined by Student’s $t$ test, (****) $P < 0.0001$.

Figure 4. **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 (gray) and pH 5.0 (black). 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 unencapsulated COH1 was used as a control. The mean values of three independent experiments and standard deviation are shown. Asterisks denote statistically significant difference, determined by Student’s $t$ test, (***$) P < 0.001$ and (****$) P < 0.0001$.

Figure 5. **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 measuring the absorbance at 540 nm. The mean values of three independent experiments and standard deviation are shown.
A

![Graph showing saccharide levels](image)

- **COH1Δcapsule**
- **COH1**
- **1**
- **2**
- **3**
- **4**

**Serotype III (ST-17)**

**Serotype V**

B

![Graph showing Crystal Violet levels](image)

- **COH1Δcapsule**
- **COH1**
- **1**
- **2**
- **3**
- **4**

**Serotype III (ST-17)**

**Serotype V**
Table 1. Effect of acidic pH and glucose biofilm formation on different GBS serotypes.

GBS biofilm formation was quantified by Crystal Violet (CV) staining. Note that the percentage of biofilm formers with a CV value >3 is referred to the number of strains of each serotype (e.g. 156 for serotype III).