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RESEARCH ARTICLE

The effect of altered pH growth conditions on the production, composition, and proteomes of *Helicobacter pylori* outer membrane vesicles

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Abstract

Gram-negative bacteria release outer membrane vesicles (OMVs) that contain cargo derived from their parent bacteria. *Helicobacter pylori* is a Gram-negative human pathogen that produces urease to increase the pH of the surrounding environment to facilitate colonization of the gastric mucosa. However, the effect of acidic growth conditions on the production and composition of *H. pylori* OMVs is unknown. In this study, we examined the production, composition, and proteome of *H. pylori* OMVs produced during acidic and neutral pH growth conditions. *H. pylori* growth in acidic conditions reduced the quantity and size of OMVs produced. Additionally, OMVs produced during acidic growth conditions had increased protein, DNA, and RNA cargo compared to OMVs produced during neutral conditions. Proteomic analysis comparing the proteomes of OMVs to their parent bacteria demonstrated significant differences in the enrichment of beta-lactamases and outer membrane proteins between bacteria and OMVs, supporting that differing growth conditions impacts OMV composition. We also identified differences in the enrichment of proteins between OMVs produced during different pH growth conditions. Overall, our findings reveal that growth of *H. pylori* at different pH levels is a factor that alters OMV proteomes, which may affect their subsequent functions.

KEYWORDS
acidic growth conditions, bacterial membrane vesicles, *Helicobacter pylori*, outer membrane vesicles, proteome

Abbreviations: BMVs, bacterial membrane vesicles; OMVs, outer membrane vesicles; MVs, membrane vesicles; 5.3U-OMVs, OMVs produced during growth at pH 5.3 with urea; 7.2U-OMVs, OMVs produced during growth at pH 7.2 with urea; 7.2-OMVs, OMVs produced during growth at pH 7.2 in the absence of urea; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; HBA, horse blood agar; BHI, brain heart infusion; HCl, hydrochloric acid; PES, polyethersulfone; DPBS, Dulbecco's PBS; TEAB, triethylammonium bicarbonate; FA, formic acid; AGC, automatic gain control; maxLFQ, label free quantification; PSM, peptide spectrum match; DAVID, database for annotation, visualization, and integrated discovery bioinformatics.

1 | INTRODUCTION

Bacterial membrane vesicles (BMVs) are nanoparticles that are released by bacteria as part of their normal growth. BMVs are broadly classified into outer membrane vesicles (OMVs) when produced by Gram-negative bacteria, and membrane vesicles (MVs) when produced by Gram-positive bacteria, with different subtypes of BMVs existing as a result of various mechanisms of biogenesis [1]. BMVs collectively have been shown to package cargo derived from their parent bacteria including nucleic acids [2–6], lipids [6, 7], and proteins [8–11], and BMVs produced via different mechanisms of biogenesis have altered cargo packaging [1]. Due to their biological cargo, BMVs can serve a range of functions that include facilitating interbacterial communication and mediating pathogenesis in the host [1, 12–14]. However, there are several fundamental factors that have been shown to affect BMV production, composition, and their biological functions, which includes environmental factors [15, 16], bacterial growth stage and bacterial growth conditions [5, 8]. For example, bacterial growth stage was shown to affect the proteome of *Helicobacter pylori* OMVs, as OMVs produced during different growth conditions differed in their proteome, and to that of their parent bacteria, revealing that OMV composition is regulated throughout bacterial growth [8]. Despite our growing understanding of factors that regulate OMV composition, we have minimal knowledge regarding how pH growth conditions affect the proteome of OMVs produced by bacteria.

H. pylori is a Gram-negative pathogen that colonizes the human stomach of approximately half of the world's population. *H. pylori* within the gastric mucosa has been shown to produce OMVs [17] that can contribute to mediating pathogenesis via their interactions with host epithelial cells [18, 19]. During colonization of the human stomach, *H. pylori* initially encounters a low pH environment, and subsequently begins producing the enzyme urease, which catalyses the hydrolysis of urea to ultimately neutralize the pH of the local environment surrounding the organism [20–22]. Although *H. pylori* can remain viable during acidic conditions in the absence of urea, the organism becomes non-replicative, highlighting the requirement of urea for optimal bacterial growth during acidic conditions [23, 24]. Despite our knowledge of the adaptations of *H. pylori* to survive and colonize the acidic environment of the stomach, the effect of the acidic gastric pH environment on the production and composition of *H. pylori* OMVs has not been examined.

In this study, we isolated OMVs produced by *H. pylori* grown during acidic and neutral growth conditions and characterized their production, size, and composition. When investigating their cargo composition, we found that *H. pylori* OMVs produced during acidic conditions packaged significantly more protein, DNA, and RNA compared to OMVs produced during neutral conditions. Proteomic analysis revealed that the proteomes of *H. pylori* OMVs produced during acidic and neutral pH conditions differed significantly to that of their parent bacteria, indicating selective packaging of proteins into *H. pylori* OMVs. Furthermore, we found that *H. pylori* OMVs produced during acidic and neutral pH conditions differed in their proteome and the type of proteins packaged within OMVs. Overall, our findings reveal that pH growth conditions alters the packaging of protein within *H. pylori* OMVs, allowing *H. pylori* to generate bespoke OMVs during challenging growth conditions.

Significance Statement

In this study, we examined the effect of bacterial growth at different pH levels on the production, composition, and proteomes of *H. pylori* OMVs. We identified that growth of *H. pylori* at an acidic pH resulted in the production of fewer and smaller OMVs, that had a significantly different proteome when compared to their parent bacteria. Importantly, we identified different enrichment of proteins within OMVs produced during pH 5.3 growth conditions compared to OMVs produced at a pH of 7.2. The enrichment of proteins within OMVs derived from pH 5.3 conditions, such as HomA and TlpC that contribute to bacterial adherence and chemotaxis respectively, suggest that OMVs produced during different pH growth conditions may have altered functions. In contrast, OMVs produced at a neutral pH were enriched in VacA and the putative beta-lactamase HcpE, indicating their potential roles in pathogenesis. These findings contribute to our growing knowledge of the effect of bacterial growth conditions on the regulation of OMV production and cargo packaging by *H. pylori*. Overall, gastric pH is a regulator of *H. pylori* proteomes as well as the production, composition and proteome of *H. pylori* OMVs which may contribute to the survival and subsequent colonization and pathogenesis of *H. pylori* in their host.

2 | MATERIALS AND METHODS

2.1 | Bacterial culture conditions

H. pylori 26695 was maintained using horse blood agar (HBA; Oxoid, USA) supplemented with 8% horse blood (Australian Ethical Biologicals, Australia) and 0.2% Skirrow's selective supplement (0.0155% (w/v) polymyxin B, 0.625% (w/v) vancomycin, 0.3125% (w/v) trimethoprim, 0.125% (w/v) amphotericin B, Sigma–Aldrich, USA) and incubated at 37°C in microaerophilic conditions (CampyGen 2.5L, Oxoid, USA) as previously [8, 18]. For the production of *H. pylori* OMVs, brain heart infusion broth (BHI; BD Biosciences, USA) was supplemented with 0.2% Skirrow's selective supplement, and 0.2% (w/v) β-cyclodextrin (Thermo Fisher Scientific, USA), with or without the addition of 5 mmol/L urea (Sigma–Aldrich, USA). The pH of the supplemented BHI media was either adjusted using hydrochloric acid (HCl) to pH 5.3, or not adjusted and left at pH 7.2, and subsequently inoculated with *H. pylori* at an OD_{600nm} of 0.05 as previously described [25–27]. Liquid cultures of *H. pylori* were grown at 37°C for 16 h with shaking

at 120 rpm in microaerophilic conditions (CampyGen, Oxoid, USA), as previously [8, 18]. Viable counts were enumerated by plating bacteria on HBA and incubating at 37°C for 5 days.

2.2 | OMV isolation and purification

OMVs were isolated from broth cultures using established techniques [5, 8, 28–30]. Briefly, *H. pylori* 26695 liquid cultures that were grown in BHI at pH 5.3 in the presence of urea, or pH 7.2 in the presence or absence of urea and incubated using microaerophilic conditions at 37°C for 16 h were used for the isolation of OMVs. Bacteria were pelleted by centrifugation at 4000 × *g* for 15 min at RT, and the bacterial free supernatant was filtered using a 0.22 µm filter. Crude OMVs were pelleted from the bacterial free supernatant by ultracentrifugation at 100,000 × *g* for 2 h at 4°C (CP100NX Ultracentrifuge, Hitachi, Japan). OMVs were then purified using an iodixanol (OptiPrep, Sigma–Aldrich, USA) density gradient as previously described [8, 28, 29, 31]. Briefly, OMVs were resuspended in 45% (v/v) OptiPrep and underlaid in a discontinuous OptiPrep density gradient, consisting of 20%, 25%, 30%, 35% and 40% OptiPrep solution diluted using sterile Dulbecco's phosphate buffered saline (DPBS; Gibco, USA). The discontinuous gradient was ultracentrifuged at 100,000 × *g* for 16 h at 4°C, and a total of twelve 1 mL fractions were collected, with OMVs being contained within fractions 4–10. Fractions 4–10 containing OMVs were then pooled and washed twice using sterile DPBS by ultracentrifugation at 100,000 × *g* for 2 h at 4°C and were resuspended in DPBS and stored at −80°C until required.

2.3 | Transmission electron microscopy

Transmission electron microscopy (TEM) was performed as previously [9, 29]. Briefly, carbon-coated copper grids (Ted Pella, USA) were treated with poly-L-lysine (Sigma–Aldrich, USA). OMV samples were coated onto grids and fixed using 1% glutaraldehyde (Sigma–Aldrich, USA) diluted using DPBS, stained using 2% (w/v) uranyl acetate (Electron Microscopy Sciences, USA) pH 7.0, and subsequently stained using 0.4% uranyl acetate pH 4.0 in 2% (w/v) methyl cellulose (Sigma–Aldrich, USA) for 10 min. Samples were air dried, and imaged using a JEOL JEM-2010 transmission electron microscope (JEOL, Japan) operated at 200 kV, with a Valeta 4 MP CCD camera (Emsis, Germany).

2.4 | Nanoparticle tracking analysis

The size and concentration of OMVs was determined using the ZetaView Quatt PMX-420 NTA (Particle Metrix, Germany) nanoparticle tracking analyzer as previously [29]. OMVs were diluted in DPBS and approximately 100–500 particles were analyzed by NTA from 1 mL of the diluted OMV sample at 11 positions, and the final concentration was calculated with respect to the dilution factor. Instrument calibration was performed using 102 nm polystyrene beads (Thermo Fisher Scientific, USA) according to the manufacturer's guidelines. Measurements were performed using a 405 nm, 68 mW laser and CMOS camera. Measurements were captured at 60 frames per position for each of the 11 positions at 25°C, with camera sensitivity 80, shutter speed 100, autofocus and automatic scattering intensity. Data were analyzed using the ZetaView software version 8.05.12 SPI with the following parameters: maximum area 1000, minimum area 5, maximum brightness 255, minimum brightness 40, and minimum trace length 15. The mean of three biological replicates was plotted as particle size versus number of particles per ml using GraphPad Prism v9.4.1.

2.5 | Qubit fluorometric quantification

The DNA, RNA, and protein content of OMVs was quantified using the Qubit high sensitivity DNA assay, high sensitivity RNA assay or protein assay respectively (Thermo Fisher Scientific, USA), and measured using the Qubit 3.0 fluorometer as previously [5, 9, 28, 29, 31]. The DNA, RNA, and protein concentrations of OMVs were normalized to 1×10^{10} OMVs as determined using NTA.

2.6 | Proteomics sample preparation

H. pylori bacteria grown at pH 5.3 in the presence of urea, and at pH 7.2 with and without urea supplementation, as well as their respective OMVs isolated from each growth condition ($n = 3$ biological replicates of each) were lysed using 1% (v/v) sodium dodecyl sulphate (SDS), 50 mM HEPES pH 8.0 and quantified by microBCA (Thermo Fisher Scientific, USA). Samples were normalized (10 µg protein) and reduced using 10 mM dithiothreitol (DTT) for 45 min at 25°C, alkylated using 20 mM iodoacetamide for 30 min at 25°C in the dark, before the Sera-Mag-based workflow [32]. Magnetic bead slurry was prepared by mixing SpeedBeads magnetic carboxylate modified particles (Cytiva, USA; 65152105050250, 45152105050250) at a 1:1 (v:v) ratio, washing with MS-grade water and reconstituted to a final concentration of 100 µg/µL. The beads were added to samples at 10:1 beads-to-protein ratio and ethanol (EA043; ChemSupply, Australia) added to a final concentration of 50% (v/v). Protein-bound magnetic beads were washed three times with 200 µL of 80% ethanol and reconstituted in 50 µL of 50 mM triethylammonium bicarbonate (TEAB) pH 8.0. Protein digestion was performed with sequencing grade trypsin (enzyme:substrate 1:50, V5113, Promega, USA) overnight at 37°C with agitation at 1000 rpm. Peptide digests were acidified to a final concentration of 2% formic acid (FA), centrifuged at 20,000 × *g* for 1 min, and the supernatant extracted, frozen at −80°C for 30 min, and dried by vacuum centrifugation. Samples were then reconstituted in 0.07% trifluoroacetic acid (TFA), and quantified by Fluorometric Peptide Assay (23290; Thermo Fisher Scientific, USA) with samples normalized for immediate analysis of stored at −80°C.

2.7 | Liquid chromatography and data-independent acquisition mass spectrometry

Tryptic peptides for one-shot analyses were analyzed on a Dionex UltiMate NCS-3000RSLC nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with nanospray ion source in data-dependent acquisition analysis and positive mode as described [33, 34]. Peptides were loaded (Acclaim PepMap100 C18 3 µm beads with 100 Å pore-size, Thermo Fisher Scientific, USA) and separated (1.9 µm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd, Japan) with a gradient of 2%–28% acetonitrile containing 0.1% FA over 95 min followed by 28%–80% from 95–98 min at 300 nl/min at 55°C (butterfly portfolio heater, Phoenix S&T, USA). MS1 full scan was set to 60,000 resolution, 3e6 AGC target and maximum IT of 50ms in 350–1100 m/z scan range MS2 was set to 15,000 resolution, 1e6 AGC target and 27ms maximum IT. A total of 63 scan windows with staggered 12 m/z isolation window from 350 to 1100 m/z were applied with 28% normalized collision energy. The MS-based proteomics data have been deposited to the ProteomeXchange Consortium via the MassIVE repository and are available via identifier MassIVE MSV000092295.

2.8 | Database searching and analysis

RAW MS data was processed using DIA-NN (v1.8.1) in silico library-based analysis (also known as DIA-NN "library free" mode) searched as a single batch against an in silico library-based analysis of *H. pylori* (strain ATCC 700392; 26695; Oct 2022; 1564 entries). Search parameters were as follows: carbamidomethylated cysteine as fixed modification, oxidation of methionine and N-terminal protein acetylation as variable modifications, trypsin/P as proteolytic enzyme (C-terminal to arginine and lysine) with ≤2 missed cleavage sites, and all default settings kept as defined at a proteome level, including a 1% false discovery rate on protein/peptide spectrum match (PSM) levels. 'Match between run' was performed. For clustering analysis, PCA analysis was applied (missing values were imputed from normal distribution; width 0.3, downshift 1.8). For differential analysis, One-way ANOVA and Student's *t* test were applied. 1D-enrichment analysis (Gene Ontology) was based on the log2 fold change for sample groups (FDR < 0.01). For enrichment analysis the NIH Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (DAVID) was utilized using recommended analytical parameters [35]. Perseus and R (ggplot2) package, Microsoft Excel and GraphPad Prism were utilized for generating boxplots, volcano plots, heatmaps, and bar charts.

2.9 | Statistical analysis

Data analysis was performed using GraphPad Prism 9.4.1. All data are represented as the mean ± standard error of the mean (SEM) of three biological replicates unless otherwise stated. Statistical analyses were performed using data from three biological replicates, using One-way ANOVA with Tukey's multiple comparisons test or Student's *t* test.

3 | RESULTS AND DISCUSSION

3.1 | OMVs produced by *H. pylori* during growth in acidic pH conditions are smaller than OMVs produced during neutral pH conditions

To examine the effects of bacterial growth in different pH levels on the production and composition of *H. pylori* OMVs, OMVs were isolated and purified from *H. pylori* bacterial cultures grown in pH 5.3 or pH 7.2 growth conditions, either in the presence or absence of urea, which is necessary for urease to neutralize the acidic pH and supplement *H. pylori* growth [23, 24]. *H. pylori* was unable to grow at pH 5.3 in the absence of urea, as reported previously [24], and therefore we were unable to include OMVs produced during these conditions in our analyses. Thus, we examined OMVs produced by *H. pylori* during pH 5.3 and 7.2 growth conditions in the presence of urea (5.3U-OMVs and 7.2U-OMVs, respectively), or during pH 7.2 growth conditions in the absence of urea (7.2-OMVs) as a control, to determine the effect of pH on the size and composition of OMVs. OMVs produced by *H. pylori* during pH 5.3 and during pH 7.2 growth conditions in either the presence or absence of urea were similar in morphology when visualized using transmission electron microscopy (TEM; Figure 1A–C). Examination of the size and concentration of *H. pylori* OMVs produced during each growth condition by ZetaView nanoparticle tracking analysis (NTA) revealed that 5.3U-OMVs had a narrower size distribution (Figure 1D–F), and were significantly smaller than 7.2U-OMVs and 7.2-OMVs ($p < 0.001$, Figure 1G), with significantly fewer 5.3U-OMVs in the 200–300 nm size range compared to 7.2U-OMVs and 7.2-OMVs ($p < 0.01$, $p < 0.05$ respectively, Figure 1G). In comparison, 7.2U-OMVs and 7.2-OMVs were similar in size (Figure 1G), suggesting that the addition of urea to the growth media did not significantly alter the size of *H. pylori* OMVs produced during neutral pH conditions. Although the addition of urea did not affect the number of OMVs produced by *H. pylori* grown during pH 7.2 conditions, fewer OMVs were produced by *H. pylori* grown at pH 5.3 compared to during pH 7.2 conditions in the presence of urea ($p < 0.05$, Figure 1H). The decrease in the number of OMVs produced by *H. pylori* during pH 5.3 conditions in the presence of urea may also be attributed to reduced bacterial growth during acidic conditions, and thus reduced OMV production ($p < 0.05$, Figure 1I). This finding is consistent with previous studies that demonstrated decreased growth of *H. pylori* when grown during acidic conditions [23, 24]. Collectively, these findings reveal that culture pH affects the growth of *H. pylori*, as well as the size and quantity of OMVs produced. Similarly, *Streptococcus mutans* MVs produced during pH 5.5 growth conditions were also found to be smaller than MVs produced during neutral pH conditions using NTA [36], suggesting that pH may be a regulator of the size of BMVs produced by a range of bacteria.

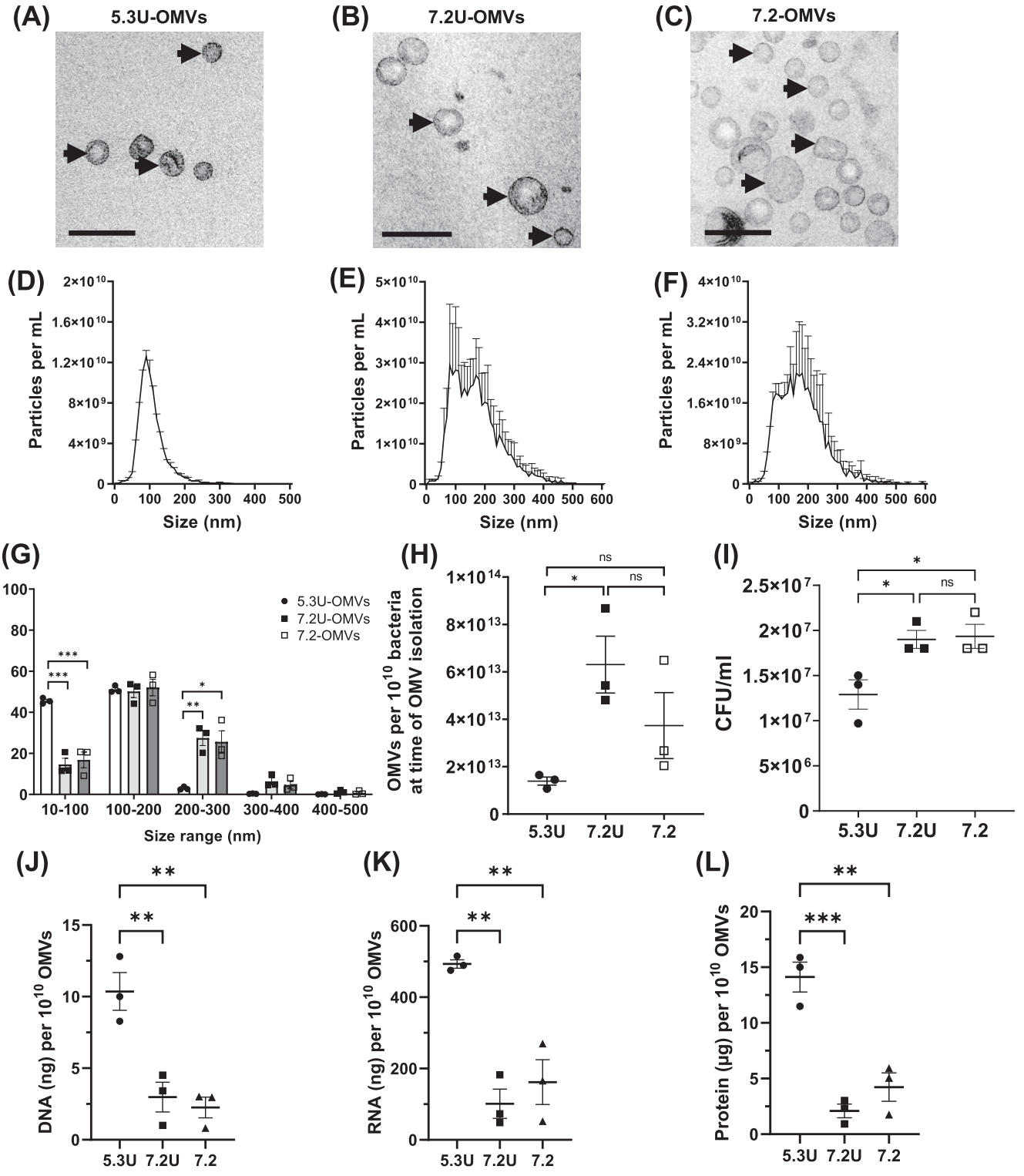

FIGURE 1 OMVs produced by *H. pylori* grown during acidic pH conditions differ in size, number and cargo compared to OMVs produced during neutral pH conditions. Transmission electron micrographs of OMVs produced by *H. pylori* 26695 grown in the presence of urea at a pH of (A) 5.3 and (B) 7.2, and (C) at a pH of 7.2 in the absence of urea. Images are representative of 3 biological replicates. Scale bar represents 200 nm. ZetaView nanoparticle analysis tracking (NTA) depicting the size and concentration of OMV particles produced by *H. pylori* 26695 grown in the presence of urea during (D) pH 5.3, (E) pH 7.2, and (F) in the absence of urea at pH 7.2. Data show the mean + SEM of 3 biological replicates. (G) The size range of *H. pylori* 26695 OMVs derived from pH 5.3+urea (5.3U-OMVs; filled circles), pH 7.2+urea (7.2U-OMVs; filled squares), or pH 7.2 (7.2-OMVs; open squares) growth conditions represented as a percentage of the total OMV population. (H) The number of OMVs produced during *H. pylori* growth in each condition per 10^{10} bacteria at the time OMVs were collected was determined by NTA. (I) The number of viable *H. pylori* present in individual cultures at the time of OMV isolation, represented as colony forming units (CFU) per ml for *H. pylori* grown in pH 5.3 in the

We next determined the quantity of DNA, RNA and protein associated with *H. pylori* OMVs produced during each condition. We observed that 5.3U-OMVs contained significantly more DNA and RNA than OMVs produced by *H. pylori* grown during pH 7.2 conditions in the presence and absence of urea ($p < 0.01$, Figure 1J,K). We also found that 5.3U-OMVs were associated with significantly more protein per 10^{10} OMVs compared to 7.2U-OMVs and 7.2-OMVs ($p < 0.001$, $p < 0.01$ respectively, Figure 1L). Similarly, *S. mutans* MVs were also found to package approximately 10-fold more protein when produced during acidic conditions compared to MVs produced during neutral conditions [36], suggesting that different bacterial species may have a conserved mechanism to produce BMVs with increased protein cargo during growth in acidic conditions. Alternatively, it is known that explosive cell lysis can contribute to increased packaging of bacterial cargo within OMVs [9] and may be a contributing factor to the increased packaging of cargo within OMVs produced by *H. pylori* grown at a low pH. Collectively, these findings reveal that culture pH affects the size and quantity of OMVs in addition to the quantity of cargo associated with OMVs produced by *H. pylori*. Similarly, the DNA, RNA, and protein quantity associated with *H. pylori* 26695 OMVs was previously reported to be altered during different bacterial growth stages [8, 30] indicating that in addition to bacterial growth stage, bacterial growth conditions such as pH also affect OMV packaging of cargo.

3.2 | *H. pylori* OMVs produced during different pH growth conditions differ in their proteome compared to their parent bacteria

Bacteria are known to package proteins selectively into OMVs to alter their biological functions when interacting with neighboring bacteria or host cells [8, 9, 37, 38]. To determine if *H. pylori* OMVs produced during different pH growth conditions differ in their proteome, we performed a quantitative global protein comparison of OMVs produced during pH 5.3 or pH 7.2 conditions in the presence or absence of urea, as well as their parent bacteria (Table S1). A total of 1206 proteins were identified within bacteria grown during all growth conditions (Table S2), covering approximately 76% of the *H. pylori* 26695 predicted coding sequence [39], of which 455 proteins were only detected in bacteria compared to OMVs (Figure 2A). In comparison, 760 proteins were identified within *H. pylori* OMVs, with only 9 proteins being detected in OMVs produced during all growth conditions compared to their parent bacteria, of which most were predominantly outer membrane proteins (Figure 2A, Table S3). Furthermore, 751 proteins were common to both bacteria and OMVs (Figure 2A). We next compared the proteome of OMVs produced during all growth conditions to the previously identified proteomes of OMVs isolated from *H. pylori* 26695 produced during early, mid and stationary phase of bacterial growth [8] to determine if *H. pylori* OMVs contained a core proteome. Analysis of the proteomes of *H. pylori* OMVs produced during pH 5.3 or pH 7.2 growth conditions in the presence or absence of urea, with the proteome of *H. pylori* OMVs produced during various stages of bacterial growth [8], revealed that OMVs from both studies shared approximately 84% of proteins (Table S4). This indicated that there may be conservation in the core proteome of OMVs produced by *H. pylori* grown in different growth stages as well as during different pH growth conditions, and may indicate a core proteome packaged within *H. pylori* OMVs during a range of bacterial growth conditions.

We next examined in greater detail the proteome of *H. pylori* bacteria and their OMVs produced using pH 5.3U, pH 7.2 and pH 7.2U growth conditions. Principal component analysis (PCA) of the proteins present in the proteomes of bacteria grown in pH 5.3U, pH 7.2 and pH 7.2U conditions demonstrated clustering, indicating a high similarity in their protein compositions compared to OMVs (Figure 2B). In comparison, PCA demonstrated that the global proteome of OMVs produced during each individual pH growth condition were distinct when compared to their parent bacteria, and to OMVs produced during each growth condition (Figure 2B). Comparison of the global proteome of bacteria produced during each growth condition to one another revealed that they shared highly similar proteomes (Figure S1A), however there was differential proteomic enrichment between bacteria grown in acidic conditions compared to bacteria grown in neutral conditions (Figure S1B–C, Table S2). Based on log2 fold change analysis comparing bacterial proteome enrichment, we found that bacteria grown using pH 5.3 growth conditions were enriched in proteins for ATP binding and translation, whereas bacteria grown during neutral pH growth conditions both in the presence and absence of urea contained similar proteins, including the enrichment of proteins with beta-lactamase activity (Figure S1C).

Comparison of the global proteome of *H. pylori* 26695 bacteria to their respective OMVs produced during different pH growth conditions was performed. We found that *H. pylori* grown using pH 5.3 conditions in the presence of urea (5.3U-BAC) contained 587 proteins that were not detected in their OMVs (Figure 2C). In comparison, 5.3U-OMVs contained 35 proteins that were not detected to significant levels in their parent bacteria, with 368 proteins shared between OMVs and their parent bacteria (Figure 2C, $p < 0.05$). OMVs derived from pH 7.2 growth conditions in the presence or absence of urea contained 442 and 462 proteins respectively that were also detected in their parent bacteria. *H. pylori* grown during pH 7.2 conditions in the presence (7.2U-BAC) or absence of urea (7.2-BAC) were enriched in 691 and 692 proteins respectively that were not detected in their

presence of urea (5.3U; filled circles), pH 7.2 in the presence of urea (7.2U; filled squares), and pH 7.2 in the absence of urea (7.2; open squares). (J) DNA, (K) RNA and (L) protein associated with OMVs produced during 5.3U, 7.2U, and 7.2 growth conditions was quantified using Qubit and represented as micrograms (μg) or nanograms (ng) per 10^{10} OMVs. Data are mean of 3 biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, One-way ANOVA with Tukey's multiple comparisons.

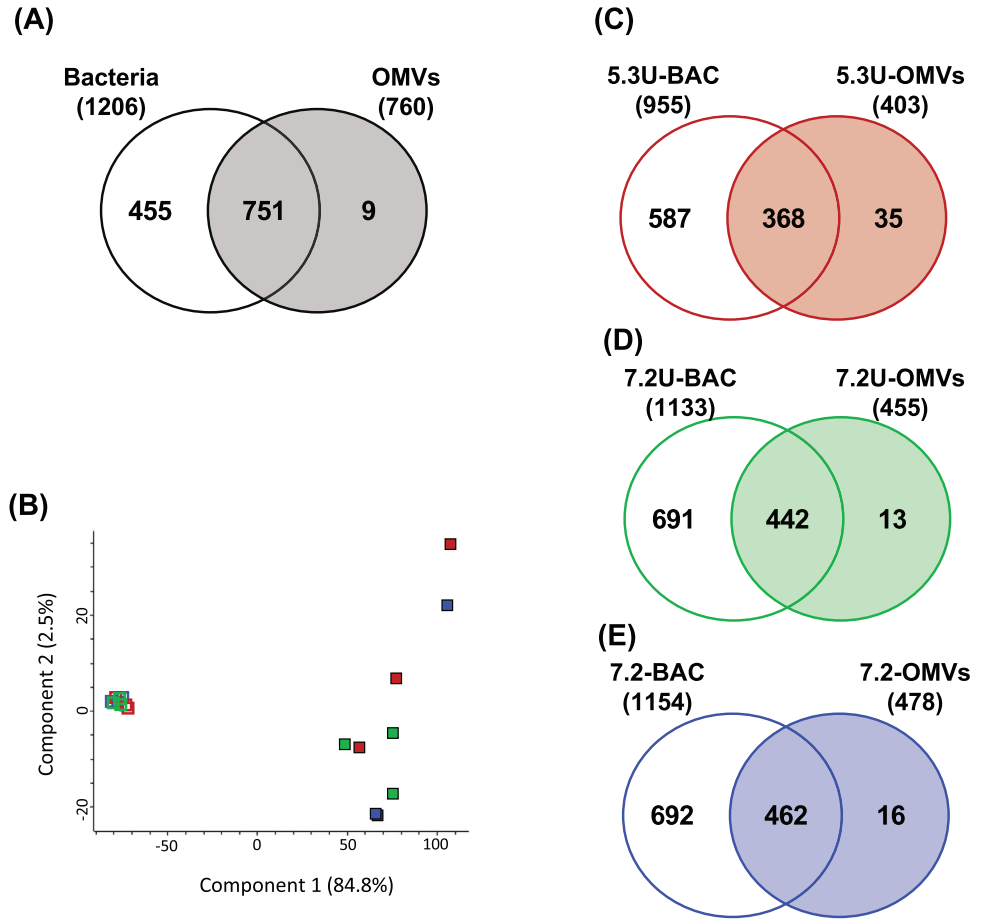

FIGURE 2 *H. pylori* and their OMVs produced during different pH conditions have significantly different proteomes compared to one another. (A) Protein identifications for *H. pylori* bacteria and OMVs produced during all growth conditions were combined and comparison between all bacteria and all OMVs was performed (normalized at protein/peptide levels). For differential expression LFQ ratio >2, $p < 0.05$. (B) Principal component analysis (PCA) for proteome profiling of *H. pylori* bacteria grown during pH 5.3 conditions with urea (red open boxes), pH 7.2 with urea (green open boxes), or pH 7.2 conditions without urea (blue open boxes), as well as their respective OMVs (green, red, and blue filled boxes). (C–E) Proteome identifications of *H. pylori* bacteria (BAC) and their OMVs (OMVs) was performed for each pH growth condition (C) pH 5.3 with urea, (D) pH 7.2 with urea, and (E) pH 7.2 without urea and protein identification was compared. For differential expression LFQ ratio >2, $p < 0.05$. Shown is the combined data from $n = 3$ biological replicates.

OMVs, and their OMVs (7.2U-OMVs, 7.2-OMVs) contained 13 and 16 proteins respectively that were not detected to significant levels in their parent bacteria (Figure 2D,E, $p < 0.05$).

Some proteins were detected to significant levels only in OMVs compared to their parent bacteria grown in at different pH levels. For example, outer membrane proteins, beta-lactamase and a metal resistance protein comprised some of the 35 proteins detected only in 5.3U-OMVs compared to their parent bacteria grown at pH 5.3 (Figure 2C, Table 1). Furthermore, of the 13 and 16 proteins detected only within 7.2U-OMVs and 7.2-OMVs respectively, 10 of these proteins were common to both OMV types, which consisted of mostly uncharacterized proteins in addition to outer membrane proteins such as Omp6, Omp27, and Omp3 (Figure 2D,E, Table 1). Additionally, 5.3U-OMVs contained 11 proteins that were also detected at significant levels in OMVs from pH 7.2 conditions compared to their parent bacteria, indicating that OMVs from all growth conditions contained a common proteome (Figure 2C–E, Table 1).

3.3 | OMVs produced during different pH growth conditions are enriched in unique proteins compared to their parent bacteria

We next examined the proteomes of *H. pylori* OMVs and their parent bacteria to identify differences in their protein cargo enrichment. As much of the *H. pylori* proteome remains uncharacterized, many proteins are not annotated and are therefore not included in gene ontology analyses [39]. Overall, we identified that bacterial growth conditions altered the proteome of OMVs compared to their parent bacteria (Figure 3A–C). Specifically, *H. pylori* grown in pH 5.3 and pH 7.2 media supplemented with urea, and in pH 7.2 media without urea were all enriched in proteins associated with translation compared to their respective OMVs (Figure 3D,E). In comparison, OMVs produced by *H. pylori* from all growth conditions were enriched in a unique subset of proteins compared to their parent bacteria (Figure 3A–C). Although many of the proteins enriched in *H. pylori* OMVs are uncharacterized,

TABLE 1 Abundant proteins detected only in OMVs isolated from pH 5.3 and pH 7.2 growth conditions in the presence of urea, and pH 7.2 growth conditions in the absence of urea compared to their parent bacteria.

Growth condition	Protein ID	Gene name	Protein description
pH 5.3 + urea	O24911; O24925; **O25321**	HP_0082; HP_0099; HP_0599	Methyl-accepting chemotaxis transducer (TlpC), hemolysin secretion protein (hylB)
	O25028	flgI	Flagellar P-ring protein
	O25075	HP_0304	Alginate_lyase domain-containing protein
	O25256	HP_0519	Beta-lactamase
	O25281	HP_0555	Uncharacterized protein
	O25353	HP_0636	Uncharacterized protein
	O25374	HP_0660	Uncharacterized protein
	O25470	HP_0781	Uncharacterized protein
	O25623	HP_0970	Nickel-cobalt-cadmium resistance protein (NccB)
	O25672	HP_1028	DUF2147 domain-containing protein
	O25740	HP_1113	Outer membrane protein (Omp24)
	O25891	HP_1333	Uncharacterized protein
	O25967	HP_1424	Uncharacterized protein
	O26055	HP_1527	Uncharacterized protein
	O25188	HP_0440	DNA topoisomerase
	O25472	HP_0783	Uncharacterized protein
	O25734	HP_1106	Uncharacterized protein
	O25885	HP_1327	Uncharacterized protein
	O26003	HP_1467	Uncharacterized protein
	O26053	HP_1525	Uncharacterized protein
	O25504	HP_0833	Uncharacterized protein
	O26012	HP_1477	Flagella basal body P-ring formation protein FlgA
	O25137	HP_0373	Uncharacterized protein
	O25464	HP_0772	N-acetylmuramoyl-L-alanine amidase
	O25601	HP_0947	Uncharacterized protein
	O25324	HP_0603	Uncharacterized protein
	O25344	HP_0627	Uncharacterized protein
	O25015; O34523	HP_0227; HP_0229	Outer membrane protein (Omp6)
	O25000	HP_0209	Uncharacterized protein
	O25495	HP_0817	Uncharacterized protein
	O25782	HP_1167	Uncharacterized protein
	P64653	HP_0122	Uncharacterized protein HP_0122
	O24927	HP_0101	Uncharacterized protein
	O25329	HP_0608	Uncharacterized protein
	O25706	HP_1066	Uncharacterized protein
pH 7.2 + urea	O25102	HP_0335	Uncharacterized protein
	O25137	HP_0373	Uncharacterized protein
	O25324	HP_0603	Uncharacterized protein
	O25344	HP_0627	Uncharacterized protein
	O25015; O34523	HP_0227; HP_0229	Outer membrane protein (Omp6)
	O25000	HP_0209	Uncharacterized protein
	O25495	HP_0817	Uncharacterized protein

(Continues)

TABLE 1 (Continued)

Growth condition	Protein ID	Gene name	Protein description
	O25782	HP_1167	Uncharacterized protein
	P64653	HP_0122	Uncharacterized protein HP_0122
	O25137; O25414	HP_0373; HP_0710	Uncharacterized protein
	O24927	HP_0101	Uncharacterized protein
	O25329	HP_0608	Uncharacterized protein
	O25706	HP_1066	Uncharacterized protein
pH 7.2	O25791; O34523	HP_0227; HP_1177	Outer membrane protein (Omp27)
	O25994	HP_1455	Uncharacterized protein
	O25710	HP_1078	Toprim domain-containing protein
	O25324	HP_0603	Uncharacterized protein
	O25344	HP_0627	Uncharacterized protein
	O25000	HP_0209	Uncharacterized protein
	O25495	HP_0817	Uncharacterized protein
	O25782	HP_1167	Uncharacterized protein
	P64653	HP_0122	Uncharacterized protein HP_0122
	O24908	HP_0079	Outer membrane protein (Omp3)
	O25137; O25414	HP_0373; HP_0710	Uncharacterized protein
	O26100	HP_1580	acidPPc domain-containing protein
	O24870; O25086; O25556; O25791; O25840	HP_0025; HP_0317; HP_0896; HP_1177; HP_1243	Outer membrane protein (Omp2)
	O24927	HP_0101	Uncharacterized protein
	O25329	HP_0608	Uncharacterized protein
	O25706	HP_1066	Uncharacterized protein

our proteomic analysis revealed that pH growth conditions regulated the proteomes of *H. pylori* bacteria and their OMVs (Figure 3A–C, Table S5). Specifically, gene ontology analysis revealed that *H. pylori* was enriched in proteins with gene ontology cellular compartment (GOCC) terms associated with the outer membrane, intracellular, and cytoplasmic regions, in comparison to OMVs produced during each pH growth condition, which were enriched in periplasmic and outer membrane proteins, as well as external encapsulating structure and cell outer membrane proteins, as expected (Figure 3D). In addition, OMVs were also depleted in proteins with GOCC terms for cytoplasm and intracellular parts (Figure 3D). Furthermore, *H. pylori* grown in each pH growth condition were enriched in proteins with gene ontology biological process (GOBP) terms associated with translation and protein metabolic functions, which were not enriched in their OMVs (Figure S2A, Table S5). Furthermore, *H. pylori* bacteria from each growth condition were enriched in proteins involved in biological processes, such as translation, protein metabolic process, and cellular homeostasis compared to their OMVs (Figure S2A).

In comparison, *H. pylori* OMVs were enriched in several proteins compared to their parent bacteria, including outer membrane proteins and the antigenic protein LPP20 (Figure S2B–D, Table S5). LPP20, identified in OMVs produced during all pH growth conditions, is a conserved *H. pylori* lipoprotein and has been identified as a target for vaccines against *H. pylori* [40]. This may indicate a role for *H. pylori* OMVs in activating the host adaptive immune system during colonization and infection. Furthermore, *H. pylori* OMVs produced during pH 5.3 growth conditions were enriched in proteins with gene ontology molecular function (GOMF) terms including tetrapyrrole binding and heme binding compared to 7.2U-OMVs and 7.2-OMVs, and to their parent bacteria (Figure 3E, Table S5). Tetrapyrroles and heme binding proteins such as uroporphyrinogen decarboxylase, and the heme oxygenase protein HugZ associated with OMVs produced during pH 5.3 growth conditions may facilitate the binding and transfer of heme for iron acquisition by *H. pylori* [41] by sequestration and association of this enzyme with OMVs. This may facilitate heme delivery via OMVs to other *H. pylori* bacteria during infection. Furthermore, OMVs derived from *H. pylori* grown using pH 7.2 growth conditions with and without urea were enriched in putative beta-lactamases compared to their parent bacteria and compared to 5.3U-OMVs (Figure 3E, Table S5), suggesting that once *H. pylori* is growing in a neutral environment it may harness OMVs to prevent antimicrobial activity, and promote persistence in the host. In addition, 7.2U-OMVs were also enriched in hydrolase activity compared to all other OMVs and their parental bacteria (Figure 3E, Table S5).

Although both OMVs and bacteria produced during all growth conditions contained the putative beta-lactamase proteins HcpA, HcpC,

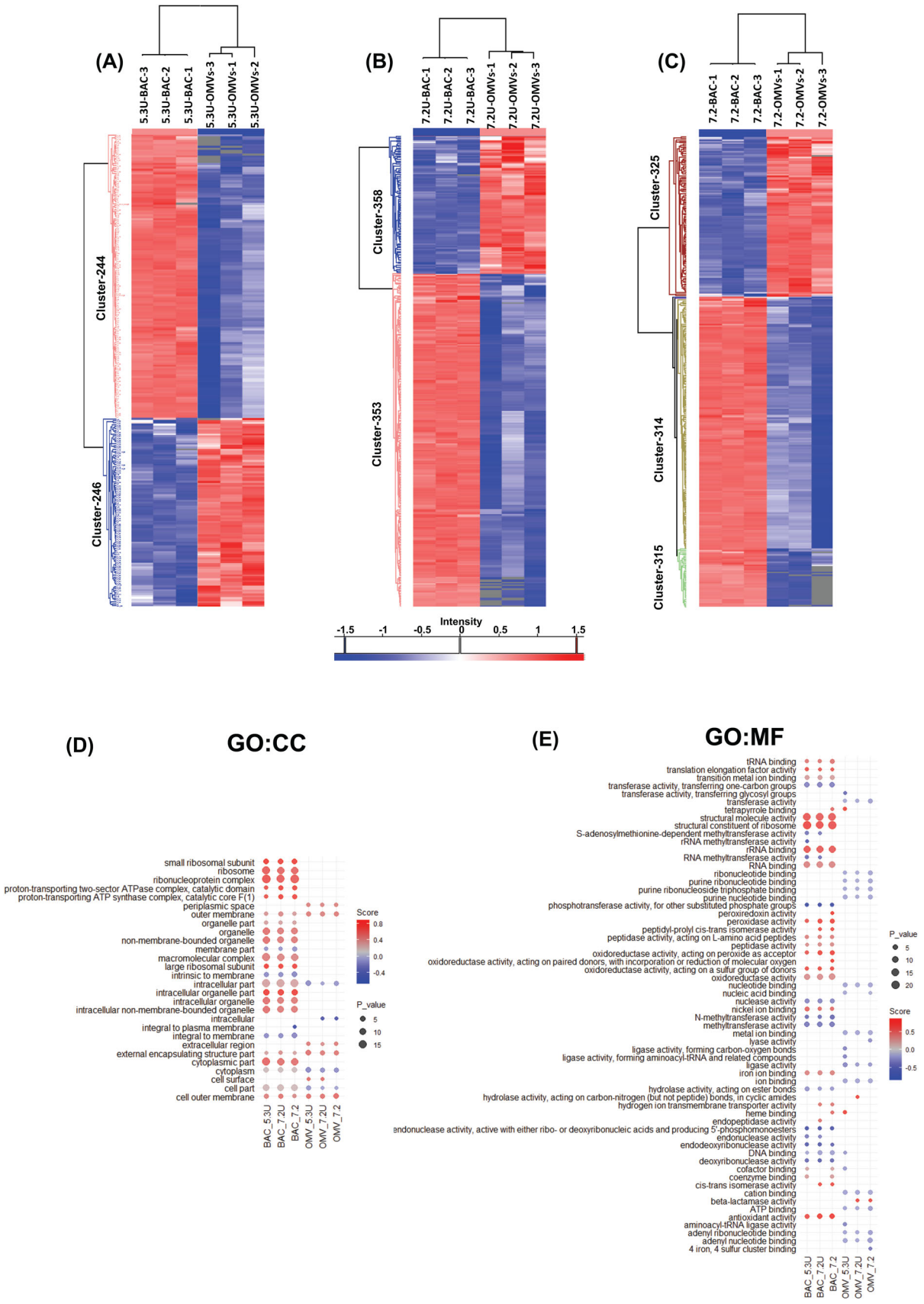

FIGURE 3 *H. pylori* OMVs produced during different pH growth conditions are specifically enriched in proteins compared to their parent bacteria. (A–C) Protein identification and abundance heatmap of *H. pylori* bacteria (BAC) grown during (A) pH 5.3 with urea, (B) pH 7.2 with urea, and (C) pH 7.2 without urea and their respective OMVs ($p < 0.05$, One-way ANOVA). (D) Gene ontology cellular component (GOCC) and (E) gene

HcpD, and HcpE, further examination revealed that OMVs were significantly enriched in these putative beta-lactamases compared to their parent bacteria (Table S5). However, HcpA, HcpC, HcpD, and HcpE were significantly enriched in bacteria grown during pH 7.2 conditions with and without urea, compared to bacteria grown in pH 5.3 conditions with urea (Table S5). In comparison, HcpA, HcpC, and HcpD were significantly enriched in OMVs produced during all growth conditions compared to their parent bacteria, with HcpE also being significantly enriched in both 5.3U-OMVs and 7.2U-OMVs compared to their parent bacteria (Table S5). These findings indicate the selective packaging and enrichment of potential beta-lactamases within *H. pylori* OMVs produced during both acidic and neutral pH growth conditions compared to their parent bacteria, which may enhance bacterial survival during colonization of the stomach. Collectively, these findings indicate that pH growth conditions define the proteomes of *H. pylori* OMVs and may dictate the molecular functions of OMVs compared to their parent bacteria.

3.4 | *H. pylori* OMVs produced during different pH growth conditions are differentially enriched in proteins compared to one another

We next compared the enrichment of proteins within *H. pylori* OMVs produced during growth in pH 5.3 and pH 7.2 with urea, and pH 7.2 without urea (Figure 4, Table S6). We found that OMVs produced during all pH growth conditions shared 362 proteins that were packaged in OMVs (Figure 4A) and may therefore comprise a common OMV proteome. Furthermore, OMVs produced during each growth condition were enriched in proteins not detected in their parent bacteria or in OMVs produced during each growth condition, as 5.3U-OMVs contained 10, 7.2U-OMVs contained 39, and 7.2-OMVs contained 51 significantly abundant proteins respectively compared to their parent bacteria (Figure 4A). Additionally, there were 44 shared proteins between 7.2U-OMVs and 7.2-OMVs, whereas 5.3U-OMVs contained 10 and 21 shared proteins when compared to 7.2U-OMVs and 7.2-OMVs respectively (Figure 4A).

PCA was performed to examine the distribution of the OMV proteomes, which demonstrated that there were differences in OMV proteomes produced during different pH growth conditions (Figure S3A). Gene ontology analysis of proteins packaged within OMVs from different pH growth conditions revealed that OMVs obtained from all conditions were not enriched in proteins relating to any known biological processes (Figure S3B). Similarly, 5.3U-OMVs were not enriched in proteins with GOMF terms compared to 7.2U-OMVs which were enriched in proteins with catalytic and binding activity, and 7.2-OMVs which were enriched in proteins for transferase activity, as well as nucleotide and ATP binding (Figure S3C). Furthermore, 5.3U-OMVs and 7.2-OMVs were enriched in proteins with GOCC associated terms such as outer membrane and external encapsulating structure part as expected, whereas 7.2U-OMVs did not contain proteins relating to cellular components (Figure S3D), which may indicate differences in the mechanisms of OMV biogenesis between different growth conditions [9]. Additionally, gene ontology analysis may have been limited due to the large number of unidentified and uncharacterized proteins present within *H. pylori* OMVs. Of the proteins identified with known characterizations, we found that 5.3U-OMVs contained proteins that clustered into ion binding, peptidoglycan binding, and molecular and endopeptidase activity (Figure 4B, Table S6). In comparison, 7.2U-OMVs contained proteins that clustered into surface enzyme activity and ion binding and molecular activity (Figure 4B). Finally, proteins within 7.2-OMVs clustered mainly into surface enzyme activity (Figure 4B). These results demonstrate the altered packaging of proteins within *H. pylori* OMVs during growth in different pH conditions, and therefore may indicate altered functions of OMVs when derived from varying pH growth conditions.

Further examination revealed that 5.3U-OMVs were significantly enriched in HomA (HP_0710) but not HomC (HP_0373) compared to 7.2U-OMVs and 7.2-OMVs (Figure 4C,D). The Hom family of proteins have been described for their ability to promote bacterial adherence to human epithelial cells [42], and the packaging of Hom proteins in OMVs produced in acidic conditions may alter their ability to facilitate bacterial adherence. *H. pylori* 5.3U-OMVs were also enriched in GmhA, a phosphoheptose isomerase that facilitates bacterial adhesion to human epithelial cells [43] and TlpC (Table S6), a methyl-accepting chemotaxis transducer that promotes *H. pylori* colonization by allowing utilization of lactate [44] compared to 7.2U-OMVs and 7.2-OMVs. This suggests 5.3U-OMVs may have a role in bacterial colonization compared to OMVs from neutral pH growth. Furthermore, we identified the presence of proteins related to the pentose phosphate pathway, important for bacterial metabolism [45, 46], including transketolase and glucose-6-phosphate isomerase within 5.3U-OMVs, in addition to QueH which is important for tRNA modification (Figure 4C). This may further indicate the role of OMVs produced during acidic growth conditions to mediate metabolic functions [47].

Furthermore, *H. pylori* OMVs produced during neutral conditions were enriched in the iron-regulated outer membrane and metal trafficking protein FrpB (HP_1512) [48] compared to 5.3U-OMVs (Figure 4C,D, Table S6). In addition, OMVs produced during neutral pH growth conditions were also enriched in proteins such as NccB, which is within the gene cluster encoding Czc-type metal export pump homologs responsible for metal resistance, essential for gastric colonization [49]. Comparatively, 7.2-OMVs were enriched in Omp21, which may contribute to bacterial adhesion [50] compared to

ontology molecular function (GOMF) functional enrichment analysis of proteins based on log2 fold change of proteins identified in bacteria produced during pH 5.3 (BAC_5.3U), pH 7.2U (BAC_7.2U), and pH 7.2 (BAC_7.2) growth conditions and their respective OMVs (OMV_5.3U; OMV_7.2U; OMV_7.2). Enriched terms (score > 0) are demonstrated in red. Depleted terms (score < 0) are shown in blue. Shown is the combined data from $n = 3$ biological replicates.

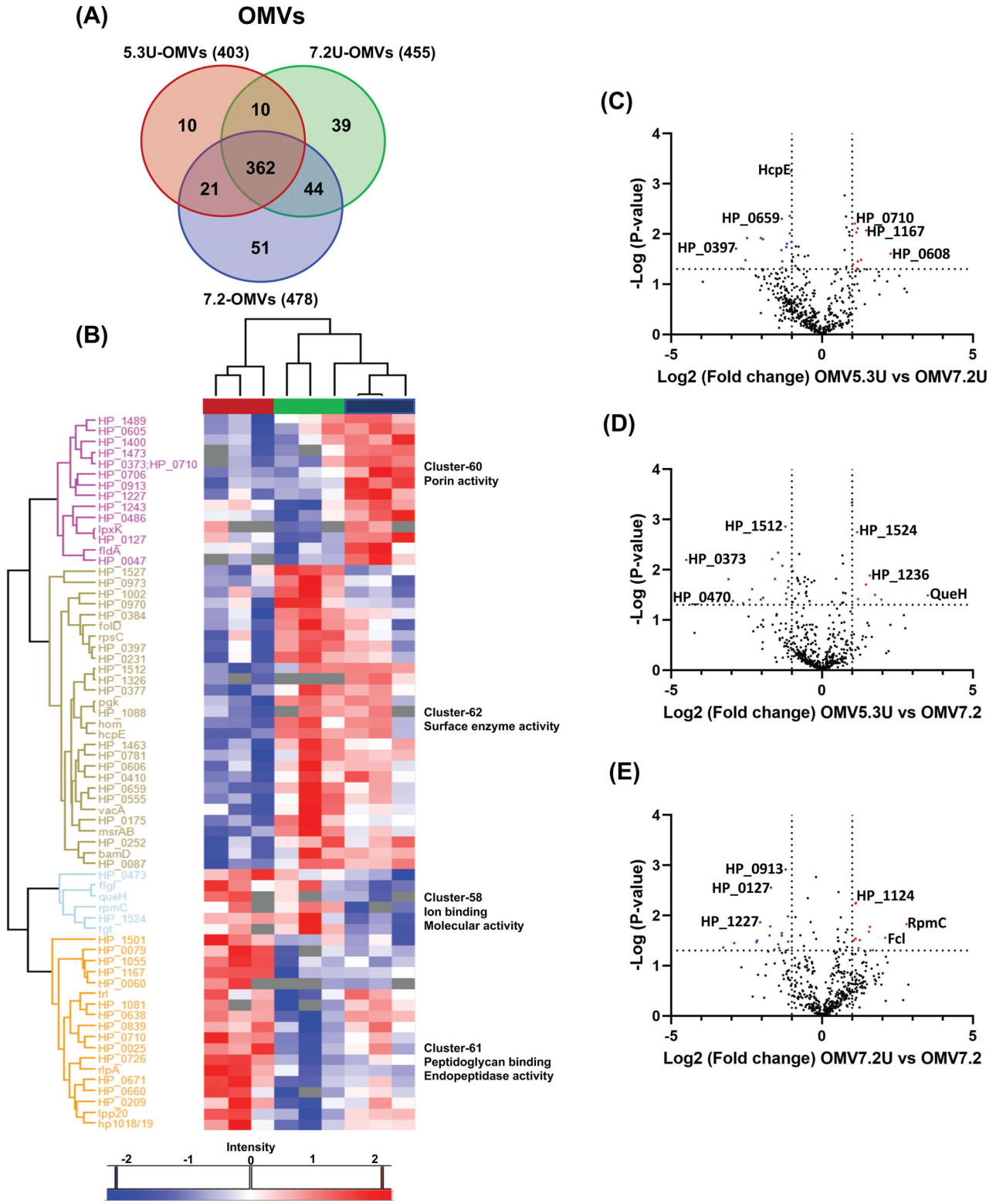

FIGURE 4 Altered pH growth conditions influences the proteomes of *H. pylori* OMVs. (A) Proteomic profiling of *H. pylori* OMVs produced during pH 5.3 with urea (red), pH 7.2 with urea (green) and pH 7.2 without urea (blue) conditions was performed and protein identification for each growth condition was compared. For differential expression LFQ ratio >2, $p < 0.05$. (B) Protein identification and abundance heatmap of *H. pylori* OMVs from pH 5.3 (red; 5.3U-OMVs) and pH 7.2 with urea (green; 7.2U-OMVs), and pH 7.2 without urea (navy; 7.2-OMVs) growth conditions ($p < 0.05$, One-way ANOVA), red shows significantly increased abundance and blue indicates significantly decreased abundance. (C–E) Volcano plots of differentially abundant proteins in (C) 5.3U-OMVs versus 7.2U-OMVs, (D) 5.3U-OMVs versus 7.2-OMVs, and (E) 7.2U-OMVs versus 7.2-OMVs. $p < 0.05$ & log2 fold change < –0.5 (blue); $p < 0.05$ & log2 fold change > 0.5 (red), Student's *t* test. Shown is the combined data from $n = 3$ biological replicates.

7.2U-OMVs (Figure 4E), suggesting that growth in the presence of urea may alter the expression of this adhesin.

OMVs from all pH growth conditions were also found to contain the putative beta-lactamases HcpA, HcpB, HcpC, HcpD, and HcpE. The gene encoding for HcpA is paralogous to a known penicillin binding protein (PBP) [51]. HcpB and HcpD, have been shown to function as PBPs [51, 52], whereas there are no known functions for HcpC and HcpE which are thought to be PBPs, however patients infected with *H. pylori* have been shown to produce antibodies against these proteins [53]. When comparing the enrichment of Hcp proteins between OMVs produced during different pH growth conditions, the putative beta-lactamase HcpE was significantly enriched in 7.2U-OMVs and 7.2-OMVs compared to 5.3U-OMVs (Table S6). OMVs produced during neutral pH growth conditions were also significantly enriched in MtrC (Table S6) which may act to hydrolyse clarithromycin [54]. Therefore, these findings suggest that OMVs produced during neutral conditions package antibiotic resistance proteins, in addition to metal resistance and metal trafficking proteins to potentially promote the survival of *H. pylori* once it has generated a neutral environment for itself within the host.

In addition, OMVs produced during neutral pH conditions were enriched in proteins attributed to promoting pathogenesis and virulence. Specifically, 7.2U-OMVs and 7.2-OMVs were enriched in a SurA N-domain containing protein compared to 5.3U-OMVs (Table S6). SurA has been demonstrated to contribute to bacterial pathogenicity in *Pseudomonas aeruginosa* as well as resistance to antibiotics [55], and therefore may potentially contribute to the ability of *H. pylori* OMVs to promote antimicrobial resistance and survival in the stomach, however this remains to be elucidated. VacA was also enriched in OMVs produced during neutral pH conditions (Table S6). VacA is a well described *H. pylori* toxin and virulence factor [56]. Although OMVs from all growth conditions were each enriched in proteins for metabolism and for chemotaxis only 7.2-OMVs contained Cag pathogenicity island proteins including Cag1 and Cag17, and therefore may have increased roles in pathogenesis compared to OMVs produced in the presence of acid or urea (Table S6). Ultimately, OMVs produced during different pH growth conditions and in the presence or absence of urea have altered proteomes, suggesting they may have altered functions during either the colonization or pathogenesis process of *H. pylori* infection.

4 | CONCLUDING REMARKS

H. pylori has been shown to produce OMVs with altered proteomes during different stages of bacterial growth [8], however the effect of altered pH conditions on OMV production, their proteome, and functions are yet to be fully elucidated. This study demonstrates that *H. pylori* OMVs produced during acidic growth conditions were smaller in size compared to OMVs produced during neutral growth conditions, and they contained more protein, DNA, and RNA cargo. As *H. pylori* OMVs that are smaller in size have been shown to enter host epithelial cells more readily via caveolin-mediated endocytosis [57], the production of smaller OMVs during acidic pH growth conditions in addition to the increased packaging of cargo including protein, DNA, and RNA may be a mechanism whereby *H. pylori* OMVs may efficiently mediate inflammation in the host. Additionally, bacteria produce different types of OMVs, including outer inner membrane vesicles that package cytoplasmic cargo via explosive cell lysis [1], and this mechanism of OMV biogenesis may account for the increased packaging of cargo within OMVs produced during pH 5.3 growth conditions, and is the focus of future studies. We identified that the proteomes of *H. pylori* OMVs were significantly different to that of their parent bacteria, as *H. pylori* bacteria grown during different pH growth conditions were significantly enriched in proteins for translation, metabolic processes and molecular activity, whereas OMVs were enriched in outer membrane proteins, putative beta-lactamases and the cytotoxin VacA. Furthermore, OMVs produced during acidic growth conditions were enriched in beta-lactamases, outer membrane proteins, and metal resistance proteins compared to their parent bacteria, suggesting they may have potential functions in promoting bacterial survival within the host. These findings reveal that pH growth conditions impact the composition of *H. pylori* OMVs, which may have bespoke functions in facilitating bacterial survival, colonization and inflammation in the host.

Furthermore, *H. pylori* OMVs produced during neutral growth conditions, such as those experienced once infection is established at the gastric mucosal surface, packaged virulence proteins including VacA toxin and cag pathogenicity island proteins, that may contribute to the production of pro-inflammatory cytokines by host epithelial cells [56, 58]. In comparison, OMVs produced during acidic growth conditions were enriched in HomA and GmhA that contribute to bacterial adhesion to host epithelial cells [42, 43], and TlpC that has a role in bacterial chemotaxis [44], suggesting that proteins packaged in OMVs produced during acidic growth conditions may contribute to the initial chemotaxis, adhesion and colonization of *H. pylori* in the gastric mucosa. This finding is supported by earlier studies showing that environmental stress such as pH and bile salts can also change the composition of MVs produced by *S. mutans*, *Vibrio fischeri and Campylobacter jejuni* which subsequently altered their biological functions [36, 59–61]. Collectively, these findings indicate that pH growth conditions regulate the packaging of protein within BMVs which may also subsequently affect their biological functions within the host.

Overall, this study highlights the significance of pH on *H. pylori* growth and their proteomes, and on the production, composition, and proteome of their OMVs. OMVs produced during acidic and neutral pH growth conditions may have altered functions which may facilitate bacterial competition, survival, and may promote colonization and pathogenesis in the host. By regulating the content and proteome of OMVs, *H. pylori* can potentially enhance its survival and persistence while transitioning between the acidic and hostile gastric environment of the gastric lumen towards the neutral pH conditions found in closer proximity to the epithelial cell lining. This study sheds light on the dynamic nature of OMVs produced by *H. pylori* during different pH growth conditions and highlights the ability of *H. pylori* to regulate cargo packaging within OMVs during different pH growth conditions,

which may have implications for the survival and pathogenesis of *H. pylori* in the host.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The MS-based proteomic data has been deposited to the ProteomeXchange Consortium via the MassIVE repository and are available via ProteomeXchange with identifier MSV000092295.

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SUPPORTING INFORMATION

Additional supporting information may be found online https://doi.org/10.1002/pmic.202300269 in the Supporting Information section at the end of the article.

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