ORIGINAL ARTICLE

Phnx Med J. December 2024, Volume 6 No 3
DOI:10.38175/phnx.1455085

# Evaluation of *In Vitro* Biofilm Formation of *Helicobacter pylori* in Different Culture Media

*Helicobacter Pylori* Farklı Kültür Ortamlarında *İn Vitro* Biyofilm Oluşumunun Değerlendirilmesi

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***ABSTRACT***

***Objective:*** *Biofilms are surface-attached cell communities that play a role in the survival of bacteria. Helicobacter pylori, a gram-negative pathogen that colonizes the human gastric mucosa, forms biofilms, causing treatment failure and the risk of developing peptic ulcers, gastritis and gastric cancer in infected individuals. The aim of the study is to evaluate the biofilm formation abilities of H. pylori ATCC 26695 and three clinical strains in different culture media.*

***Material and Method:*** *Biofilm formation characteristics of H. pylori strains using different culture media were evaluated, and the crystal violet (CV) staining method (measured at OD 595 ) was used. Various media were used for incubating H. pylori strains: Brucella broth (BB), TSB with 10% FBS, BHI with 10% FBS, BB with 10% FBS, BB with 10% FBS + 0.25% glucose, and BB with 10% FBS + 1% glucose (incubated for 3 days). Additionally, BB with 1% FBS, BB with 10% FBS, and BB with 5% inactivated human serum were incubated for 2 and 4 days at 37°C under microaerophilic conditions.*

***Results:*** *It was observed that 5% inactived human serum was more effective in biofilm formation of H. pylori ATCC 26695 and three clinical strains. However, there was no biofilm production in the strains cultured with Brucella broth alone and that the strains cultured with TSB + 10% FBS could not form a strong biofilm compared to other media.*

***Conclusion:*** *Different culture media used for H. pylori ATCC 26695 and three clinical strains affect biofilm formation. It is thought that in vitro experiments to prevent biofilm formation may provide a solution to the prevention of H. pylori infection.*

***ÖZET***

***Amaç: B****iyofilmler, bakterilerin hayatta kalmasında rol oynayan yüzeye bağlı hücre topluluklarıdır. İnsan mide mukozasını kolonize eden gram negatif bir patojen olan Helicobacter pylori, biyofilm oluşturarak tedavi başarısızlığına ve enfekte bireylerde peptik ülser, gastrit ve mide kanseri gelişme riskine neden olur. H. pylori ATCC 26695 ve üç klinik suşun farklı kültür ortamlarında biyofilm oluşturma yeteneklerinin değerlendirilmesi amaçlanmıştır.*

***Gereç ve Yöntem:*** *H. pylori suşlarının biyofilm oluşturma özellikleri farklı kültür ortamları kullanılarak biyofilm oluşturmaları Kristal viyole (CV) yöntemi ile spektrofotometrede (OD595) değerlendirildi. Kullanılan ortamlar; Brucella broth (BB) besiyeri, TSB + %10 FBS, BHI + %10 FBS, BB + %10 FBS, BB + %10 FBS + %0.25 glukoz ve BB + %10 FBS + %1 glukoz-(3 gün inkübasyon) ve BB + %1 FBS, BB + %10 FBS, BB + %5 inaktive insan serumu 2 ve 4 gün 37°C de mikroaerofilik koşullarda inkübasyon gerçekleştirildi.*

***Bulgular:*** *H. pylori ATCC 26695 ve üç klinik suşun biyofilm oluşumunda %5 inaktive edilmiş insan serumunun daha etkili olduğu görüldü. Sadece Brucella besiyeri ile biyofilm üretiminin olmadığı TSB + %10 FBS ile suşların diğer ortamlara göre güçlü bir biyofilm oluşturmadığı gözlendi.*

***Sonuç:*** *H. pylori ATCC 26695 ve üç klinik suş için kullanılan farklı kültür ortamları biyofilm oluşumunu etkilemektedir. Biyofilm gelişiminin önlenmesine yönelik in vitro deneylerin yapılmasının H. pylori enfeksiyonlarının önlenmesinde çözümler sağlayabileceği düşünülmektedir.*

***Keywords:***
*ATCC 26695*
*Biofilm*
*Crystal violet*
*Helicobacter pylori*
*Infection*

***Anahtar Kelimeler:***
*ATCC 26695*
*Biyofilm*
*Kristal Viyole*
*Helicobacter pylori*
*Enfeksiyon*

## INTRODUCTION

*Helicobacter pylori* is a spiral-shaped, gram-negative, microaerophilic bacterium that colonizes the human stomach (1). It has been observed that in developing countries, the prevalence of *H. pylori* can reach 90% of the population and the infection can last a lifetime. The study group of the World Health Organization's International Agency for Research on Cancer concluded in 1994 that *H. pylori* is classified as a group I carcinogen in humans. *H. pylori* is associated with several gastric disorders, including peptic ulcer disease, which is a major cause of both duodenal ulcers and gastric ulcers. Additionally, it is linked to chronic active gastritis, Mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma. Long-term infection with *H. pylori* increases the risk of developing gastric cancer

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**Cite as:** Gülseroğlu S, Akpınar H, Soytürk M, Yılmaz Ö. Evaluation of *In Vitro* Biofilm Formation of *Helicobacter pylori* in Different Culture Media. Phnx Med J. 2024;6(3):e10-e16.
**Received:** 19.03.2024 **Accepted:** 23.07.2024 **Online Published:** 30.07.2024

(2). Most people infected with *H. pylori* are asymptomatic However, infected people are at high risk of developing related diseases. *H. pylori* exhibits 2 morphological forms. One appears as a spiral form, while the other is a coccoid form that cannot be cultured but is viable the spiral shape is the most common form in colonization of the human stomach. This microorganism has ability to change its spiral shape in to a coccoid form in order to survive when the environmental conditions deteriorate (3). Most bacteria can adapt to life under environmentally restrictive conditions by developing different mechanisms.

Bacteria form surface-attached communities defined as "bacterial biofilms" to protect themselves from adverse environmental conditions. Biofilms are ubiquitous in natural, industrial and clinical environments and have a critical role in the establishing persistent infection (4). Biofilms are composed of dead and living microbial cells and extracellular polymeric substances (EPS), which are proteins, nucleic acids and polysaccharides synthesized and secreted by these microbial cells (5). The EPS matrix constitutes approximately 90% of the biofilm biomass. The first binding phase is formed by specific bacterial surface molecules as well as hydrophobic or electrostatic interactions. The next stage is the proliferation of bacteria. In the third stage (maturation stage), as the number of bacteria increases, the biofilm forms a thick tower-like structure. The expanding biofilm releases planktonic bacterial cells to dissolve and spread to other sites (6,7). Stark et al. conducted the first research on biofilm formation for *H. pylori*. First demonstration of the ability of *H. pylori* to form biofilms *in vitro*, the presence of a water-insoluble polysaccharide at the liquid–air interface was reported that *H. pylori* strain NCTC 11637 was grown continually in a glass fermentor (8). Later, in the study with clinic isolate laboratory strains of *H. pylori*, Cole and colleagues reported that biofilms formed over glass surfaces only in the air-liquid interface, which was an indicator of microaerobicity (9). Several studies have mentioned in ability of *H. pylori* to form biofilms when grown upon a glass surface, observing a biofilm whose content is polysaccharide in the air-liquid interface (10,11). Biofilm formation is also critical for bacteria to survive and establish a successful infection. Biofilms exhibit the ability to survive exposure to antibacterial agents. Bacteria within biofilms are able to become 10-1000 times resistant to the effects of antimicrobial agents. This is because EPS is a diffusion barrier that prevents substances that inhibit bacterial growth from entering the biofilm. Even if some antibacterial agents can penetrate freely, protection will be provided by EPS blocking the diffusion reaction of the antibacterial agent, bringing it to a sublethal concentration before it reaches all bacterial cells in the biofilm. Thus, bacteria are found deep within the biofilm, creating an opportunity for them to develop resistance to the drug (12). *H. pylori*, uses biofilm production as a strategy to evade the host's immune system and protect itself from the harsh environment and antimicrobial agents found in the stomach (13,14). The aim of this study is to investigate the effectiveness of the biofilm-forming ability of *H. pylori* clinical strains, which is the reason for treatment failure, in different culture environments.

## MATERIAL AND METHOD

This study was carried out by Dokuz Eylül University Faculty of Medicine Ethics Committee with the decision dated 20.02.2019/20.07.2022 and numbered 2019/4-35;2022/04-16 at Dokuz Eylül University Faculty of Medicine, Department of Medical Microbiology.

### Bacterial strains and culture conditions

ATCC 26695 and three clinical isolates were used in the study. *H. pylori* ATCC 26695 was obtained from the American Type Culture Collection (Rockville, MD, USA) and was later used in this study. Clinical isolates were retrospectively obtained from previously studies including the patients antrum and corpus gastric biopcies Rapid urease test (RUT) and histopathology were also performed. Three clinical isolates in stock culture medium stored in aliquots in BHI medium containing 20% glycerol at -80°C, were brought to room temperature and cultured on Columbia Blood agar (Oxoid) medium containing 7% Defibrined Horse Blood with *H. pylori* Selective Supplement (DENT, Oxoid) for three to five days at 37°C in microaerophilic condition using the GasPak Campy Container System (Becton Dickinson and Company) in an anaerobic jar (Oxoid). Subcultures were made from the colonies that grew as a result of incubation.

### Biofilm formation and its quantification

Bacteria growing over Columbia Blood agar (Oxoid) medium containing 7% Defibrinated Horse Blood, and *H. pylori* Selective Supplement (DENT, Oxoid) were collected in 1 mL Brucella broth (BB; Biolife, Italiana). Bacterial suspensions with a standard turbidity of 3.0 McFarland (~6x108 CFU/ml) were obtained using the Densimat device (Biomerieux SA, France). 20 µl of *H. pylori* bacterial suspension and 180 µl BB supplemented with 1% FBS were added to each well of a sterile flat-bottom 96-well polystyrene microtiter plate (Greiner bio-one Austria); 20 µl of *H. pylori* bacterial suspension and 180 µl BB supplemented with 10% FBS were added to the second plate; 20 µl of *H. pylori* bacterial suspension and 180 µl BB supplemented with 5% inactivated human serum were added to the third plate; The environmental and time-dependent biofilm formation were evaluated by incubation in an anaerobic jar in a microaerophilic environment for 2 days and 4 days in an anaerobic jar in a microaerophilic environment at 37°C using the GasPak Campy Container System (Becton Dickinson and Company). Each plate was run on consecutive days and the experiments were repeated 3 times.

In the other study; Biofilm formation of the strains were evaluated using different culture media and contents. Culture media used; alone Brucella Broth (BB), Tryptic Soy Broth (TSB) (LAB M, UK) supplemented with 10% Fetal bovine serum (FBS ), Brain Heart Infusion broth (BHI) (Oxoid Ltd., England) supplemented with 10% FBS, BB supplemented with 10% FBS, BB supplemented with 10% FBS and 0.25% glucose (AppliChem, Germany), and BB supplemented with 10% FBS and 1% glucose (15).

Using six separate plate for each culture medium from a sterile flat-bottomed 96-well polystyrene microtiter plate (Greiner bio-one Austria), 180 µL of the culture medium and 20 µl of *H. pylori* suspension were added to each well. The microplates were incubated for 3 days in an anaerobic

jar in a microaerophilic environment at 37°C using the GasPak Campy Container System (Becton Dickinson and Company). Each plate was run on consecutive days and the experiments were repeated 3 times.

The planktonic cell suspension was removed by washing the microplate three times with sterile phosphate-buffered saline (PBS, pH 7.3). The microplate was air dried for 1 hour. The cells adhering to the microplate were fixed by adding 200 µl methanol (Merck, Germany) to each well and waiting for 15 minutes. The wells were emptied and left at room temperature for 1 hour to dry. Then, each well was stained with 200 µl 1% (w/v) crystal violet (CV) (Merck, Germany) on account of 5 minutes, and the wells were emptied. Washed 2 times with PBS. It was air dried for 15 minutes. Then, 200 µl of ethanol-acetic acid (ethanol: acetic acid = 95:5) (Merck, Germany) was added to the wells stained with crystal violet and waited for 1 minute. The quantity of biofilm was obtained from absorbance measurements with a spectrophotometer (BioTech Synergy HT, USA) at a wavelength of 595 nm. The experiments were performed in triplicate. In this study, absorbance measurement of the well containing bacteria-free medium was used as a negative control.

Biofilm formation was evaluated in 3 classes: a negative biofilm-former ODT < control ODC, a weak biofilm-former ODT ≥ the control ODC, and a strong biofilm-former ODT ≥ 2 times of the control ODC (16).

## RESULTS

### Effect of cell growth on biofilm formation

Two different concentrations of FBS (1%, 10%) and 5% inactivated human serum were added to the BB medium of *H. pylori* ATCC 26695 standard strain and 3 clinical strains, and 2-day and 4-day biofilm formations were compared.

Total biomass of biofilm formation (time and %FBS) under different settings was evaluated among three clinical strains and *H. pylori* ATCC 26695 standard strain. At the end of the study, a significant increase in growth was observed in all the strains on day four BB supplemented with 5% inactivated human serum. This was chosen as the ideal condition because of the development due to the biofilm model of *H. pylori* strains (Figure 1,2).

The ability of *H. pylori* strains to model biofilms was

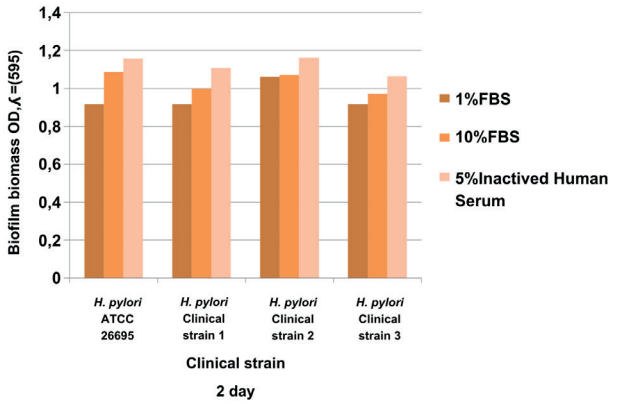



**Figure 1:** Two-day biofilm evaluation of *H. pylori* in culture medium with 1% FBS, 10% FBS, and 5% inactivated human serum.

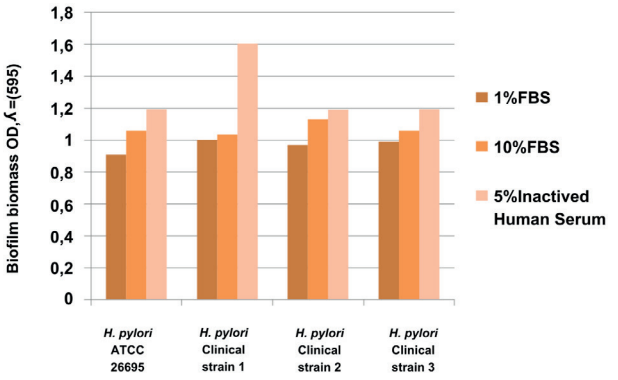



**Figure 2:** Four-day biofilm evaluation of *H. pylori* in culture medium with 1% FBS, 10% FBS, and 5% inactivated human serum.

determined by a microplate-based assay using flat-bottom polystyrene microtiter plates (Greiner bio-one Austria) with CV (w/v) staining. The results were evaluated by spectrophotometric measurement (OD595). An ıncrease in biofilm biomass of strains was observed between the 1% FBS, 10% FBS and 5% inactivated human serum used (Table 1,2).

Biofilm formation capacity due to *H. pylori* strains in six varied culture media was determined by a microplate-

**Table 1:** Average ODT of *H. pylori* two-day biofilm formation.

| | *ODT of BB+1%FBS (2 day) | ODT of BB+10%FBS (2 day) | ODT of BB+5% inactivated human serum (2 day) |
|---|---|---|---|
| **ODC | 0.919 | 0.900 | 0.978 |
| ***H. pylori* ATCC 266695** | 0.917 | 1.087 | 1.157 |
| ***H. pylori* clinical strain 1** | 0.916 | 0.999 | 1.107 |
| ***H. pylori* clinical strain 2** | 1.062 | 1.071 | 1.162 |
| ***H. pylori* clinical strain 3** | 0.917 | 0.971 | 1.064 |

**ODT = Optical Density of the isolates*

***ODC = Optical Density of the controls (BB+1%FBS, BB+10%FBS, BB + 5% inactivated human serum)*

**Table 2:** Average ODT of *H. pylori* four-day biofilm formation.

| | *ODT of BB+1%FBS (4 day) | ODT of BB+10%FBS (4 day) | ODT of BB+5% inactivated human serum (4 day) |
|---|---|---|---|
| ****ODC** | 1.015 | 1.093 | 1.246 |
| ***H. pylori* ATCC 266695** | 1.014 | 1.240 | 1.212 |
| ***H. pylori* clinical strain 1** | 0.959 | 1.024 | 1.263 |
| ***H. pylori* clinical strain 2** | 0.979 | 0.971 | 1.256 |
| ***H. pylori* clinical strain 3** | 1.052 | 1.139 | 1.340 |

**ODT = Optical Density of the isolates*
***ODC = Optical Density of the controls ( BB+1%FBS, BB+10%FBS, BB + 5% inactivated human serum)*

based assay using flat-bottom polystyrene microtiter plates (Greiner bio-one Austria) with CV (w/v) staining. The results were evaluated by spectrophotometric measurement (OD595) (Table 3,4) .

In all experiments, three *H. pylori* clinical strains and one *H. pylori* ATCC 26695 standard strain (n = 4) were used. The results obtained from the experiments were as follows: BB was 100% (n=4) non biofilm, TSB + 10% FBS produced 75% (n = 3) weak biofilm and 25% (n=1) non biofilm, BHI + 10% FBS 75% (n = 3) strong biofilm and 25% (n=1) weak biofilm BB + 10%FBS 75% (n = 3) strong biofilm and 25% (n=1) weak biofilm, BB + 10%FBS + 0.25% glucose It was observed that three was 75% (n=3) strong biofilm and 25% (n=1) weak biofilm, and BB+10% FBS+1% glucose was 50% (n=2) strong biofilm and 50% (n=2) weak biofilm producers (Figure 3,4).

**DISCUSSION**

Bacterial biofilms are a serious global health problem due to their ability to evade host defence systems, antibiotics, and other external stresses, thereby contributing to persistent chronic infections (17). Several studies have reported that *H. pylori* is able to form biofilms on abiotic surfaces *in vitro* and on the surface of human gastric

**Table 3:** H. pylori Biofilm Formation on BB, TSB + 10 % FBS, BHI + 10 % FBS, BB + 10 % FBS, BB + 10 % FBS + 0,25 % glucose and BB + 10 % FBS + 1 % glucose culture media.

| ***Helicobacter pylori* strains** | **BB** | **TSB+ 10%FBS** | **BHI+ 10%FBS** | **BB+1 0%FBS** | **BB+10%FBS +0,25%glucose** | **BB+10%FBS +1%glucose** |
|---|---|---|---|---|---|---|
| **ATCC 26695** | non | weak | strong | strong | strong | strong |
| **Clinical strain 1** | non | non | weak | weak | weak | weak |
| **Clinical strain 2** | non | weak | strong | strong | strong | weak |
| **Clinical strain 3** | non | weak | strong | strong | strong | strong |
| **Number ( n) and % for Biofilm Formation on Culture Media** | non-biofilm (n=4) (100%) | non-biofilm (n=1) (25%)<br>weak (n=3) (75%) | weak-biofilm (n=1) (25%)<br>strong (n=3) (75%) | weak-biofilm (n=1) (25%)<br>strong (n=3) (75%) | weak-biofilm (n=1) (25%)<br>strong (n=3) (75%) | weak-biofilm (n=2) (50%)<br>strong (n=2) (50%) |

**Table 4:** Average ODT of *H. pylori* Biofilm Formation on BB, TSB + 10 % FBS, BHI + 10 % FBS, BB + 10 % FBS, BB + 10 % FBS + 0,25 % glucose and BB + 10 % FBS + 1 % glucose culture media.

| ***Helicobacter pylori* strains** | ***ODT of BB** | ***ODT of TSB+ 10%FBS** | ***ODT of BHI+ 10%FBS** | ***ODT of BB+10%FBS** | ***ODT of BB+ 10%FBS +0,25%glucose** | ***ODT of BB+ 10%FBS+ 1%glucose** |
|---|---|---|---|---|---|---|
| ****ODC** | 0,126 | 0,132 | 0,124 | 0,142 | 0,145 | 0,148 |
| **ATCC 266695** | 0,110 | 0,158 | 0,403 | 0,353 | 0,420 | 0,426 |
| **Clinical strain 1** | 0,123 | 0,130 | 0,172 | 0,182 | 0,169 | 0,196 |
| **Clinical strain 2** | 0,116 | 0,146 | 0,248 | 0,320 | 0,342 | 0,215 |
| **Clinical strain 3** | 0,108 | 0,136 | 0,362 | 0,332 | 0,326 | 0,384 |

**ODT = Optical Density of the isolates*
***ODC = Optical Density of the controls (BB, TSB+10%FBS, BHI+10%FBS, BB+10%FBS, BB+10%FBS+0,25%glucose, BB+10%FBS+1%glucose)*

**Figure 3:** *H. pylori* Biofilm Formation Percentages on BB, TSB + 10 % FBS, BHI + 10 % FBS, BB + 10 % FBS, BB + 10 % FBS + 0,25 % glucose and BB + 10 % FBS + 1 % glucose culture media.

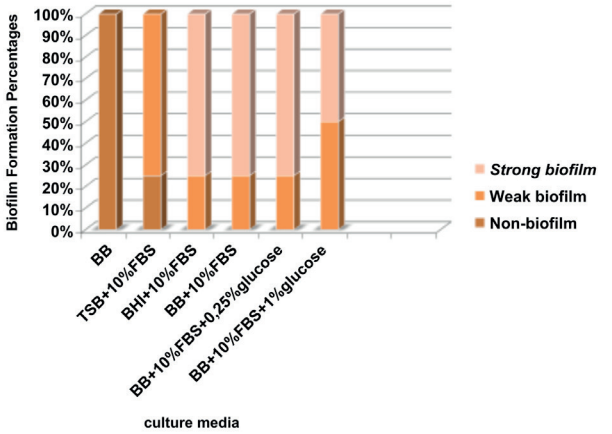
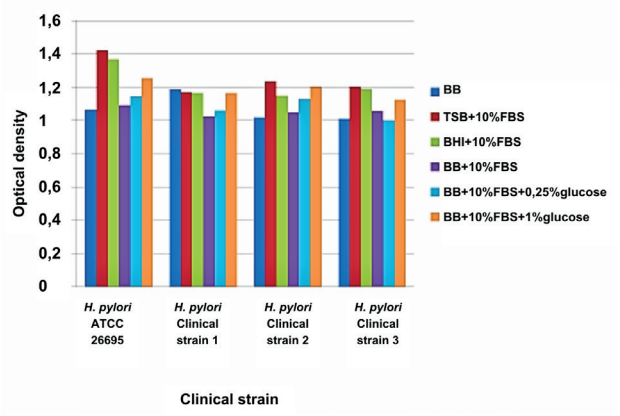



**Figure 4:** *H. pylori* ODT of H. pylori Biofilm Formation on BB, TSB + 10 % FBS, BHI + 10 % FBS, BB + 10 % FBS, BB + 10 % FBS + 0,25 % glucose and BB + 10 % FBS + 1 % glucose culture media.




mucosal (18,19). Studies have shown that some bacterial biofilms consist of two processes: (i) initial adhesion to the surface via adhesins; (ii) formation of multilayered cell clusters by spontaneous production of extracellular matrix by cell growth (20,21). Our results showed that *H. pylori* biofilm formation proceeds in the same way. The results showed that for 1% FBS, the number of viable bacteria in the biofilm matrix was low despite the time-related increase. However, 5% inactivated human serum was found to be more effective as the most suitable environmental condition for *H. pylori* viability and growth. It resulted in the highest amount of biofilm biomass among the conditions tested. Accordance with the habitat due to *H. pylori* with consideration of the media used due to standard culture, some of the most common rich growth media were used for biofilm formation (22). Despite the differences in *H. pylori* medium compositions, it formed biofilms at similar levels in each medium. The data obtained indicating that slight changes in rich growth media did not significantly influence *H. pylori* biofilm formation and that our determination of biofilm formation was not an creation of medium selection. Pinho et al., in their study with 1% FBS, 5% FBS and 10% FBS, found that the decrease in FBS supplementation (normal 10% versus 5% FBS) resulted in higher biofilm biomass production.They showed that under 10% FBS supplementation, bacteria did not have a defense strategy as in biofilm formation (23). In the study conducted by Cole et al., the effect of mucin on biofilm formation was investigated and it was shown that it significantly increased the number of planktonic *H. pylori* in the mucus-rich stomach environment, but did not affect biofilm formation (24). Many published studies have demonstrated the amino acid requirement of *H. pylori* and have shown that amino acids are used instead of sugar as a carbon source. In his study, Mends et al. showed that *H. pylori* was able to survive by using amino acids as its main nutrient, converting arginine, asparagine, aspartate, glutamine, and serine, which are used as substrates, into the metabolic products acetate, succinate, and lactate (25). In the study of Reynolds et al., it was found that the addition of alanine in the presence of glucose in the environment induces growth using it as a nitrogen or carbon source (26). In another study designed by Abdollahi et al., it was found that *H. pylori* isolates showed positive chemotaxis and negative chemotaxis (repellency) towards the tested sugars and amino acids phenylalanine, aspartic acid, glutamic acid, isoleucine and leucine, and tyrosine, since the ability to move for biofilm plays an important role in *H. pylori* pathogenicity (27). Commonly used in tissue culture media, Ham's F-12 has been shown to promote the growth of *H. pylori* in the absence of serum or protein (28). Although F-12 promotes *H. pylori* growth, the addition of fetal bovine serum (FBS) to the medium has been shown to significantly increase growth; This showed that further optimization or supplementation with identified nutrients was required (29).

Another environmental situation that affected biofilm creation was the surface on which the biofilm formed. Initiating biofilm formation necessity bacterial cells attached to a surface and shape microcolonies. These microcolonies turn into a grown biofilm structure. In biofilm formation, bacterial cells adhere to a surface or interface. This duration conditional on provided that the bacteria come into direct touch with the surface and on appropriate cell-surface interactions to overcome the repellent forces generated between the two surfaces. Windham et al. in their study, they showed that although various enriched growth media did not significantly affect biofilm formation, surface selection had a significant effect on biofilm mass (15). There is evidence to suggest that the microorganism can sense contact with a superficies and in answer change gene expression to promote stable cell-surface interactivity. However, the exact molecular mechanisms are still unknown. For this reason, uncertainties about biofilm development remain on the agenda (30,31).

*H. pylori* has the capacity to form biofilms in the environment, on abiotic surfaces, and in the human stomach. *H. pylori* can be found embedded in drinking water biofilms in water distribution systems in countries (32,33). Various studies have shown that understanding *H. pylori* biofilm formation is important for in the

prevention and control of *H. pylori* infection (34,35). The emergence of drug resistance in *H. pylori* has become a serious clinical problem. Biofilm formation is considered an important factor contributing to antibiotic resistance in humans. Therefore, it is essential to develop anti-biofilm agents with strong antagonistic effects against bacterial biofilms. These agents can enhance *H. pylori* eradication by reducing drug resistance, and offering a promising approach to combat a key challenge related to antibiotic resistance.

## CONCLUSION

Assessment of the biofilm formation ability of *H. pylori* plays an significant role in controlling and biofilm preventing the formation of antibiotic resistance. Therefore, it is thought that investigating *H. pylori* biofilm creation may be efficient in understanding the infection and colonization of this microorganism. There are differences in how biofilm is carried out. Although studies have been conducted to examine factors related to *H. pylori* biofilm formation, it is not yet resolved where it may vary depending on conditions. It is thought that awareness of *H. pylori*, an overlooked bacterium, is also important in the protection of public health and multidisciplinary studies are needed to understand the complexity of biofilm formation.

**Conflict of Interest:** No conflict of interest was declared by the authors.
**Ethics:** This study was conducted after approval by the Ethics Committee of the Faculty of Medicine of Dokuz Eylül University (Decision No: 2019/4-35;2022/04-16, Date 20.02.2019;20.07.2022).
**Funding:** This research project was carried out as part of a doctoral thesis and was funded by the Scientific Research Projects Coordination Unit of Dokuz Eylül University with grant number 4505 GOA.
**Approval of final manuscript:** All authors.

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