

Effect of Serum Proteins on Planktonic Growth and Biofilm Formation of *Helicobacter pylori* in vitro

Tovar Robles Clara Lourdes¹, Romo Lozano Yolanda¹, Cervantes García Daniel^{1,2}, González Segovia Rodolfo^{1,*}

¹Universidad Autónoma de Aguascalientes, Aguascalientes, México

²Consejo Nacional de Humanidades, Ciencias y Tecnologías, Ciudad de México

Abstract

The growth of the gastric bacteria *Helicobacter pylori* can occur both planktonic and by developing biofilm (BF). Fetal calf serum (FCS) is used for *in vitro* growth of *H. pylori* in the liquid medium but is also considered in protocols for BF formation. However, evidence suggests that high percentages of FCS may impair *H. pylori* BF formation. The study focused on assessing the effect of protein saturation of FCS on *H. pylori* BF formation *in vitro*. A clinical isolate of *H. pylori* characterized genotypically by PCR as *vacA s2/m2*, *cagA*⁺ was used. Bacterial growth and BF formation were measured spectrophotometrically in Brain-Heart-Infusion (BHI) and Brucella-Broth (BB), alone or in combination with β -Cyclodextrin (β -CD) and FCS. The effect of different percentages of FCS (0, 2.5, 5.0, and 7.5%) and bovine serum albumin (BSA) at equivalent protein concentrations on BF formation was evaluated. The results indicated that the best planktonic growth occurred in BB, with a 4-fold increase compared to BHI when β -CD/FCS was used and that this growth was also dependent on the concentration of FCS or BSA used. BB medium was also the most suitable for BF generation; however, increasing FCS or BSA concentrations proportionally inhibited its formation. It is concluded that *in vitro* serum protein saturation generates a steric effect that inhibits BF structuring, using a *cagA*⁺ *H. pylori* strain with a lower virulence version of the *vacA* gene (*s2/m2*), establishing that the absence of serum improves BF formation but decreases planktonic growth.

Keywords: *H. pylori*, biofilm, *in vitro*, FCS, serum, inhibitory effect.

Резюме

Растежът на *Helicobacter pylori*, бактерия която живее в стомаха, може да се случи както планктонно, така и чрез развитие на биофилм (БФ). Фетален телешки серум (ФТС) се използва за *in vitro* растеж на *H. pylori* в течна среда, но се разглежда и в протоколи за образуване на БФ. Въпреки това, данните сочат, че високите проценти на ФТС могат да нарушат образуването на биофилми от *H. pylori*. Проучването се фокусира върху оценката на ефекта от насищането с протеини на ФТС върху образуването на биофилми от *H. pylori* *in vitro*. Използван е клиничен изолат на *H. pylori*, генотипно характеризиран чрез PCR като *vacA s2/m2*, *cagA*⁺. Бактериалният растеж и образуването на БФ са измерени спектрофотометрично в Brain-Heart-Infusion и Brucella-Broth, самостоятелно или в комбинация с β -циклодекстрин (β -CD) и ФТС. Оценен е ефектът от различни проценти ФТС (0, 2.5, 5.0 и 7.5%) и говежди серумен албумин при еквивалентни протеинови концентрации върху образуването на БФ. Резултатите показват, че най-добър планктонен растеж се наблюдава в Brucella-Broth, с 4-кратно увеличение в сравнение с Brain-Heart-Infusion, когато е използван β -CD/ФТС, и че този растеж зависи от използваната концентрация на ФТС или говежди серумен албумин. Средата Brucella-Broth е най-подходяща за генериране на БФ. Въпреки това, увеличаването на концентрациите на ФТС или и говежди серумен албумин пропорционално инхибира образуването му. Заключение е, че *in vitro* насищането на серумните протеини генерира стеричен ефект, който инхибира структурирането на БФ, използвайки щам *cagA*⁺ *H. pylori* с версия на *vacA* гена с по-ниска вирулентност (*s2/m2*), което установява, че отсъствието на серум подобрява образуването на БФ, но намалява растежа на планктона.

*Corresponding author: rgonzals@correo.uaa.mx
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Introduction

Helicobacter pylori is a microaerophilic, Gram-negative, slow-growing, helical-shaped bacterium with abundant flagella. *H. pylori* infection is a risk factor for chronic gastritis and peptic ulcer. It affects 50% of the world's population and is recognized as a class I carcinogen by the World Health Organization (WHO) (Zeng *et al.*, 2022).

The pathogenicity mechanisms of the bacteria have been extensively studied, with the CagA and VacA molecules standing out as important virulence factors. The CagA protein is associated with the pathogenicity island *cagPAI* and is considered a marker of the most virulent *H. pylori* strains (Takahashi-Kanemitsu *et al.*, 2020). It has been shown that the translocation of CagA to gastric cells induces various alterations such as increased cell proliferation, IL-8 expression, and cell elongation, among others (Ansari *et al.*, 2019). The VacA molecule is identified as an important cytotoxin that causes endosomal osmotic disorders by inducing the formation of cytoplasmic vacuoles in gastric cells (Sharndama *et al.*, 2022). Although this pathogenicity factor is constitutive of all *H. pylori* strains, its gene presents high genetic variability with important polymorphisms in the “s” region (amino-terminal signal sequence) and the “m” region (middle region of the p33 domain of the toxin) that generate the *vacA s1/s2* and *vacA m1/m2* subfamilies which combine to originate variants of the VacA toxin with different vacuolating activity (Ansari *et al.*, 2019).

As occurs in various bacterial species, *H. pylori* growth can develop both in a planktonic form and in a biofilm (BF) state (Yonezawa *et al.*, 2013). The planktonic form is a free-floating state in which the bacteria is able to move, allowing its dispersion and colonization of new sites. In some bacteria, planktonic growth may be strongly related to components present in the medium in which the microorganism develops. In the bacterium *Prevotella intermedia*, the steroid hormone estradiol regulates planktonic growth (Fteita *et al.*, 2014). The ability of *H. pylori* to develop BF has been fully described *in vitro* and *in vivo* in human gastric mucosa (Hessey *et al.*, 1990; Azevedo *et al.*, 2006). Due to the relevance of BF as a potential mechanism of pathogenicity, its study has increased in recent years (Fauzia *et al.*, 2024). BF are defined as microbial communities adhered to living or abiotic surfaces through a matrix of extracellular polymers composed of polysaccharides (EPS), lipids, nucleic acids, and proteins (Liu *et al.*, 2023). The

extracellular polymers of the BF offer protection to resident microorganisms by limiting the entry of toxic and biocidal molecules and also allowing nutrients to be concentrated and distributed (Rather, *et al.*, 2021). Bacterial cells in BF are phenotypically and metabolically heterogeneous. In the case of *H. pylori*, the presence of two morphological states can be distinguished: spiral forms and coccoid forms. Spiral forms are live, culturable, and have a strong colonization capacity while coccoid forms are mainly associated with viable non-culturable states (VBNC), which represent persistent forms of bacteria (Krzyk *et al.*, 2018). The *H. pylori* BF is characterized by a reduced metabolic activity that is associated with quorum-sensing processes induced by molecules such as the N-acyl homoserine lactone Al-2 generated from the expression of the *luxS* gene. (Yonezawa *et al.*, 2013; Percival *et al.*, 2014; Anderson *et al.*, 2015).

The formation of BF is a complex process that requires multiple steps, with reversible adhesion to a surface being the first key process for a planktonic microorganism to initiate the formation of this structure (Rather *et al.*, 2021). Among the different adhesion mechanisms that have been identified in *H. pylori*, outer membrane proteins (OMPs) play an important role in abiotic cell-to-cell or cell-to-surface adhesion processes (Yonezawa *et al.*, 2009). The *H. pylori* OMP AlpB is strongly associated with adhesion processes and has been identified as an important contributor to BF formation (Yonezawa *et al.*, 2017). The *H. pylori* flagellum has been shown to promote BF adhesion in addition to its motility functions, allowing anchoring that maintains the architecture of this structure (Elshenawi *et al.*, 2023).

In vitro, BF formation can be developed by growth in nutrient-rich culture media commonly used for the planktonic growth of the bacteria, but considering the incorporation of high carbon sources for the biosynthesis of exopolysaccharides, being β -cyclodextrin and glucose commonly used supplements (Stark *et al.*, 1999). Fetal calf serum (FCS) is a component that significantly contributes to the growth of the planktonic state of *H. pylori* in liquid media, but in different percentages, it is frequently considered in the protocols for BF formation (Attaran *et al.*, 2017). However, there is evidence suggesting that FCS at high concentrations affects the formation of *H. pylori* BF (Yonezawa *et al.*, 2009). Furthermore, it has been demonstrated that serum has a significant dose-dependent inhibitory effect on the adherence capacity of *H. pylori* to

various abiotic surfaces, a situation that could alter the development of BF (Williams *et al.*, 2008).

Using a genotypically characterized *H. pylori* isolate, the present study focused on the assessment of the effect of serum protein saturation on the formation of the BF of this bacterium, seeking to establish adequate conditions for the formation of this structure *in vitro*.

Materials and Methods

Culture of H. pylori, bacteria characterization, and cagA/vacA genotyping

A clinical isolate of *H. pylori* previously obtained in the laboratory identified as A97 was used. The bacteria were grown in agar base (BD Bioxon 21728) with 10% defibrinated sheep blood, at 37 °C under microaerophilic conditions. The identity of bacteria was confirmed by Gram staining, biochemical tests (urease and catalase), as well as PCR to identify the *ureC* (phosphoglucosamine mutase, *glmM*) gene (Fig. 1 and Table 1) (Osman *et al.*, 2015).

Additionally, by PCR, the virulence gene *cagA* was identified (Wagih *et al.*, 2015) and genotyping of the signal region (*s*) and middle region (*m*) of the *vacA* gene was performed using pairs of previously reported oligonucleotides (Table 1) (Atherton *et al.*, 1995). Bacterial DNA used in PCR analyses was obtained using the following protocol: bacteria were digested with lysis buffer (5% SDS, 0.15% Tris HCl, and 0.5M EDTA pH 8.0) and 0.4 mg/ml of proteinase K (Invitrogen cat. 25530) in a water bath at 60 °C overnight. Subsequently, DNA

extraction was carried out with 500 µl saturated phenol (Invitrogen cat. 15513) and the sample was centrifuged at 14,000 rpm. The aqueous phase was mixed with 500 µl of chloroform/isoamyl alcohol (24:1) (Sigma-Aldrich, St. Louis, MO, USA), and was centrifuged at 14,000 rpm. The DNA contained in the aqueous phase was precipitated with 500 µl of isopropanol (Sigma), and the pellet obtained by centrifugation was washed with 70% ethanol (Sigma). The extracted DNA was adjusted to a concentration of 100 ng/ml in double-distilled water. The PCR reactions were carried out with Master Mix K0171 (Thermoscientific), primers were obtained from T4oligo (ADN Sintético SAPI de CV), and the working concentration used was 0.05 pmol/ml of reaction. The amplification conditions were: (1) initial denaturation for 2 min at 95°C, (2) 40 cycles of 95°C for 1 min, 60°C for 40 s, and 72°C for 1 min, and (3) final elongation for 2 min (72°C). An endpoint thermal cycler (3Primebase, Techne Scientific Ltd) was used. In the amplification of *vacA* and *cagA* genes, the alignment was carried out at 54°C. Identification of amplification products was performed by electrophoretic separation in 2.5% agarose gels in Tris-borate-EDTA (TBE) solution and staining with ethidium bromide 1 µg/mL (JT Baker). The result of the genotypic characterization of the clinical isolate A97 is described in Fig. 1.

Bacterial growth quantification and BF in vitro formation

Bacterial growth and BF formation were carried out in 24-well culture plates (Celltreat) from

Table 1. Primer sequence for *glmM* genes and virulence genes *vacA* and *cagA* in *H. pylori*

Gene	Name	Sequence (5'-3')	Amplicon
<i>glmM</i>	<i>ureC</i> F	CTTTCTTCTCAAGCGGTTGTC	204 nt
	<i>ureC</i> R	CAAGCCATCGCCGGTTTATGC	
<i>vacA s1/s2</i>	VA1F	ATGGAAATACAACAAACACAC	s1: 259 nt
	VA1R	CTGCTTGAATGCGCCAAAC	s2: 286 nt
<i>m1</i>	VA3F	GGTCAAAATGCGGTCATGG	290 nt
	VA3R	CCATTGGTACCTGTAGAAAC	
<i>m2</i>	VA4F	GGAGCCCCAGGAAACATTG	352 nt
	VA4R	CATAACTAGCGCCTTGAC	
<i>cagA</i>	<i>cagA</i> F D008	ACAATGCTAAATTAGACAACCTTGAGCGA	298 nt
	<i>cagA</i> R R008	TTAGAATAATCAACAAACATCACGCCAT	

*Pairs of sense/antisense oligonucleotides (F/R) for the amplification of PCR are indicated. Differentiation of the genotypes of signal region (*s1/s2*) and middle region (*m1/m2*) of *vacA* gene is carried out according to indicated amplicon size.

bacterial suspensions adjusted to 1.5×10^6 bacteria/ml, using the McFarland scale turbidity standard. Suspensions were obtained from 72 h bacterial cultures on agar plates (BD Bioxon 21728) with 5% defibrinated sheep blood, incubated at 37°C under microaerophilic conditions, and harvested with a swab. BHI (BD Difco 211200) and BB (BD BBL) culture media were considered. The final volume per well was 1000 µl, with the following components: culture medium (BHI or BB), adjusted bacterial suspension (20 µl), with and without heat-inactivated FCS (Sigma Aldrich F2442) at 5%, 0.1% β-CD (Sigma C-4767) and 0.3% glucose (Chemical Technique 1895). Also, 0.4% of selection antibiotics for *H. pylori* (Selective supplement DENT, Oxoid SR0147E) were added. Culture plates were incubated at 37°C in a microaerophilic environment for up to 5 d (120 h). For each combination of components, three independent tests were carried out.

Bacterial growth quantification was determined from culture supernatant in each well at 72 and 120 h using spectrophotometric measure at 600 nm (Jenway 7305, Cole Parmer, USA). The corresponding culture medium (BHI or BB) with supplements (β-CD, FCS, glucose, and selection antibiotic) without bacteria was used as a calibration target for the equipment.

Identification and quantification of BF

BF formation in the culture plate was quantified based on the protocol proposed by O'Toole (2011). The supernatant was discarded and the BF was gently washed twice with phosphate-buffered saline (PBS, 1.75 mM NaH₂PO₄, 8 mM Na₂HPO₄, and 75 mM NaCl and pH 7.2). Then, 125 µl of crystal violet (Karal, cat. 7006) at 0.1% in water was added to each well. The dye was incubated for 15 min and then rinsed with distilled water 3 times. The plate was left to dry overnight. Subsequently, to solubilize the dye retained by BF at the bottom of the wells, 1.0 ml of acetic acid (JT Baker cat. 9507-05) at 30% in water was added and incubated at room temperature for 15 min. Spectrophotometric measurements were carried out at 550 nm, using 30% acetic acid as a calibration blank. Optical density (OD) indicated the formation of BF.

FCS and bovine serum albumin in different concentrations

To know the effect of FCS on BF formation, experiments were carried out at 120 h, in a 24-well microplate with different concentrations of FCS: 0, 2.5, 5.0, and 7.5 % (v/v) in 1000 µl of BB medium supplemented with β-CD at 0.1%, glucose at 0.3%,

selection antibiotic for *H. pylori* (Selective supplement DENT) at 0.4%, and 20 µl of the bacterial suspension (obtained according to previously mentioned procedure). In addition, tests were performed with bovine serum albumin (BSA) (Sigma A2153), as a protein supplement with characteristics similar to FCS. Albumin concentrations of 0, 0.10, 0.20 and 0.30% were used, equivalent to the protein content present in the different percentages of FCS used. Three independent tests were conducted.

BF in vitro visualization in scanning electron microscopy (SEM)

SEM analysis was performed from the BF formed at the bottom of the microplate wells under each condition previously mentioned. The culture supernatant was gently discarded and then two washes with PBS were performed. Subsequently, 1 ml of 2.5% glutaraldehyde was added to each well and incubated for 3 h at room temperature, fixation solution was removed and a wash with PBS was performed. After, the BF was dehydrated with increasing concentrations of ethanol (50–100%). The bottom of the wells is carefully cut with a curved knife. Subsequently, the samples were brought to a critical drying point with liquid carbon dioxide at 99% (Samadri-PVT-3D, TOUSIMIS Research Corporation, Rockville, MD, U.S.A.). The samples were coated 100 Å with gold (Denton Vacuum Desk II, NJ, U.S.A) and analyzed in a scanning electron microscope (JEOL, JSM-5900LV, Musashino, Tokyo, Japan).

Statistic analysis

Statistical analyses were performed in Minitab® 19.1.1 software. For growth kinetics and BF formation kinetics analysis. Student's t-tests were performed. For BF *in vitro* development and growth of bacteria, analysis of variance (ANOVA) and comparison of means by Tukey were performed. A significance level of $\alpha=0.05$ was used in all analyses. Graphics were made by Prism 10.2.3.

Results

Identification and genotyping of the H. pylori strain

Characterization of the bacteria confirmed its characteristic staining and morphology (Gram-negative and bacilli with some coccoid forms) (Fig. 1A). Biochemical tests were also positive for both catalase and urease (Fig 1B). Molecular tests showed the presence of the *ureC* gene confirmed the species *Helicobacter pylori* (Fig. 1C), and a band for the *cagA* pathogenicity gene was also present

(Fig. 1D), identifying strains associated with the development of gastric cancer. The strain studied had an *s2/m2* genotype for the VacA cytotoxin (Fig. 1D), which is associated with the ability to form vacuoles and is present in all strains.

H. pylori bacterial growth quantification

Bacterial multiplication was analyzed using

growth kinetics from day 2 to day 5, finding that in both media used, bacterial population showed logarithmic growth up to five days of culture, since it increased according to time of culture elapsed (Fig. 2A). With optical density values of 0.067 ± 0.010 on day 2 to 0.378 ± 0.035 on day 5 for BHI, and 0.111 ± 0.012 to 0.625 ± 0.048 for BB, growth of

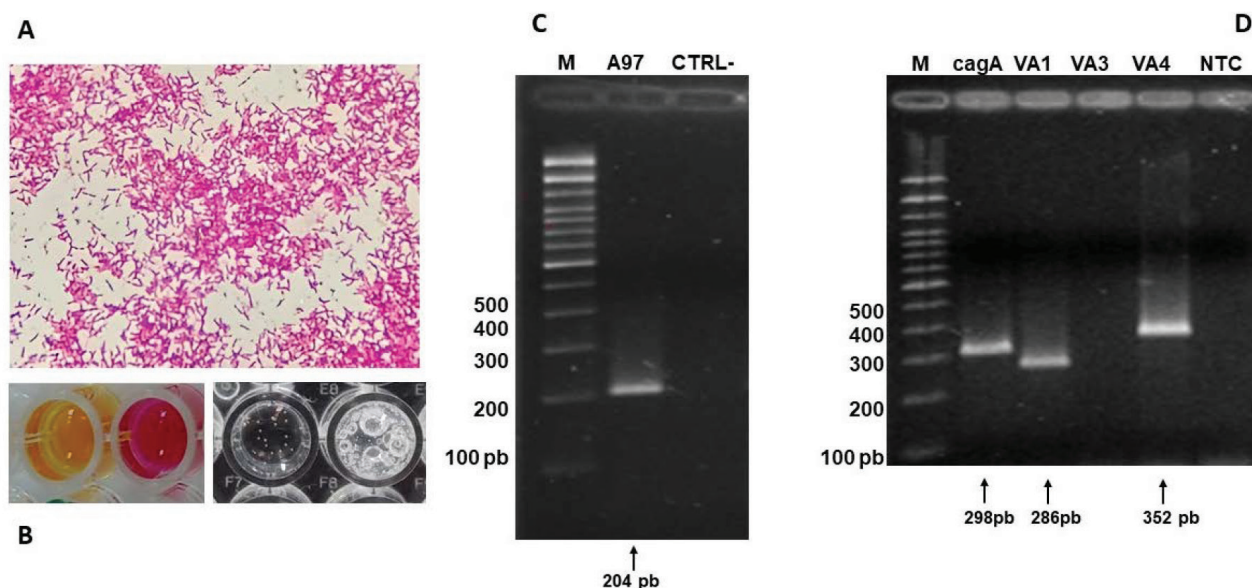


Fig. 1. Characterization tests for the *H. pylori* species of clinical isolate A97. (A) Gram stain Gram-negative bacteria of spirillar form and some coccoid forms. Magnification 100X. (B) Enzymatic tests for urease (left) and catalase (right) positive. (C) PCR amplification of *ureC* gene (204 bp). Weight marker (M) indicated; DNA from clinical isolate (A97); negative control (CTRL-) DNA from a culture of *Lactobacillus rhamnosus*. (D) Characterization by PCR of *cagA* and *vacA* genes in DNA of isolate A97. Virulence gene *cagA* (298 bp) and fragments VA1 with 286 bp corresponding to the *s2* signal region and VA4 with 352 bp corresponding to middle *m2* region are identified. According to the analysis performed, clinical isolate A97 was identified as *H. pylori cagA*⁺, *vacA s2/m2*.

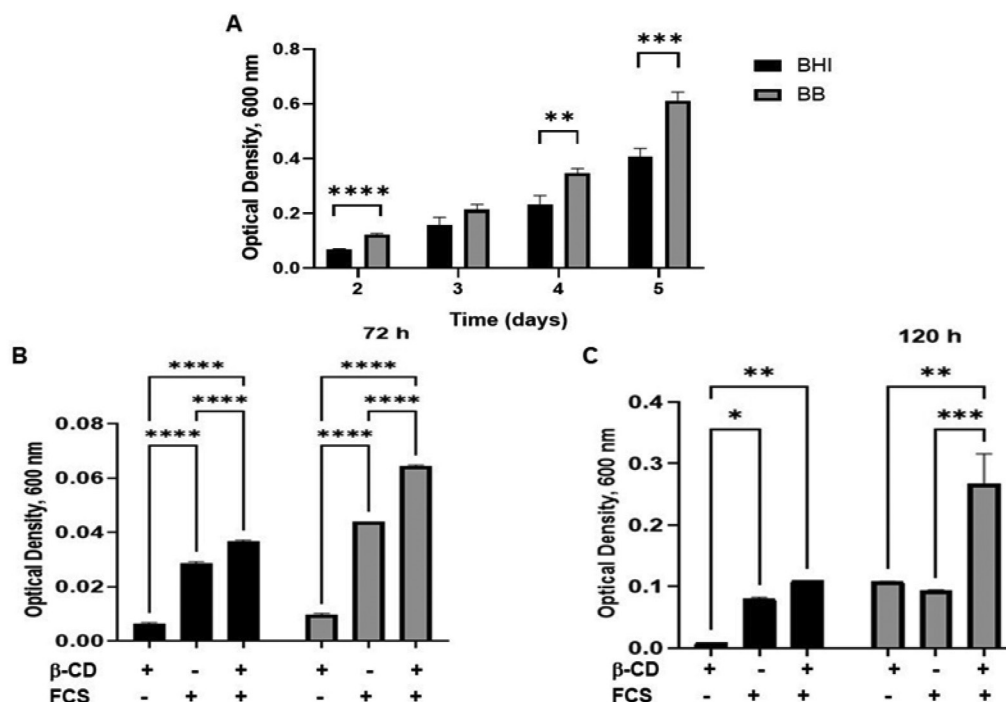


Fig. 2. Spectrophotometric analysis of *H. pylori* planktonic growth using BHI or BB. (A) Growth kinetics considering 2, 3, 4 and 5 days with the addition of b-CD 0.1% and FCS 5%. Growth analysis at 72 h (B) and 120 h (C) evaluating the absence (-) or incorporation (+) of both b-CD and FCS. Each condition was performed in triplicate. Error bars represent the standard error for each average value. Student's t tests were performed in (A) and one-way ANOVA with Tukey's *post hoc* test in (B) and (C) ($P \leq 0.05^*$, $P \leq 0.01^{**}$, $P \leq 0.001^{***}$ and $P \leq 0.0001^{****}$).

bacteria was always higher for the BB medium, with values greater than 1.5 times compared to the BHI medium at most of the analyzed times (days 2, 4 and 5) (Fig. 2A).

Subsequently, the effect of 5% FCS on the growth of *H. pylori* in both media with or without β -CD was evaluated. Bacterial growth was significantly increased when both supplements were added to the culture media, compared to the individual addition of FCS or β -CD (Fig. 2B and 2C). In 72-h cultures, the absence of FCS was related to very low growth, obtaining values less than 0.01 of optical density in both media (OD of 0.006 ± 0.001 and 0.009 ± 0.001 in BHI and BB, respectively) (Fig. 2B).

Additionally, bacterial growth at 72 h in BB medium was twice that in BHI in all culture conditions. At 120 h of culture, the presence of FCS in the BHI medium considerably increases OD (growth) with respect to β -CD, but is lower than that of the culture including both supplements (Fig. 2C). The supplements added individually to the BB medium generated an equivalent growth, but the simultaneous incorporation of both supplements allowed significantly greater growth (approximately 4 times) than the individual application of β -CD and FCS ($p \leq 0.05$). This indicates a possible synergism of β -CD and FCS that also allows to extend the logarithmic growth of the bacteria to 120 h of culture (Fig. 2C).

Identification and quantification of BF

In the generation of BF, the conditions evaluated in bacterial growth analysis were also used. BF formation was significantly increased when only

β -CD was included in both, the BHI and BB medium (0.019 ± 0.007 and 0.54 ± 0.003 OD respectively). The addition of 5% FCS in both media caused a significant reduction in BF formation. In the case of BB medium, an 80% decrease in BF was identified when FCS was added compared to the culture with only β -CD (0.545 ± 0.003 vs. 0.110 ± 0.003 OD, treatment with FCS and β -CD respectively). While in the BHI medium, a decrease of approximately 44% of BF was identified when the medium is supplemented with FCS and β -CD, compared to the addition of only β -CD (0.300 ± 0.006 vs. 0.545 ± 0.003 OD, treatment with FCS or β -CD respectively). These results indicate a possible inhibitory effect of FCS on BF formation, a phenomenon contrary to the bacterial growth-promoting effect of serum previously identified (Fig. 3A).

After, BF kinetics was compared between both culture media, BHI and BB, under optimal conditions of bacterial growth. Although BF development showed an increment at each culture time with both culture media, in BB media, BF was significantly increased since 2 d. The difference in BF formation with BB was more marked on the fifth day since a 2-fold increase was observed when compared to BHI (2.062 ± 0.273 vs. 1.037 ± 0.186 OD) (Fig. 3B).

Effect on growth and formation of BF under different concentrations of fetal calf serum

In order to confirm the inhibitory activity of serum on BF formation, a dose-response curve was performed with different concentrations of FCS (0, 2.5, 5.0, and 7.5 %), BF formation and the growth of the bacteria were determined. The high-

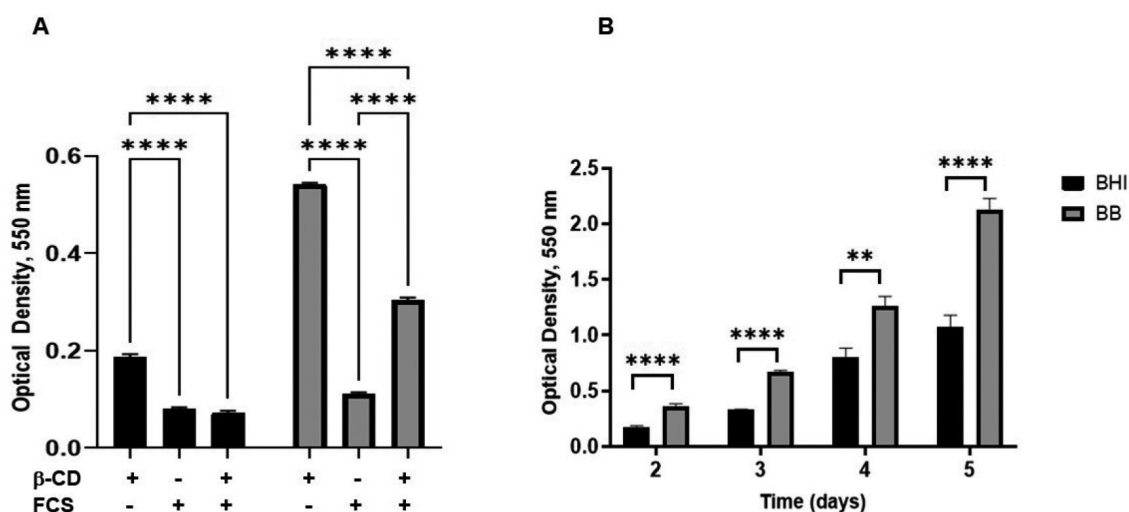


Fig. 3. Spectrophotometric analysis of BF formation in BHI and BB media. (A) Addition (+) or absence (-) of both β -cyclodextrin (β -CD) and 5% fetal calf serum (FCS) is indicated. BF quantification was performed after 72 h of culture. (B) Kinetics for BF formation comparing BHI medium vs. BB. Each condition was performed in triplicate. Error bars represent standard deviations for each average value. In (A) one-way ANOVA with Tukey's *post hoc* test was performed. In (B) comparison of means using Student's *t* test is indicated ($P \leq 0.05^*$, $P \leq 0.01^{**}$, $P \leq 0.001^{***}$ and $P \leq 0.0001^{****}$).

est OD-BF formation was obtained in the absence of FCS (1.057 ± 0.072) and as the concentration of FCS increased, the development of BF decreased, 7.5% serum caused the lowest BF formation (0.032 ± 0.001) (Fig. 4A). In contrast, the growth of the bacteria was increased in an FCS concentration-dependent manner. Then, the lowest growth of bacterial multiplication was observed in the absence of serum, and the incorporation of 7.5% FCS led to an increase of around 8 times concerning the growth observed without FCS (absorbance 2.756 ± 0.101 vs. 0.321 ± 0.001 , respectively) (Fig. 4A).

In order to identify a possible relation between the inhibitory effect of FCS and the concentration of proteins, a test was carried out where the effect on the formation of BF and bacterial growth with BSA was evaluated at concentrations equivalent to those added in FCS in terms of protein concentration. Greater BF formation was obtained in the absence of BSA and growth from 0.758 ± 0.008 OD without FCS to 4.276 ± 0.041 with 7.5% (Fig. 4B).

BF in vitro visualization in scanning electron microscopy (SEM)

Finally, using SEM, different percentages of FCS on the formation of the BF effect, were observed. It was identified that, as the percentage of FCS decreased, there was an increase in the generation of the BF structure. In the absence of serum, the highest BF structuring was achieved, which was decreased after FCS application (2.5%) and maintained a low formation in the presence of higher

percentages of serum (5 and 7.5%) (Fig. 5A, 5B, 5C and 5D).

In the absence of FCS, a varied morphology of the bacteria could be distinguished within the BF that included coccoid forms and spiral bacillus, identifying the presence of flagella in some bacilli. BF by SEM was identified as the presence of irregular structures that are different from a cellular morphology and in which, in addition, different bacterial forms are immersed (Fig. 5E).

Discussion

Genotypic characterization of the clinical isolate used in this study determined that it corresponds to a *cagA*-positive *H. pylori* strain. Pathogenicity factor CagA is responsible for the induction of proinflammatory responses in the gastric mucosa and the induction of carcinogenesis (Šterbenc *et al.*, 2019). Evidence suggests that the CagA protein participates in processes such as biofilm formation, by interacting with the *luxS* gene (Fauzia *et al.*, 2023) and efflux pump function (Karbalaei *et al.*, 2022). Then, the expression of the *luxS* gene is related to mechanisms of adhesion, motility (Lucio-Sauceda *et al.*, 2019) and promoted *H. pylori* biofilm dispersion in which bacteria migrate and adhere to microcolonies (Elshenawi *et al.*, 2023). On the other hand, the virulence gene *vacA* was characterized in the strain as *s2/m2*. All *H. pylori* strains have the *vacA* gene, and the *vacA* *s2* variant is considered less pathogenic than *s1* (Šterbenc *et al.*, 2019)., in turn, the *m2* allele is considered non-cytotoxic (Castillo-Rojas *et al.*, 2004). The working strain for

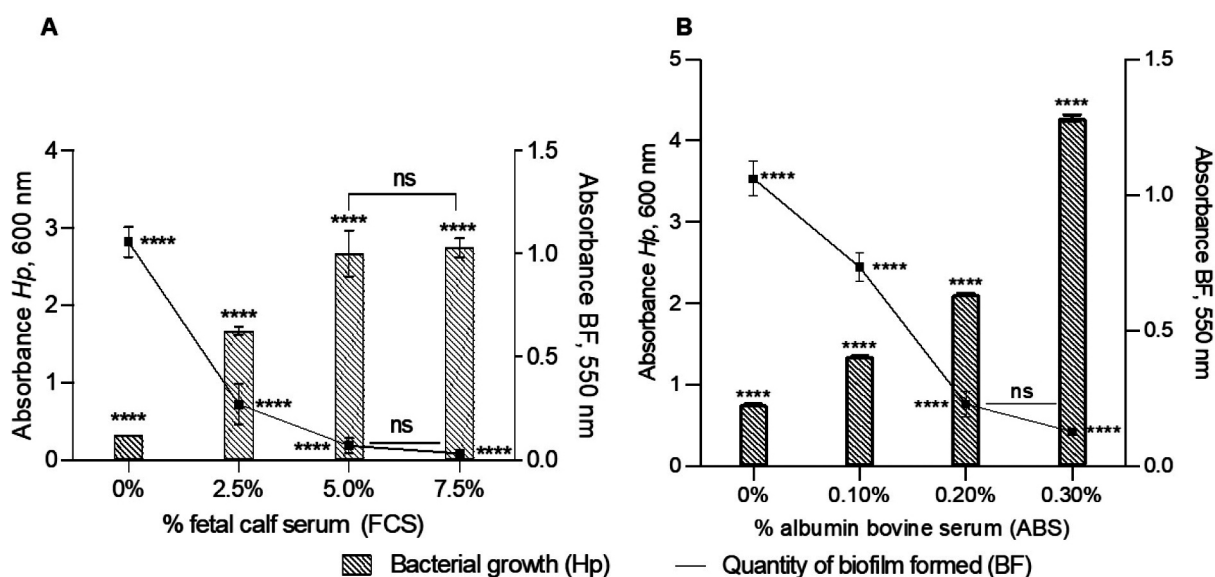


Fig. 4. Effect of FCS and bovine serum albumin concentration on biofilm growth and formation. (A) Different concentrations (0, 2.5, 5.0 and 7.5% v/v) of FCS were tested. Cultures were carried out for 5 days of incubation in BB medium with 0.3% glucose and 0.1% β -CD). (B) FCS was replaced by bovine serum albumin (BSA) at the same concentration in terms of protein content (equivalent to 0, 0.10, 0.20 and 0.30%). Each condition was performed in triplicate. Error bars represent the standard error for each average value. One-way ANOVA was performed with Tukey's *post hoc* test ($P \leq 0.05^*$, $P \leq 0.01^{**}$, $P \leq 0.001^{***}$ and $P \leq 0.0001^{****}$).

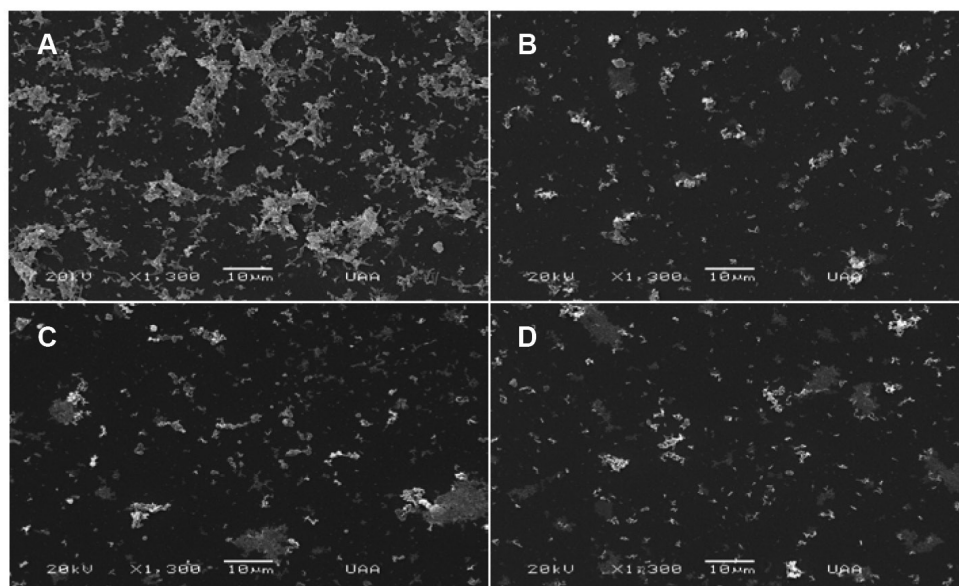


Fig. 5. SEM analysis of effect of FCS on *Helicobacter pylori* biofilm formation. Micrographs (1300X magnification) of BF formed *in vitro* culture in BB and application of 0, 2.5, 5.0 and 7.5% FCS (A, B, C and D respectively). As the concentration of FCS increases, the structuring of the BF decreases.

experiments carried out in this article was characterized as *s2/m2* and showed the ability to develop BF, which represents an effective strategy in antibiotic resistance by *H. pylori*. Although Karbalaee and collaborators state that strains of the *vacA s1m1* genotype show a greater capacity for BF formation (Karbalaee *et al.*, 2022).

According to Krzyżek and collaborators since 1999, knowledge about BF formation by *H. pylori* has expanded significantly, and the presence of *H. pylori* BF has been demonstrated in studies using plastic or glass surfaces, cell cultures, gastric biopsies of animals or humans, and even plant surfaces or water (Krzyżek *et al.*, 2020). Various *H. pylori* strains have shown the ability to develop BF *in vitro* on polystyrene support of the microplate (Elshenawi *et al.*, 2023). The strain used in this study generated BF formation adequately on this type of material.

Common liquid media for culturing *H. pylori* start with a complex base containing animal tissue extracts and include media such as BB, BHI broth, Mueller-Hinton broth, or Columbia broth. Although previous studies do not establish a consensus on conditions and media that are particularly advantageous over others in terms of bacterial growth (Bessa *et al.*, 2013, Molina-Castro *et al.*, 2022), in the experiments carried out in our laboratory, BB was better not only for planktonic growth but also for BF formation with respect to BHI. Both BHI and BB media are used for the growth of demanding microorganisms, being used interchangeably for *H. pylori*, although BB is recommended for subcultures and strains from clinical isolates (Yo-

kota *et al.*, 2022). Furthermore, for better growth, it is reported that the addition of supplements is required, such as fetal bovine serum, β -CD, horse serum, BSA, and yeast extract as additional carbon sources (Bessa *et al.*, 2013; Hathroubi *et al.*, 2018).

Planktonic growth *in vitro* was considerably improved by the addition of FCS, which is the most used in most/many studies (Shibayama *et al.*, 2006). Even, it is known that *H. pylori* cells cultured in a cholesterol-free medium adsorb cholesterol from serum (Ansorg *et al.*, 1992). In the tests carried out in our laboratory, simultaneous use of FCS and β -CD supplements allowed greater development of the bacteria, by extending exponential or log phase of growth.

At 120 h there was significantly greater growth than when using β -CD and FCS separately. β -CD, for its part, is a cyclic oligosaccharide that when added to the medium would favor the growth of the bacteria as a source of carbon and energy, although in addition and according to a study with *Pseudomonas aeruginosa* by Berkl and collaborators, β -CD interacted with N-hexanoyl homoserine lactones, which is the quorum-sensing signals produced in Gram-negative bacteria and therefore influences the activation of BF formation genes, such as *luxS*, *flaA*, *omp18*, and *lpxD* (Lucio-Sauceda *et al.*, 2019; Berkl *et al.*, 2022).

In contrast to bacterial growth, BF formation was affected by the presence of FCS since when it was removed or decreased in media, increased its formation, as observed in the experiments carried out with different concentrations of serum in BB. In previous works, it was found that *H. pylori*

form significantly more BF in BB than in BHI and Ham's, although in this case FCS was added at 2% (Bessa *et al.*, 2013).

In our experiments was found that, in coincidence with Hathroubi and collaborators the serum is used to enrich liquid media, promoting the planktonic growth of *H. pylori* (Hathroubi *et al.*, 2018). However, it seems that the use of rich and complex media does not favor BF *in vitro* formation since they do not necessarily reflect the natural environment in which *H. pylori* develops.

The inhibitory activity of serum to reduce BF formation has been studied in bacteria such as *Staphylococcus aureus*. In this bacterium, a serum component has been identified that causes transcriptional alteration of genes related to BF biosynthesis (Abraham and Jefferson, 2010). However, it has also been shown that serum proteins such as lactoferrin, apotransferrin, and albumin inhibit the adhesion and aggregation of *S. aureus*, an important step in the structuring of bacterial BF (Ardehali *et al.*, 2002; Singh *et al.*, 2002; Ardehali *et al.*, 2003).

This condition could also happen for *H. pylori* since our studies with different concentrations of FCS and BSA appeared equivalent. The above would indicate that the inhibition of BF is probably related to a steric saturation effect of the FCS (or BSA) proteins that blocks the adhesion process or to the existence of some specific inhibitory component in serum.

Conclusions

The ability of *Helicobacter pylori* to develop biofilms *in vitro* was confirmed using polystyrene supports (microplate). Consistent with what was previously reported, the liquid media BHI and BB allowed the development of BF. But specifically, the use of BB as a growth medium, supplemented with glucose, and β -CD as an energy source, constitutes one of the best conditions for the formation of BF in this bacterium. As has been fully reported, the incorporation of FCS favors the planktonic growth of the bacteria, but on the other hand, our results showed that the presence of this component in the liquid cultures of *H. pylori* significantly affects the formation of BF. Found that the best conditions for the development of BF *in vitro* were obtained with the total absence of serum. The inhibitory activity of FCS on BF formation should be studied in greater depth although our results indicate that it could be related to a saturation effect by serum proteins such as albumin. Results of this work were obtained with the analysis of an *H. pylori* *cagA*⁺/*vacA* *s2m2* strain. Given the existence of a high gene polymor-

phism in the bacteria, it is important to extend the study with different variants of *H. pylori*.

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