Comparison of three methods for generating the coccoid form of *Helicobacter pylori* and proteomic analysis

Kyoungwon Jung[1], Haram Bae[2], Jiyeun Kate Kim[2], Bohyun Jeong[2], Moo In Park[1] and Jee Young Lee[2]*

Abstract

Background *Helicobacter pylori* changes from spiral to coccoid depending on the host state, environmental factors, and surrounding microbial communities. The coccoid form of *H. pylori* still maintains its complete cellular structure, retains virulence genes, and thus plays a role in pathogenicity. To understand the coccoid form, it is crucial to establish the in vitro generation of the coccoid *H. pylori*. Although some conditions have been studied for the generation of the coccoid form, few studies have compared these conditions for coccoid generation. Here, we generated coccoid forms via three methods and compared the differences in morphology, viability, culturability, and protein expression.

Results The coccoid *H. pylori* was generated in vitro via three methods: a starvation method, a method using amoxicillin, and a method using the culture supernatant of *Streptococcus mitis*. The morphology and viability of the cells were examined by fluorescence microscopy after staining with SYTO9 and propidium iodide. The culturability of *H. pylori* was examined by counting colony-forming units on chocolate agar plates. In the starvation group, no colonies formed after 7 days, but viable coccoids were continuously observed. In the amoxicillin-treated group, the culturability decreased rapidly after 12 h, and showed a viable but non culturable (VBNC) state after the third day. Most cells treated with *S. mitis* supernatant changed to coccoid forms after 7 days, but colonies were continuously formed, probably due to living spiral forms. We performed proteomics to analyse the differences in protein profiles between the spiral and coccoid forms and protein profiles among the coccoid forms generated by the three methods.

Conclusion Amoxicillin treatment changed *H. pylori* to VBNC cells faster than starvation. Treatment with the *S. mitis* supernatant prolonged the culturability of *H. pylori*, suggesting that the *S. mitis* supernatant may contain substances that support spiral form maintenance. Proteomic analysis revealed that the expression of proteins differed between the spiral form and coccoid form of *H. pylori*, and this variation was observed among the coccoid forms produced via three different methods. The proteins in the coccoid forms produced by the three methods differed from each other, but common proteins were also observed among them.

Keywords Coccoid form, *Helicobacter pylori*, Starvation, Amoxicillin, *Streptococcus mitis*, Proteomics

*Correspondence:
Jee Young Lee
imezee@naver.com
[1]Department of Internal Medicine, Kosin University Gospel Hospital, Busan 49267, Republic of Korea
[2]Department of Microbiology, Kosin University College of Medicine, Busan 49267, Republic of Korea

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Background

Helicobacter pylori (H. pylori) is a bacterium discovered in the stomach, an environment previously thought to be unfavourable for bacterial survival [1]. It is a major cause of peptic ulcer disease and is closely associated with atrophic gastritis and intestinal metaplasia, precursors of gastric cancer [2]. This bacterium is considered the most important risk factor for gastric cancer and was classified as a Group 1 carcinogen in 1994 [3]. This spiral-shaped, Gram-negative bacterium can transform into a coccoid form under various conditions, including host factors, environmental stress, and interactions with other microbial populations [4, 5]. The coccoid form, known as viable but non-culturable (VBNC), can survive in stressful environments by entering a dormant state, reducing metabolic activity [6]. Despite this transformation, *H. pylori* retains its complete cell structure, genomic DNA, and pathogenic genes such as vacA and cagA [6]. Morphological transformation in *H. pylori* has been suggested to contribute to gastric cancer development, with studies showing a higher prevalence of coccoid forms in gastric cancer compared to benign peptic ulcers [6, 7]. The coccoid form of *H. pylori* has been reported to reduce the rate of apoptosis of host cells through the expression of survivin, an anti-cell death protein, thereby accelerating cell proliferation and contributing to gastric cancer [8, 9]. *H. pylori* does not spontaneously disappear if left untreated and may continue to interact with other microbiota in the stomach [10]. Standard eradication therapy involves a combination of antibiotics and proton pump inhibitors (PPIs), but treatment failure rates are increasing, primarily due to antibiotic resistance. Recent studies suggest that the coccoid form of *H. pylori* may also contribute to treatment failure, as it is more tolerant to antibiotics. Additionally, this form can evade detection due to its reduced urease activity, leading to false-negative urease test results [11, 12]. The coccoid form is also implicated in the human-to-human transmission of *H. pylori*, although this has not been confirmed [13].

Research on the coccoid *H. pylori* is crucial for understanding its transmission routes, improving eradication rates, and addressing clinical challenges such as recurrent infections [5]. Furthermore, the expression of proteins related to morphological changes may influence gastric cancer development. While the environmental and experimental conditions that induce spiral-to-coccoid transformation have been described, few studies have compared the differences in coccoid formation under these conditions. Additionally, little is known about the protein expression changes accompanying *H. pylori*'s morphological transformation [14, 15].

In this study, we chose three methods that are clinically relevant to generate the coccoid form of *H. pylori in vitro*. First, we selected the method of nutritional depletion, which is thought to be the most common environmental condition inducing a VBNC state [16]. Second, we chose an antibiotic treatment method that is commonly used in clinical practice for the eradication of *H. pylori* [5, 17]. Third, we selected an exposure to the culture supernatant of *S. mitis*, as after *H. pylori* infection, *S. mitis* becomes the most prominent bacterial species in the gastric microbiota [18, 19]. We first confirmed the spiral shape of cultured *H. pylori* and then established methods to reliably induce the coccoid form and assess its viability. The morphological changes, viability, and culturability of *H. pylori* cells subjected to the three methods were compared. Finally, the protein expression of the coccoid cells generated by three methods was analysed and compared with that of spiral cells as well as with each other.
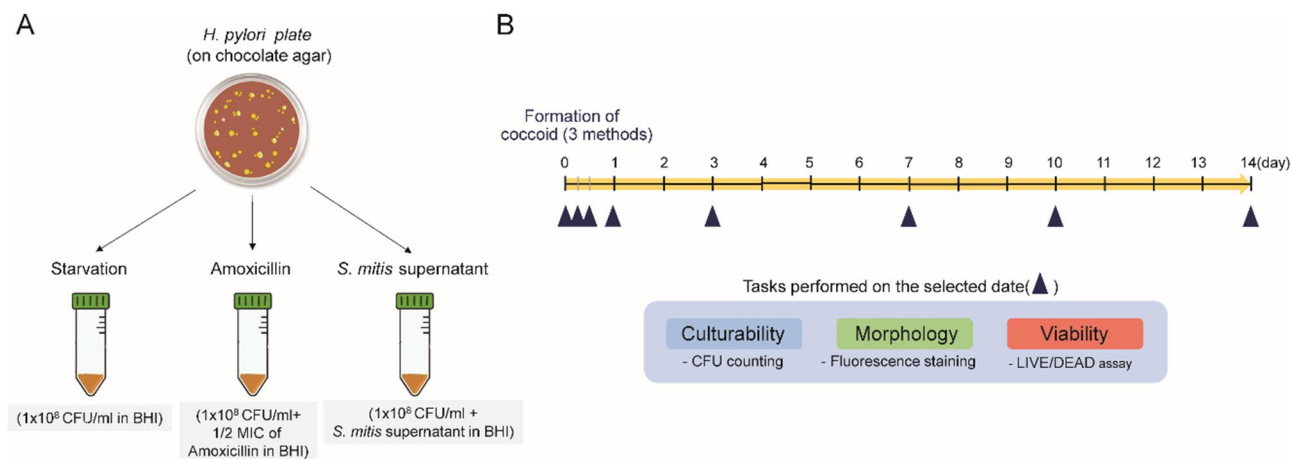
Methods

Bacterial strains and culture conditions

The *H. pylori* strain (ATCC 26695), provided by the Helicobacter Bank (Gyeongsang National University, South Korea), was cultured on chocolate agar plates (Asan Pharm. Co., South Korea) enriched with 5% sheep blood under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). For the experiment, *H. pylori* were incubated for 3days, and the bacterial cells from a plate were sub-cultured onto three new chocolate agar plates to increase the number of fresh cells. *Streptococcus mitis* (KCTC5650) was initially cultured on brain heart infusion (BHI) agar plates at 37 °C. To obtain the culture supernatant of *S. mitis*, the cells were inoculated in BHI broth and incubated at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2).

Three methods for coccoid formation

Coccoid generation by the three methods is illustrated in Fig. 1A. To prepare *H. pylori* suspensions for the three methods, the cells were cultured on chocolate agar plates for 2 days and collected in BHI broth. Ten milliliters of *H. pylori* suspension was prepared at a concentration of 6×10^8 colony forming units (CFUs)/mL and diluted at a final concentration of 1×10^8 CFUs/mL for each three methods. To induce the coccoid form by the starvation method, *H. pylori* was incubated in BHI broth without the addition of any serum. For the amoxicillin method, an amoxicillin solution was added to the *H. pylori* suspension (final concentration of 1×10^8 CFUs/mL) to have a 1/2 minimum inhibitory concentration (MIC), which was 0.03125 µg/mL. The MIC of amoxicillin was determined using the agar dilution method, and the MIC of amoxicillin for the ATCC26695 strain was confirmed to be 0.0625 µg/mL. To perform the third method with *S. mitis* supernatant, *S. mitis* strain KCTC 5650 was cultured overnight in BHI broth at 37 °C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2), diluted

Fig. 1 Study design of coccoid generation by three methods and the experimental timeline. (**A**) Coccoid forms of *H. pylori* were generated in vitro by three methods: starvation, amoxicillin treatment, and *S. mitis* supernatant treatment. (**B**) The culturability, morphology, and viability of *H. pylori* cells under the three conditions were examined at the designated time points

100 times with BHI broth and cultured for 2 days. After centrifuging the culture at 3,500 rpm, the supernatant was collected, filtered through a 0.1 μm filter, sonicated for 1 min, and filtered again through a 0.1 μm filter to exclude any bacterial cells. Two milliliters of the filtered supernatant was added to 8 mL of BHI broth containing *H. pylori* (at a final concentration of 1×10^8 CFUs/mL). The bacterial samples prepared via the three methods were incubated at 37 °C under microaerophilic conditions.

### Examination of the morphology, viability and culturability of *H. pylori*

At the designated times indicated in Fig. 1B, the bacterial samples were centrifuged, and the pellets were washed with 0.85% saline solution. Next, saline and SYTO9/propidium iodide (SYTO9/PI) staining solution (LIVE/DEAD BacLight Bacterial Viability Kit, Thermo Fisher, USA) were mixed at a 1:1 ratio and added to the pellet. After incubating for 3 min, the stained sample was placed on a glass slide, covered with cover glass, and observed with a fluorescence microscope [20]. The morphological changes of *H. pylori* were confirmed with SYTO9 staining, and viability was assessed with SYTO9/PI staining. At a designated time, 100 μL of the bacterial samples prepared via the three methods were spread on chocolate agar plates and incubated at 37 °C under microaerophilic conditions. Colony forming units (CFUs) were counted after 2 days of incubation. Because *H. pylori* colonies are transparent and small, they were counted under a microscope at 10x magnification. Morphology, viability, and culturability were checked at 6, 12, 24, and 3, 7, 10, and 14 days after incubation. In the *S. mitis* supernatant-treated *H. pylori* group, the colonies were observed to continue growing at 14 days, so morphology, viability and culturability were also checked on days 21, 24 and 27.

Preparation of proteome samples

For the preparation of protein extracts from the coccoid form of *H. pylori*, a bacterial solution was spread on chocolate agar medium and incubated for 2 days to confirm the absence of colony formation, which is characteristic of the coccoid form. The samples were then observed under a fluorescence microscope to confirm that more than 95% were coccoid. Replicate experiments were conducted, and 7-, 14-, and 30-day samples of amoxicillin, starvation and *S. mitis* supernatant, respectively, were used. Each sample was aliquoted 10 mL from the broth and centrifuged at 12,000 rpm for 10 min. The pellet was harvested, washed in saline solution and centrifuged again to harvest the pellet. For spiral form proteomic analysis, colonies were scraped from the plate, collected, suspended in saline, and the pellet was harvested after centrifugation. Each pellet was treated with lysis buffer, and the protein concentration was quantified by Bradford assay, which revealed 60.18 mg/mL in the starvation group, 21.781 mg/mL in the *S. mitis* supernatant-treated group, and 24.445 mg/mL in the amoxicillin-treated group. The protein concentration from the spiral form was 66.08 mg/mL. Whole cell protein analysis was performed by loading 20 ug of each sample on 14% SDS–PAGE (Fig. 2).

Trypsin-mediated gel digestion and peptide extraction

Protein bands from the SDS–PAGE gel were excised and in-gel digested with trypsin according to standard protocols [21]. Protein bands were excised from the stained gels and cut into pieces. Then, the gel pieces were washed for 1 h at room temperature in 25 mM ammonium bicarbonate buffer, pH 7.8, containing 50% (v/v) acetonitrile. The gel pieces were dehydrated for 10 min in a centrifugal vacuum concentrator (Biotron Inc., Incheon, Korea) and then rehydrated in 50 ng of sequencing grade trypsin
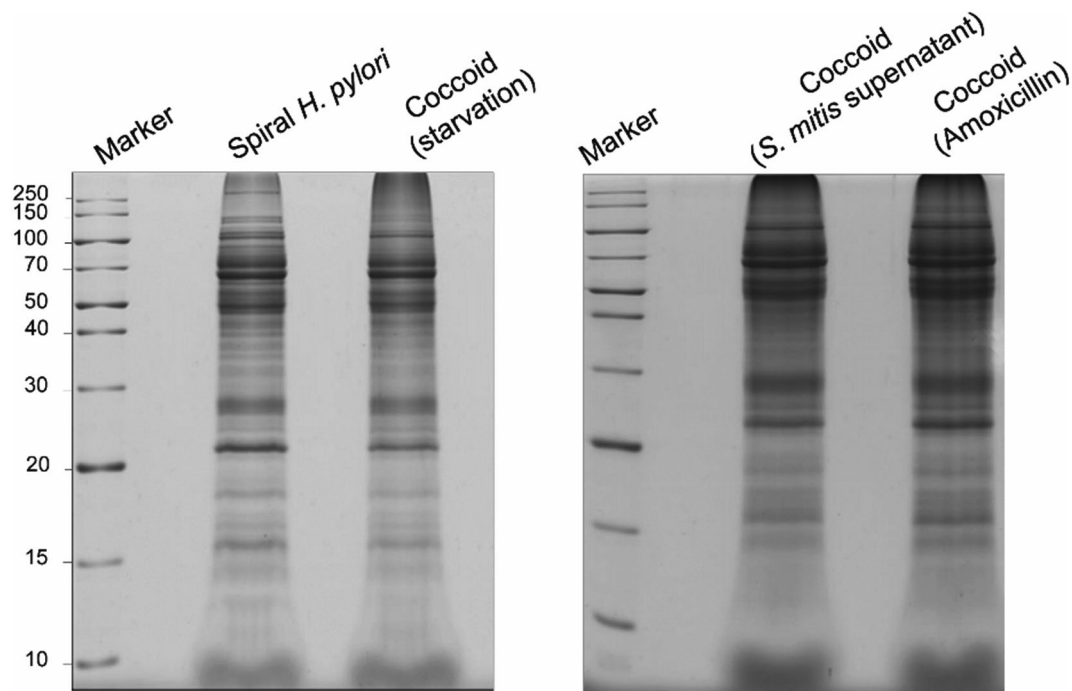
Fig. 2 SDS-PAGE analysis of whole cell proteins from *Helicobacter pylori* cell lysates. Protein patterns of spiral form *Helicobacter pylori* and coccoid form *Helicobacter pylori* generated by various methods (starvation, *S. mitis* supernatant treatment, and amoxicillin treatment) were examined on 14% SDS-PAGE. SDS-PAGE, Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis; MIC, minimum inhibitory concentration

solution (Promega, Madison, WI, USA). The tryptic peptides were extracted with 100 μL of 1% formic acid with 50% (v/v) acetonitrile by weak sonication for 20 min after incubating overnight in 25 mM ammonium bicarbonate buffer, pH 7.8, at 37 °C. The extracted solution was concentrated using a centrifugal vacuum concentrator, and the peptide solution was desalted using a reverse-phase column before mass spectrometric analysis [22]. After equilibration with 10 μL of 5% (v/v) formic acid, the peptide solution was loaded onto the column and washed with 10 μL of 5% (v/v) formic acid. The bound peptides were extracted with 8 μL of 70% acetonitrile with 5% (v/v) formic acid.

Protein identification using liquid chromatography–mass spectrometry (LC–MS/MS)

The analysis was performed by LC–MS/MS using a nano ACQUITY UPLC and an LTQ-orbitrap-mass spectrometer (Thermo Electron, San Jose, CA). The column used was a BEH C18 column (1.7 μm, 100 μm × 100 mm) (Waters, Milford, MA, USA). The mobile phase A for LC separation was deionised water containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. The chromatography gradient was set to provide a linear increase from 10% B to 40% B over 21 min, from 40% B to 95% B over 7 min, and from 90% B to 10% B over 10 min. The flow rate was 0.5μL/min. For serial mass spectrometry, mass spectra were acquired using data-dependent acquisition with MS/MS scans following full mass scans (300–2000 m/z). Each MS/MS scan was acquired with an average of 1 microscan in the LTQ, the ion transfer tube temperature was set to 275 °C, and the spray voltage was set to 2.0 kV. The normalised collision energy was set to 35% for MS/MS, and individual MS/MS spectra were processed using SEQUEST software (Thermo Quest, San Jose, CA, USA). The generated peak list was queried against the database using the MASCOT program (Matrix Science Ltd., London, UK). For MS analysis, carbamidated (C), deamidated (NQ), and oxidation (M) were modified, and the data analysis was performed by setting the peptide mass tolerance to 10 ppm, the MS/MS ion mass tolerance to 0.8 Da, the missed cleavage allowance to 2, and the charge state to +2 and +3. Only significant hits as defined by the MASCOT probability analysis were collected.

Results

Morphological changes of *H. pylori* cultured via three methods

Based on previous studies conducted in our laboratory, we observed that *H. pylori* cultured in BHI broth with serum transformed into the coccoid form within 10 to 14 days. This result confirms that long broth culture induces *H. pylori* to change into the coccoid form due to nutritional depletion [23]. In our study to induce coccoid form by starvation, we compared the coccoid transformation

of *H. pylori* in BHI broth and BHI broth with 10% FBS. We observed minimal differences in coccoid formation between the two groups (Supplementary Fig. 1). Therefore, we established a baseline condition of starvation method in BHI broth and set up an experiment to compare three groups under the same conditions: one with *S. mitis* supernatant, one with added amoxicillin, and a starvation group.

When *H. pylori* cells were collected after 2 days of culture on chocolate agar plates, most of the bacteria were spiral shaped, and some were slightly shorter (Fig. 3A).

After 6 h of broth culture, the coccoid form of *H. pylori* started to appear in all three groups, and U-shaped and donut-shaped forms were also observed (Fig. 3B). On the third and seventh days, U-shaped, donut, rod, and elongated bacterial cells were observed along with the coccoid form (Fig. 3B). The proportion of coccoid cells increased over time under all three experimental conditions: starvation, amoxicillin treatment, and *S. mitis* supernatant treatment (Fig. 3B). After 12 h of culture, coccoid cells accounted for approximately 56%, 51%, and 52% of the total bacteria in the starvation, amoxicillin,
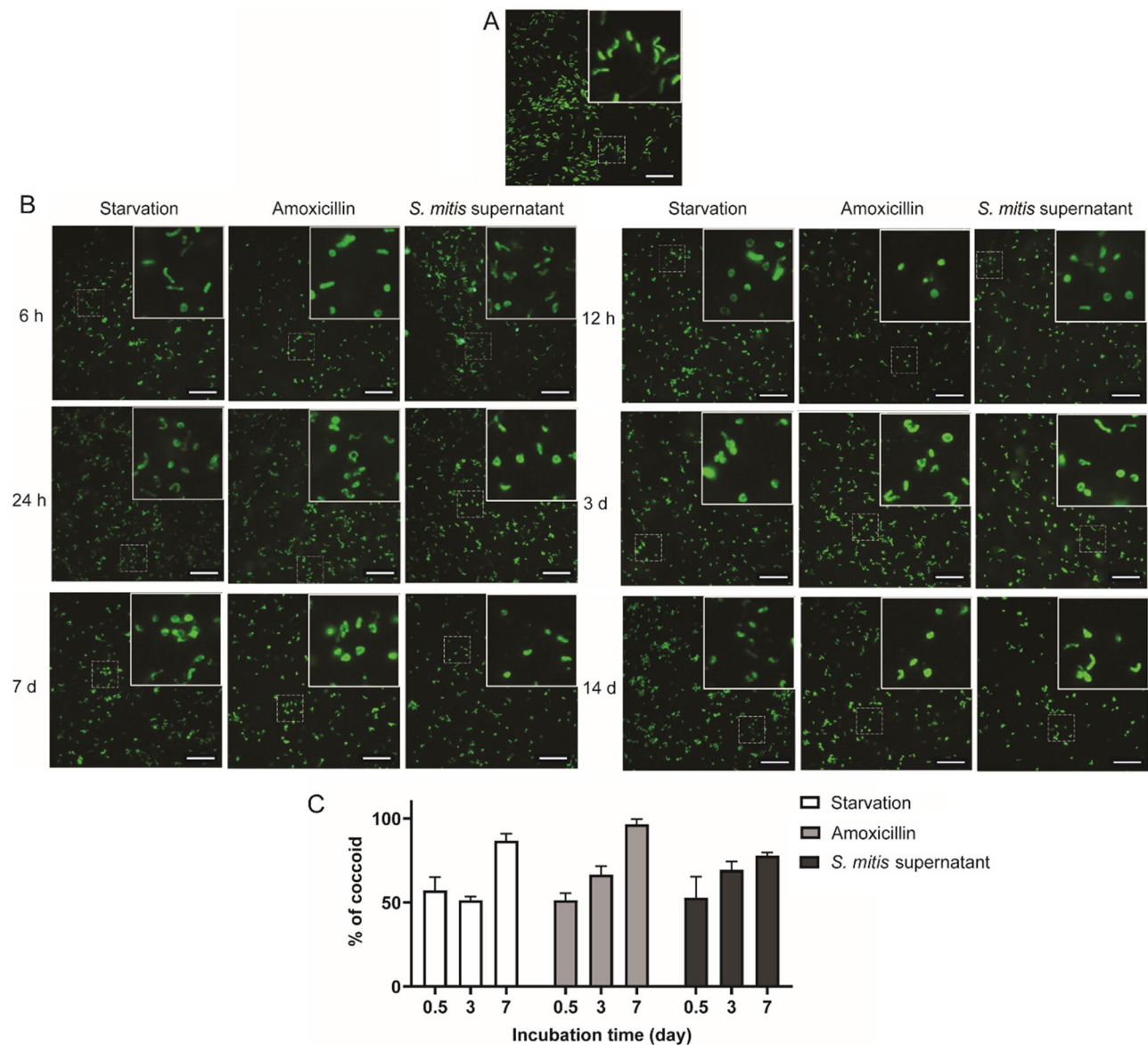
Fig. 3 Morphological changes in *H. pylori* from spiral to coccoid forms. *H. pylori* cells were collected from chocolate agar plates and suspended in BHI broth for coccoid generation via three methods. (**A**) The SYTO9 fluorescent image of the spiral form of *H. pylori* was observed by immediately staining the sample obtained from the colony. (**B**) *H. pylori* cells from samples subjected to three methods (starvation, amoxicillin, and *S. mitis* supernatant) were stained with SYTO9 to observe morphological changes. The scale bars indicate 10 µm. (**C**) Percentages of coccoid form were calculated at 12 h, 3 days, and 7 days of culture for the three methods. Means and standard deviations (SDs) ($n = 3$) ($n = 3$: technical replicates) are shown as columns and error bars, respectively

and *S. mitis* supernatant-treated *H. pylori* groups, respectively (Fig. 3C). Coccoid transformation continued as the culture time progressed, reaching 88%, 95%, and 77% of the total bacteria in the starvation, amoxicillin-treated *H. pylori* groups, and *S. mitis* supernatant-treated *H. pylori* groups, respectively, after 7 days of culture (Fig. 3C).

Culturability of coccoids from *H. pylori*

The culturability of *H. pylori* cells from the three methods was assessed by measuring colony formation on chocolate agar plates (Fig. 4A). Among the three methods, the amoxicillin group showed the most rapid decrease in CFUs after 12 h of culture, and no colonies formed since the third day of culture. In the starvation group, the number of colonies also decreased over time, and no colonies were observed after the seventh day. In contrast, CFUs in

the *S. mitis* supernatant –treated *H. pylori* group initially tended to decrease until the third day but then gradually increased (Fig. 4B). The culturability of the *S. mitis* supernatant-treated *H. pylori* group was observed until the 21st day of culture, but no colonies were observed on the 24th and 27th day.

Viability of the coccoid *H. pylori* form

SYTO9 and PI staining were used to confirm the viability of *H. pylori* cells. While SYTO9, which is membrane permeable and stains nucleic acids with green fluorescence, can stain both living and dead cells, nonmembrane permeable PI stains only dead cells and emits red fluorescence [20]. The viability of the three methods determined by SYTO9/PI staining was compared from 12 h to 14 days (Supplementary Fig. 2).
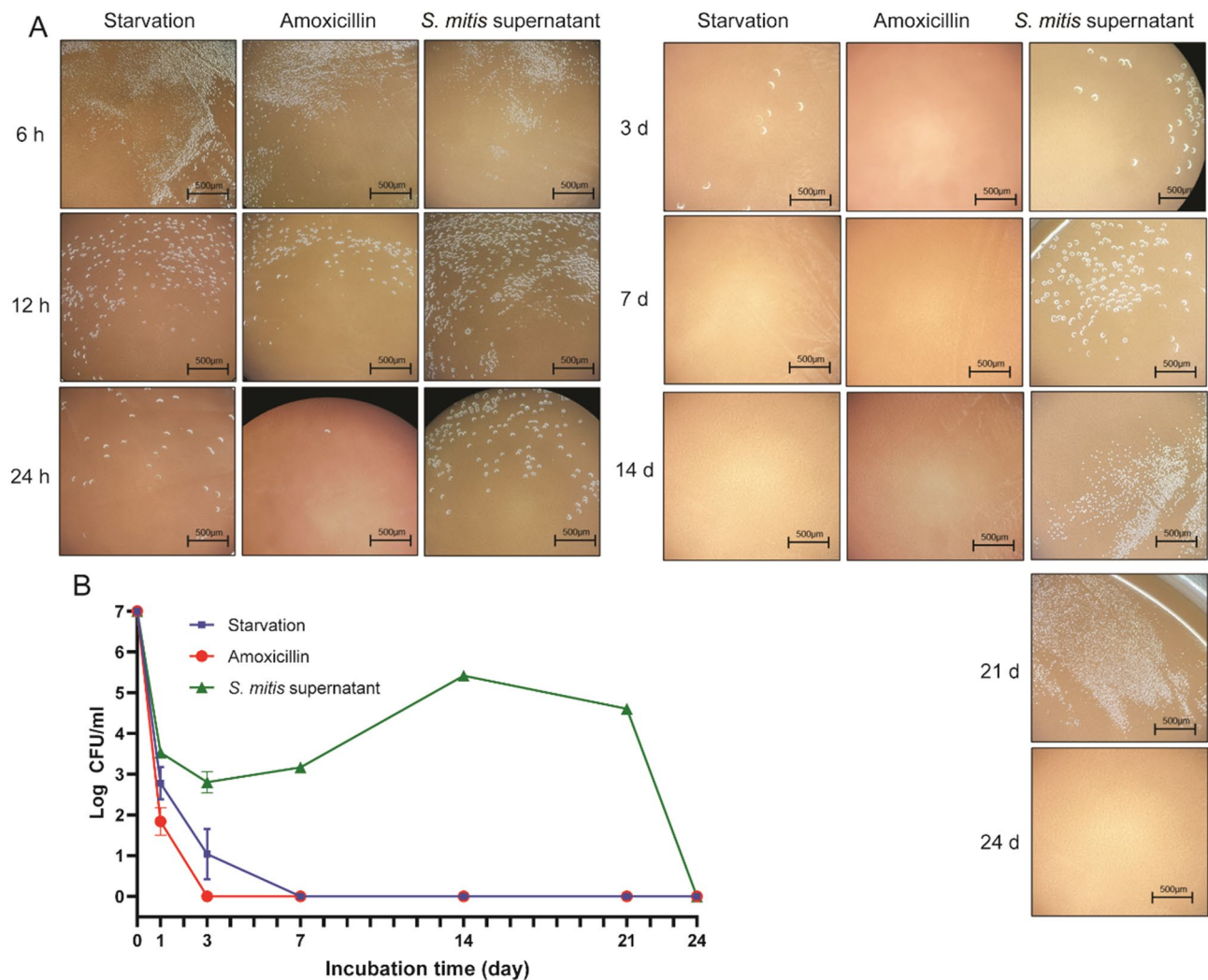
Fig. 4 Culturability of *H. pylori* cells from the three methods. (**A**) At the designated time points, *H. pylori* cells were plated on chocolate agar plates and cultured for 2 days. In the *S. mitis* supernatant-treated *H. pylori* group, colonies were observed to continue growing at 14 days, so culturability was also checked on days 21, 24 and 27 (data not shown for days 27). Images of the culture plates were obtained using a stereoscopic microscope. Colonies were observed as white due to the reflection of the light. The scale bars indicate 500 μm. (**B**) CFUs of *H. pylori* were counted to quantitatively analyse culturability. The means and SDs ($n = 3$) ($n = 3$: technical replicates) are shown as dots and error bars, respectively

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When SYTO9/PI staining was performed on the 12 h in the starvation group, the number of spiral form bacteria was comparable to that of the coccoid form. However, more than half of the spiral forms were stained with PI, indicating that they were dead. On the other hand, most coccoid forms showed only green fluorescence, indicating that they were alive. After 7 days of culture, most of the bacteria changed to coccoid forms that were viable, but the spiral forms were all stained with PI, indicating they were dead (Fig. 5A, B). In the amoxicillin-treated group that did not grow in culture by day 3, more than 50% had changed to a coccoid or U-shaped morphology. Among those that changed to coccoid forms, the bacteria were confirmed to be alive, whereas all the spiral forms were found to be dead. This finding indicates that the bacteria were viable but nonculturable (VBNC). The living coccoid form of *H. pylori* was observed on the seventh and fourteenth days in the amoxicillin group (Fig. 5A, Supplementary Fig. 2).

Approximately half of the bacteria from the *S. mitis* supernatant-treated *H. pylori* group also changed to viable coccoid forms when stained only with SYTO9 at 12 h, and the number of coccoid forms increased gradually over time. Compared to the starvation and amoxicillin groups, which showed no living spiral forms on culture day 7, the *S. mitis* supernatant group showed a greater proportion of living spiral forms (Fig. 5A, B).

Protein changes according to the coccoid transformation of *H. pylori*

We performed proteomics to analyse the differences in protein profiles between the spiral and coccoid forms and protein profiles among the coccoid forms generated by the three methods. Each score is an indication of sensitivity, with higher scores indicating better detection, but this is a relative number and cannot be measured quantitatively. We first compared the proteins between the spiral form and the starvation-induced coccoid form, and the proteins with the highest common scores were the elongation factor Tu and the 60 kDa chaperonin. The proteins that were detected in both groups but were specifically expressed with more than 2-fold higher scores in the spiral form were LPP20 lipoprotein, ATP synthase subunit beta, and CTP synthase, while proteins that were more highly expressed after the change to the coccoid form were urease subunit alpha and uncharacterised protein. There were also proteins that did not score high but were only seen in the spiral form and proteins that were only seen in the coccoid form (supplementary Table 1). When comparing the proteins expressed in the coccoid form induced by amoxicillin with those in the spiral form, the highest scoring proteins in both were Elongation factor Tu and 60 kDa chaperonin. The LPP20 lipoprotein was reduced in expression in the starvation-induced coccoid form but increased in score in the amoxicillin-induced coccoid form. The proteins that scored
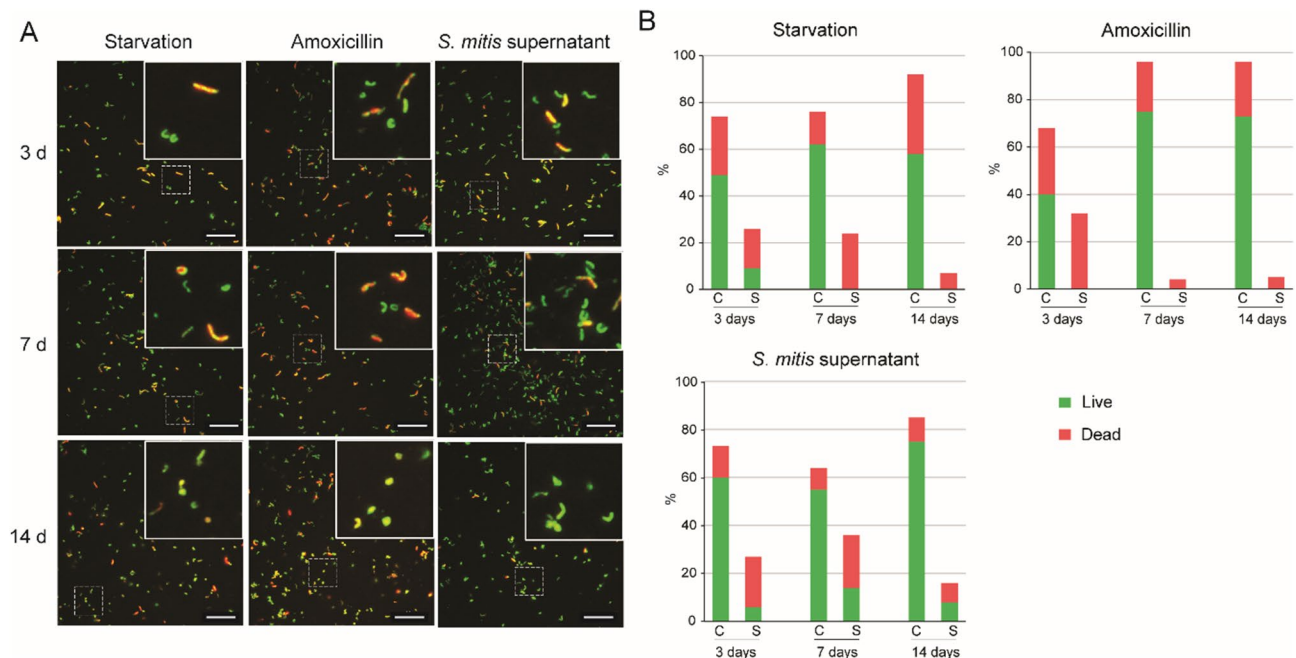

Fig. 5 Viability of *H. pylori* cells. (**A**) At the designated time points, cells from the three methods were stained with SYTO9/PI (LIVE/DEAD) staining reagents. SYTO9 stained both live and dead cells with green fluorescence. PI stained only dead cells with red fluorescence. In merged images, dead cells are shown as an orange fluorescent color. The scale bars indicate 10 µm. (**B**) Percentages of coccoid and spiral forms, along with the proportions of live and dead cells, were counted at 3, 7, and 14 days of culture for the three methods

more than twice as high in the spiral form as in the amoxicillin-induced coccoid form were CTP synthase and adhesion, ATP-dependent zinc metalloprotease FtsH, and the chaperone protein ClpB, while the proteins that scored higher in the coccoid form were LPP20 lipoprotein, Thiol peroxidase, urease subunit alpha, DUF3944 domain-containing protein, and uncharacterised protein. There were many differences in proteins seen only in the spiral form and the amoxicillin-induced coccoid form, which are summarised in Supplementary Table 2. When comparing the proteins of the spiral form and the coccoid form induced by *S. mitis* supernatant, the highest scoring proteins in both groups were the 60 kDa chaperonin and the elongation factor Tu. Among the proteins common to both groups, those whose scores decreased by less than half in the coccoid form compared to the spiral form were phosphoglycerate kinase, CTP synthase, ATP synthase subunit alpha, and the chaperone protein ClpB, while those whose scores increased by more than twofold in the coccoid form were aconitate hydratase B, urease subunit alpha, uncharacterised protein, 2-oxoglutarate ferredoxin oxidoreductase subunit alpha, 2-oxoglutarate oxidoreductase alpha subunit, 2-ketoisovalerate ferredoxin oxidoreductase, and adhesin. These proteins that were common to both groups are listed in Supplementary Table 3, along with those that were measured only in the spiral form and the *S. mitis*-induced coccoid form, respectively. Proteins detected in only the spiral form, and only the coccoid form from the three methods

are listed with scores in Supplementary Table 4, respectively. Comparing spiral and coccoid forms from each method, there were proteins detected only in the coccoid forms (Fig. 6A-C). When proteins detected only in coccoid forms were analysed among the three methods, 13 proteins were commonly detected (Fig. 6D). The protein with the highest scores in all three groups was type I glutamate-ammonia ligase. Thioredoxin reductase, and SabA_adhesion domain-containing protein were higher in the *S. mitis* supernatant groups. A list of proteins commonly detected in all three coccoid forms is shown in Table 1. Proteins not seen in the coccoid form but only in the spiral form include ATP-dependent Clp protease proteolytic subunit, tumor necrosis factor alpha-inducing protein, AAA family ATPase, DUF 1523 family protein, and threonine-t RNA ligase. The proteins that appeared only in the spiral form are listed in Supplementary Table 4, along with the proteins that appeared only in each of the coccoid forms. Among the proteins seen in both the spiral form and the coccoid form of all three groups, Elongation factor Tu and 60 kDa chaperonin showed the highest scores. Among the proteins seen in all four groups, CTP synthase showed more than twice the score in the spiral form compared to the coccoid form in the three methods, thiol peroxidase showed over double the score in the amoxicillin group, and 2-ketoisovalerate ferredoxin oxidoreductase showed more than twice the score in the *S. mitis* group. (supplementary Table 5).
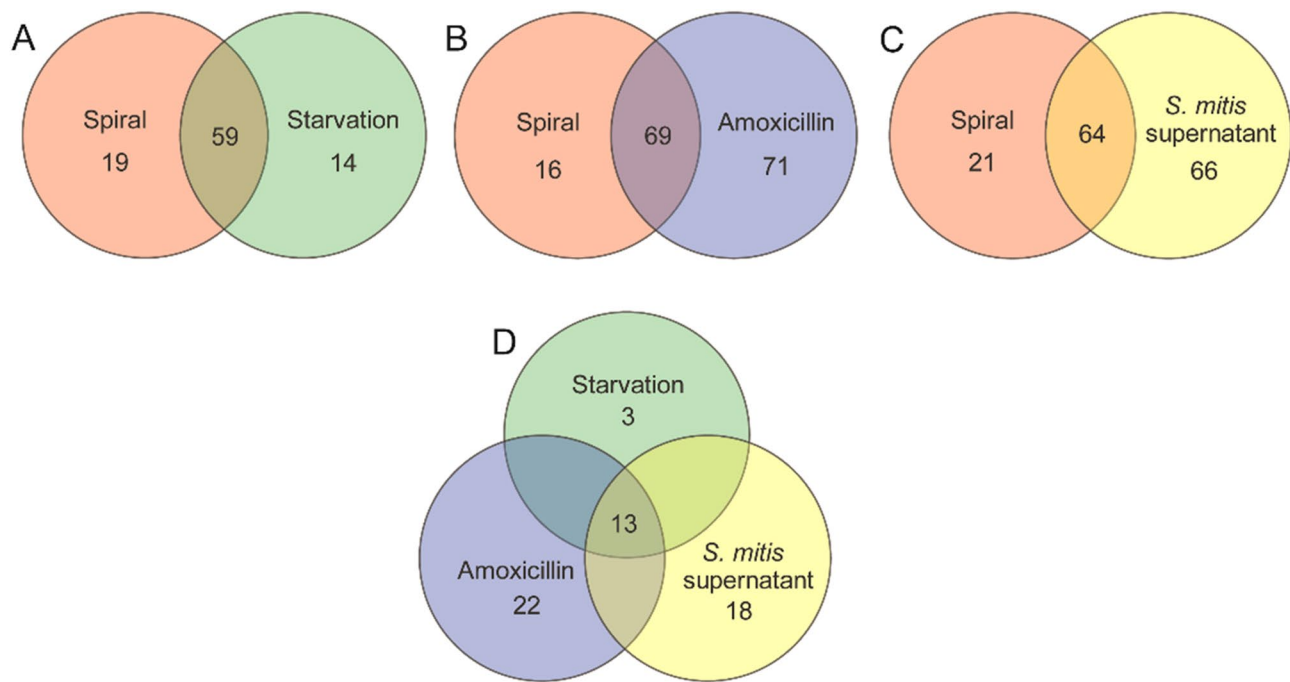
Fig. 6 Summary of proteomics results. (**A-C**) Venn diagrams showing unique and common proteins between the spiral and coccoid forms generated by starvation (**A**), amoxicillin treatment (**B**), and *S. mitis* supernatant treatment (**C**). (**D**) Venn diagram showing the unique and common proteins among the coccoid-specific proteins identified by the three methods

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Table 1 Proteins commonly detected in coccoid forms via three methods

Protein name	Gene name	Score		
		Starvation	amoxicillin	*S.mitis* supernatant
Type I glutamate–ammonia ligase	glnA	118	189	169
SabA_adhesion domain-containing protein	C2R45_08160	107	50	172
Cysteine desulfurase IscS	nifS, iscS	51	91	44
30 S ribosomal protein S1	EG65_05625	81	48	78
Chemotaxis protein CheY	EG65_00875	73	79	79
Thioredoxin reductase	EG65_02680	72	77	154
Chaperone protein HtpG	htpG	71	66	64
Succinyl-CoA:3-ketoacid-CoA transferase	EG65_03570	65	60	55
Protease	EG65_00890	54	61	51
GTP-binding protein	EG65_02360	51	76	41
7-alpha-hydroxysteroid dehydrogenase	EG65_02220	47	51	59
10 kDa chaperonin	groS	40	48	41
Ribose-phosphate pyrophosphokinase	prs	52	72	61

Discussion

Clinical significance of the coccoid form and comparison of three methods for generating the coccoid *H. pylori*

H. pylori, a gram-negative and microaerophilic bacterium, plays an important role in gastritis, peptic ulcer disease, and gastric cancer [2]. It is known as a spiral-shaped bacterium with flagella that moves in the thick gastric mucus, but changes to a coccoid form when exposed to certain situations or stresses [6]. This morphological change is considered to be one of the survival mechanisms of *H. pylori* under adverse environmental conditions, including aerobic conditions, alkaline pH conditions, high temperature, nutrient depletion, antibiotic treatment, proton pump inhibitor treatment, and co-culture with other bacteria [5, 14, 24–26]. These coccoid *H. pylori* cells are usually in the VBNC state. The important point of this state is that they have minimal metabolic activity but express virulence factors such as urease, HpaA, CagA, CagE, VacA, and BabA [27, 28]. These factors affect the development of chronic inflammation and diseases such as gastric cancer [29].

In this study, we induced morphological changes in *H. pylori* to coccoid forms using three clinically important methods: starvation, amoxicillin antibiotic treatment, and *S. mitis* supernatant treatment. We experimentally confirmed the VBNC state by comparing colony formation on culture plates and fluorescence staining with SYTO9/PI reagents (LIVE/DEAD bacterial viability staining kit).

In our preliminary study comparing the morphology of *H. pylori* by Gram stain, DAPI stain, Hoechst, and SYTO9 staining, the most distinct morphology was observed with SYTO9 staining, and SYTO9/PI staining could simultaneously confirm viability; therefore, we used this method to monitor coccoid changes. We observed that spiral-shaped *H. pylori* changed to coccoid shapes and various shapes, such as the U, donut,

and short rod forms, which are considered intermediate forms in the process of becoming coccoid forms. These shape changes are thought to be an adaptive mechanism of *H. pylori* to unfavourable conditions and are related to peptidoglycan and cytoskeleton rearrangement [11]. We also observed that some cells changed to filament shapes, which later disappeared, probably due to their septation into shorter cells. In this study, we counted and assessed only the spiral and coccoid forms (including the U and donut forms) because the significance of the rod and filamentous forms is not clear. The proportion of *H. pylori* that changed to a coccoid shape over time was greater when only living cells were considered. In the amoxicillin-treated group, some spiral *H. pylori* cells stained with SYTO9 but not with PI were observed on the third day, but they did not form colonies on chocolate agar plates, suggesting that these cells might have intact cell membranes but lost the ability to proliferate (Figs. 4 and 5B). As a survival strategy, *H. pylori* cells undergo coccoid transformation, which enables them to persist for longer periods of time with reduced metabolic activity [30].

The first method we applied to generate the coccoid form was starvation by long broth culture. Starvation can be an unfavourable condition that *H. pylori* encounters under various environmental conditions. For starvation induction, we cultured *H. pylori* in two broths: one in BHI broth and the other in BHI broth with 10% FBS. Through repeated experiments, we observed minimal differences in the rate of coccoid formation between the two groups (Supplementary Fig. 1). This finding indicates that *H. pylori* transform into the coccoid form in prolonged broth culture as nutrients are depleted, regardless of the presence of serum. Thus, broth culture itself is an unfavourable condition for bacterial growth. Consequently, we established a baseline condition of starvation method in BHI broth and compared three groups under the same conditions.

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We observed the coccoid transformation of *H. pylori* by treating the bacteria with amoxicillin, a commonly used antibiotic used for *H. pylori* eradication therapy, at a half the MIC. The clinical significance of the coccoid conversion of *H. pylori* by antibiotic treatment is that it may result in the failure of eradication therapy. In this study, we observed rapid coccoid transformation in a culture containing amoxicillin, the most potent coccoid form changer among antibiotics [17], and confirmed that the cells were in the VBNC state for more than 14 days. When *H. pylori* is exposed to amoxicillin, it can rapidly transform into a coccoid form that is tolerant to the antibiotic, potentially leading to treatment failure. Therefore, the impact of the coccoid form in *H. pylori* eradication should not be ignored.

The third method that we used to generate the coccoid form was supplementing the culture broth of *H. pylori* with the supernatant of *S. mitis*. We compared the culture conditions, we found that *S. mitis* grew much better under microaerophilic condition, which is more similar to the conditions under which *S. mitis* grows in the human body. It has been confirmed in gerbil and mouse studies that long-term infection by *H. pylori* changes the composition and diversity of the gastric microbiota due to increased gastric pH, epithelial cell destruction, and metabolic products from infection [19, 31, 32]. When the gastric microbiota is altered by *H. pylori* infection, *S. mitis*, which is part of the human oral microbiota, is significantly increased in the gastric mucosa of patients with atrophic gastritis or gastric cancer [18]. Khosravi et al. showed the coccoid transformation of *H. pylori* by *S. mitis* using an in vitro co-culturing system as well as by supplementing the culture supernatant of *S. mitis* [24]. These authors suggested that fast-growing *S. mitis* contributed to nutrient depletion and induced a morphological change in *H. pylori* from a spiral to a coccoid form when they were co-cultured. The authors asserted that *S. mitis* produced and released diffusible factor(s) that induce coccoid conversion of *H. pylori* cells. However, in our study, *H. pylori* treated with the *S. mitis* supernatant was culturable for the most extended period among the three methods, showing different results from those of Khosravi et al. Moreover, in this experiment, the group that added *S. mitis* supernatant, even though it was nutritionally deficient, remained culturable and maintained the spiral form longer. In our experiments, the number of colonies in the *S. mitis* supernatant-treated *H. pylori* group initially decreased but increase again after approximately 4 days. This suggests that the culturability of *H. pylori* initially declined due to nutrient deficiency but was later restored, possibly due to substances present in the *S. mitis* supernatant. Based on our results, we speculated that there may be some substances in the supernatant of *S. mitis* that allow *H. pylori* to maintain culturability for a long time. These findings imply that *S. mitis* may prolong the survival of *H. pylori* when they coexist in the stomach, affecting the pathogenicity of *H. pylori*. However, more research is necessary to understand the interaction of *S. mitis* with *H. pylori* and its subsequent effects on the gastric diseases.

As confirmed in this study, the coccoid *H. pylori* can survive for a long time under unfavourable conditions. The extended survival of the coccoid form in unfavourable gastric environments, potentially due to antibiotic tolerance, may contribute to eradication failure [28]. Furthermore, these coccoid *H. pylori* can affect the occurrence and progression of gastric cancer [7, 9]. This suggests that it would be necessary to eradicate both the spiral and coccoid forms to successfully eradicate and prevent gastrointestinal diseases.

Proteomic analysis of the coccoid *H. pylori*

We performed proteomics analysis of the spiral and coccoid forms of *H. pylori* generated by three methods. Through this analysis, we were able to identify proteins that are detected only in spiral form, proteins that are detected only in coccoid form, proteins that are detected in both forms, and proteins that are commonly detected in all three coccoid forms of *H. pylori*. (Fig. 6; Table 1). Although the 60-kDa chaperonin (GroEL) was detected in the spiral form, it was more abundant in the coccoid form in our study. GroEL belongs to the chaperonin family and is required for proper folding of proteins in many bacteria [33]. Protein folding, which induces protein denaturation, is important for bacterial survival under stressful conditions. In addition to its chaperone activity, GroEL is reported to bind to iron [34]. The acquisition of iron is essential for nearly all organisms and is involved in the efficiency of several metabolic processes, such as respiration and oxygen transport [35]. The abundance of these proteins in the coccoid *H. pylori* may be advantageous for the nutritional supply and stabilisation of cellular proteins. Furthermore, GroEL is a heat shock protein (HSP60) that can induce the expression and secretion of tumor necrosis factor-α (TNF-α) in host cells, contributing to disease development by producing proinflammatory cytokines [36, 37]. Tanaka et al. reported that the serum antibody levels against *H. pylori* HSP60 were greater in patients with gastritis and gastric cancer, suggesting that HSP60 may be associated with gastric carcinogenesis [37]. HSP60, which is expressed in the coccoid form, may continuously affect the occurrence of gastric cancer even after *H. pylori* becomes dormant, but this needs to be further studied in the future. Elongation factor (EF) Tu is the protein with the highest scores in the spiral and coccoid forms of *H. pylori*. It is related to transcription and translation, contributing to peptide chain elongation during protein translation. Increased EF Tu

expression levels in the coccoid form of *H. pylori* demonstrate continued protein translation [38].

Our study revealed that the CTP synthase (CtpS) score is more than twice as high in the spiral form of *H. pylori* than in its coccoid form (Supplementary Table 5). This finding is consistent with the known role of CtpS in other bacteria, such as *Caulobacter* and *E. coli*, where CtpS is involved in cell shape regulation and enzymatic activity. In *Caulobacter*, CtpS forms filaments that regulate cell curvature by interacting with crescentin, a cytoskeletal element. This interaction is crucial for maintaining the characteristic curved shape of *Caulobacter*. The higher expression of CtpS in the spiral form of *H. pylori* suggests that CtpS may play a role in maintaining the spiral shape, which is critical for its motility and colonisation in the gastric mucosa [39].

The ATP-dependent Clp protease proteolytic subunit (ClpP) and AAA family ATPases were expressed only in the spiral form of *H. pylori*, but not in the coccoid form. The Clp proteases consist of a self-compartmentalized peptidase and ATPases associated with diverse cellular activities (AAA family ATPases). These components collaborate to carry out regulated protein degradation in a variety of physiological processes, including homeostatic protein quality control, responses to environmental stress, and virulence in pathogenic bacteria [40]. The active state likely involves high metabolic activity, where the demand for protein synthesis and degradation is elevated. The presence of ATP facilitates the binding and activation of AAA+family, enabling efficient protein unfolding, translocation, and degradation. This process ensures that misfolded or damaged proteins are promptly removed, maintaining cellular homeostasis and promoting bacterial growth and virulence. In our study, in the coccoid form of *H. pylori*, these proteolytic components were not expressed. As reported, dormant *Mycobacterium* carries out less Clp-mediated protein degradation, with ClpP1 and ClpP2 existing predominantly in inactive conformations [40]. Similarly, the lack of expression of these proteins in the coccoid form of *Helicobacter pylori* would be a strategic adaptation to conserve energy during periods of low metabolic activity. The inactivity of ClpP in the absence of sufficient ATP and AAA+family binding prevents unnecessary degradation of nascent polypeptides or transiently disordered proteins [40]. By minimizing energy expenditure on protein degradation during dormancy, *H. pylori* might enhance its long-term viability and persistence.

There were proteins that were commonly expressed in the coccoid forms induced by the three methods, the highest scoring of which was type I glutamate-ammonia ligase (Table 1). Type I glutamate-ammonia ligase, also known as glutamin synthetase, plays an essential role in nitrogen assimilation [41]. This enzyme catalyses the

ATP-dependent conversion of glutamate and ammonia into glutamine. And in some bacteria, it is linked to responses to environmental stresses, such as nutrient depletion [41]. Thioredoxin reductase was commonly detected in three methods inducing coccoid forms, with the highest score observed in the *S. mitis* supernatant-treated group (Table 1). This protein increases tolerance against oxidative stress, suggesting that the coccoid *H. pylori* may survive under unfavourable conditions such as oxidative stress [42]. There were differences in protein expression in the coccoid forms induced by each method, suggesting that the predominant mechanisms of coccoid transformation in response to external stress may differ depending on the situation. Additionally, the proteins commonly observed in the spiral form and across all three groups exhibited varying scores for each method, indicating that the key proteins utilized in each group might vary. In this study, some proteins that are highly expressed in the coccoid *H. pylori* were shown to play a role in securing nutrition in extreme situations or resisting various stresses, thus promoting survival under unfavourable conditions. Further studies on the proteins expressed in the coccoid form of *H. pylori* are necessary to understand the significance of these proteins.

In this study, we set up BHI broth culture without serum as a method to induce the coccoid form by starvation. Even with the inclusion of serum, prolonged broth culture itself acts as a stress factor for the survival of *H. pylori*. In our experiments, adding serum to BHI broth did not significantly impact the transformation to the coccoid form (Supplementary Fig. 1). To ensure consistent growth conditions across the three groups, serum was not added to any of the samples. This could be a limitation of the experiment, as the absence of serum might have influenced the starvation effects on the amoxicillin and *S. mitis* groups. However, we reliably confirmed that even under nutrient-deficient conditions, the addition of *S. mitis* supernatant significantly prolonged the culturability and maintenance of the spiral form of *H. pylori*. Another limitation of this study is that the *H. pylori* strain we tested was a standard strain, so different clinical strains may yield different results. Third, the proteins expressed when *H. pylori* changes to the coccoid form in the stomach may differ from those expressed in vitro. Therefore, it may not establish a relationship between the expression of these proteins and the development of gastritis or gastric cancer.

Conclusions

We compared the transformation of *H. pylori* into a coccoid form using three methods: (i) starvation, the traditional method of coccoid generation; (ii) the amoxicillin method, which uses the most representative antibiotic for treating *H. pylori*; and (iii) the *S. mitis* supernatant

method, which uses the culture supernatant of a coexisting bacterium in a gastric environment. We compared the culturability, viability, and morphological changes of each group over time and found that the spiral form of *H. pylori* changed into the coccoid form by using all three methods.While amoxicillin and starvation induced *H. pylori* to transform into a coccoid form, culturing with *S. mitis* supernatant allowed some *H. pylori* cells to remain in a viable spiral form for an extended period and remain culturable. We speculate that the *S. mitis* supernatant may contain substances that prevent spirals from changing into coccoid forms and allow them to be cultured. Additionally, we also believe that since the extended survival of the coccoid form in unfavourable gastric environments may contribute to treatment failure, it is necessary to eradicate both the spiral and coccoid forms to achieve successful eradication. Through proteomics analysis, we identified proteins that are commonly detected in the coccoid *H. pylori* via three different methods and, also confirmed the differences from the proteins expressed in the spiral form. Although all three methods generated coccoid forms, the protein profiles of the coccoid forms varied considerably. Further studies are needed to explore the relevance of the proteins expressed in the coccoid form.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-024-03599-5.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Author contributions
KJ was involved in the study concept, drafted the manuscript, and secured funding for the project; JKK was involved in the study concept and design, data analysis and interpretation of the data, and study supervision; HB, BJ and performed the experiments and analysed the data; MIP was involved in study supervision; and JYL was involved in the data analysis and interpretation, drafted the manuscript, and approved the final version of the manuscript. The final manuscript has been reviewed and approved by all the authors.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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