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Type
Research paper

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Material and methods
This study used in vitro methods to extract anti-H. pylori peptides from caseins by the gastric protease, pepsin under environments with similar pH values to those found in the human stomach. The molecular weights and sequences of the peptides were identified by MALDI-TOF mass spectrometry and MS/MS Ion Search, respectively. Antibacterial activity tests were performed to calculate the minimum inhibitory concentration (MIC90) of the extracts.

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The findings of this study revealed that the major products of bovine milk casein digestion by pepsin are casecidin 17 and β-casein 207–224. The extracts produced promising anti-H. pylori effects with the lowest MIC90 found at pH values of 1.5 and 2.0.

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This study identified the anti-H. pylori effects of casecidin 17 and β-casein 207–224, which may help in developing therapeutic agents to modulate the effect of antibiotics on H. pylori infections.
Extraction of antibacterial peptides against *H. pylori* from bovine milk casein

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**Key words:** Antibacterial peptides, casein, casecidin 17, helicobacter pylori, minimum inhibitory concentration
Introduction

*Helicobacter pylori* is a well-known causative agent of chronic dyspepsia, peptic ulcer, gastric mucosa-associated lymphoma and gastric cancer [1-3]. Currently, the global infection rate of *H. pylori* exceeds 50% of the population [4]. The treatment of *H. pylori* mainly comprises triple therapy such as omeprazole, clarithromycin and amoxicillin [5]. However, recent studies show that the curative rate of the triple therapy is gradually reducing, mainly due to the emergence of resistant strains [6,7]. Recently, the WHO considers clarithromycin-resistant *H. pylori* to be one of the most urgently needed new antibiotic groups [8]. The mechanisms of resistance to clarithromycin is mainly caused by the mutations of the peptidyltransferase region encoded in domain V of 23s rRNA [9]. The increase in clarithromycin resistance in the last decade has reduce the efficacy to eradicate *H. pylori* [10].

In the past decade, various antibacterial peptides were discovered through the studies of organisms’ immune mechanisms [11]. These peptides showed antibacterial effects over various kinds of bacteria, including *H. pylori*. For example, Chen et al. identified the first anti-*H. pylori* peptides from amphibian skins [12]. The peptide was composed of 23 amino acids with the sequence of GLLRASSVWGRKYYVDLAGCAKA. Another recent example is the venom peptide,
bicarinalin, which has produced similar anti-\textit{H. pylori} activity as four antibiotics currently used in therapies against \textit{H. pylori} [13]. Most of the discovered antibacterial peptides have a nonspecific innate immune response against exogenous pathogens. At present, the mechanism of antibacterial activities can be divided into two groups. The first is the amphipathic \( \alpha \)-helix structure of peptides that binds to the bacterial cell membrane to form pores and destroy the cell membrane by releasing the cell contents [14]. The second is the antibacterial peptides that directly enter the bacterial cells and act on the DNA, RNA, protein, mitochondria and cell membrane, inhibiting the synthesis, transcription and translation of the cells. The peptides may also interfere with cell metabolism and inhibit the formation of the cell membrane [14]. The study also demonstrated that peptides can kill bacteria at a faster rate than that of its multiplication [15]. The combination of the physical killing mechanisms and fast killing rate mean resistant strains are potentially less likely to develop while comparing to most antibiotics [16]. Another advantage of antibacterial peptides is the highly degradable properties, which may lead to few drug residues and side effects [17].

Antibacterial peptides have been found in mammals, plants, insects and even microorganisms. This inspired us to evaluate whether food also contains anti-\textit{H. pylori} peptides. The aim of this study is to investigate whether the most common food source,
milk contains any peptides against *H. pylori*. Milk is rich in protein, of which caseins account for about 80% of the total protein [18]. Caseins, in addition to nutrition, produce numerous biologically active peptides through enzymolysis during food processing. Many of these peptides demonstrated antimicrobial activities. For example, bovine $\alpha_{s1}$-casein f (99–109) has antibacterial activities against Gram-positive bacteria *Bacillus subtilis* and *Listeria innocua* [19] and, $\alpha_{s2}$-casein f (181–207), f (175–207) and f (164–207) demonstrated antibacterial properties against both certain Gram-positive and Gram-negative bacteria [19]. This study aimed to extract antibacterial peptides from caseins and to attain their minimum inhibitory concentrations (MIC) against *H. pylori* under environments with different pH values.

**Materials and methods**

Caseins (Sigma C5890) from bovine milk were purchased from Sigma (Guangzhou, China). Antibacterial peptide extraction was performed on the caseins, followed by concentration and identification of the peptides. A study had shown that casein has anti-*E. coli* activities and hence, it was used in this study to ensure the extracted antibacterial peptides were active. The antibacterial activities of the extracts were also tested on *H. pylori* (ATCC-43504). Eight concentrations of the extracts were used to calculate the MIC values at four environments with different pH values.
eight concentrations were 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.5625 mg/mL, 0.78125 mg/mL, 0.390625 mg/mL and 0.1953125 mg/mL. They were all tested at the environments with pH values of 1.5, 2.0, 2.5 and 3.0. The H. pylori were purchased from Tin Hang Technology, Hong Kong, China. The experiments were performed with blank solutions, negative and positive controls. The blank solutions did not contain antimicrobial peptides or H. pylori. It was used to ensure there was no contamination and it also served as a measurement of the microplate spectrophotometer optical density values at 600 nm (Multiskan™ GO, Thermo Fisher Scientific). The negative controls contained H. pylori and no antibacterial peptides, whereas the positive controls contained the extracted antibacterial peptides and no H. pylori.

Recovery, storage, and identification of H. pylori

Strains of H. pylori (ATCC-43504) were recovered in accordance with the instructions provided by the supplier product information sheet (https://www.atcc.org/~ps/43504.ashx). In which, the cryopreserved strains were thawed in a 37°C water bath. The solutions (100 μl) were then inoculated with 6% sheep blood Columbia agar (Huankai Microbial, Guangdong, China) and incubated at 37°C for 3 days in a facultative anaerobic environment. In terms of storage, glycerin was added to brain heart infusion broth to prepare the cryopreservation medium, where the
*H. pylori* were added and stored at -80°C. Identification of the *H. pylori* (ATCC-43504) were performed by assessment of colony morphology by microscopy, in which conformation were suggested to be needle-like with a glassy translucent appearance of colonies formed on the Columbia blood agar plate (Huankai Microbial, Guangdong, China). This indicates the strains were gram-negative spiral bacilli. Identification of *H. pylori* (ATCC-43504) were also performed by biochemical assays, including oxidase, catalase and urease tests (Huankai Microbial, Guangdong, China). A combination of the above results served as the identification of the *H. pylori* strain.

**Extraction and identification of antibacterial peptides**

Caseins (20g) were dissolved in 300 mL of deionized water at 37°C, the pH value of the solutions was adjusted to 1.5, 2.0, 2.5, or 3.0 with hydrochloric acid. Pepsin (0.6 g) (1:10000, P-5144, BoMei Biotechnology, HeFei, China) was added to the solutions for enzymolysis, which were carried out at 37°C for 5 hours. During the experiments, hydrochloric acid was added drop-wise to maintain the pH value of the solution. After enzymolysis, the pepsins were inactivated by incubation in a water bath at 80°C for 20 minutes. The de-enzymatic solutions were then centrifuged at 3500 rpm and 9000 rpm for 30 minutes and 20 minutes, respectively. The supernatants were then collected and filtered through a 0.45 μm filter and re-filtered through a 0.22 μm filter. The mixtures
were evaporated to dryness on a rotary evaporator and freeze-dried. The dry mixture (0.1g) was sent to the National Center for Protein Sciences (Beijing, China) for molecular weight analysis and peptide sequencing analysis by MALDI-TOF mass spectrometry and MS/MS Ion Search, respectively.

Preparation of *H. pylori* and *E. coli* 0.5 McFarland Turbidity solution

Several *H. pylori* colonies from the 6% sheep blood Columbia broth were added to sterile tubes containing 0.9% normal saline, shaken for 15 seconds and adjusted to a turbidity of 0.5 in the microbial turbidimeter (DensiCHEK Plus, bioMerieux, USA). The same methods were performed for the preparation of the *E. coli* (ATCC-25922) solution.

Antibacterial activity test

Microdilution methods were used to find the inhibitory effects of each group of antibacterial peptides against *E. coli*. Sterile Brucella broth (HopeBio, Qingdao, China) was used to dilute the antibacterial peptide extracts with pH = 1.5, 2.0, 2.5 and 3.0 through the following methods. The broth (600 μl) was added to a 48-well plate and 600 μl of 50 mg/mL antibacterial peptides was added to the first well, followed by seven half-dilutions to produce the 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, 3.125 mg/mL, 1.5625 mg/mL, 0.78125 mg/mL, 0.390625 mg/mL and 0.1953125 mg/mL peptide extracts.
The E. coli 0.5 (approximately 1.5 x 10^8 CFU/mL) McFarland Turbidity solution (2 μl) was then added to each of the peptide solutions and incubated at 37°C for 24 hours in a facultative anaerobic environment. The mixtures were assigned to five categories by naked-eye observations. The categories were clearly transparent, blurred (80% suppressed), turbidity significantly reduced (50% inhibited), turbidity mildly reduced and turbidity was not reduced. Mixtures of blurred or turbidity significantly reduced were considered of having antibacterial activities against E. coli.

Minimum inhibitory concentration determination

The preparations of MIC assays of antibacterial peptides against H. pylori were the same as that for analysis of E. coli, with the difference being that each H. pylori assay was repeated 12 times for higher accuracy and consistency. Instead of performing naked-eye observation as in the E. coli assays, the H. pylori MIC assays were performed in sterile 96-well microliter plates (ChunBo Biologics, Haimen, China) and the absorbance of the spectrophotometer was set at 600 nm. The MIC_{90} values were defined as the lowest concentration of peptide that inhibits 90% of H. pylori. The inhibition percentage was calculated by the following equation:
Inhibition (%) 

\[ \text{Inhibition (\%)} = \left( 1 - \frac{\text{optical density (OD) value of sample}}{\text{optical density (OD) value of negative control}} \right) \times 100\% \]

Statistical analysis

Data analysis was performed using the paired t-test comparative method embedded in the SPSS 20.0 software to calculate the P values, which were considered to be statistically significant when \( P \) was less than 0.05. The sample groups were compared with the blank control, negative and positive control groups. Regression analysis was used to analyze the relationship between the concentration of each group of the antibacterial peptides and their OD values. Pearson correlation analysis was used to evaluate the relationship between pH changes and MIC values.

Results

Antibacterial peptide extraction rate

Casein (20 g) was hydrolyzed by pepsin at different pH values. They were then concentrated and freeze-dried to give the weight and extraction rate as shown in Table 1.

Identification of antibacterial peptides

The MALDI-TOF mass spectrometry analyzed the molecular weight of the
antimicrobial peptides at pH 2.0 and pH 3.0 environment (Figure 1 and 2). The peaks with the highest abundance at both pH values were located at 1881 m/z and 1994 m/z. Peptide sequencing analysis was performed by the MS/MS Ion Search and the resulting amino acid sequences with 1881 m/z and 1994 m/z were YQEPVLGPVRGPFPIIV and LYQEPVLGPVRGPFPIIV, corresponding to residues 208–224 and 207–224, respectively (Table 2). These sequences were matched with the corresponding peptides in the Antimicrobial Peptide Database [20], Milk Peptide Database [21] and MilkAMP Database [22]. The peptides with 1881 m/z were identified as casecidin 17 with APD (Antimicrobial Peptide Database) ID of AP01398, and there were no records in the above three databases for the peptide with 1994 m/z. Literature searches of this peptide were performed on the most common databases, including PubMed, Google Scholar, Cochrane Library Databases, Science Direct, and only a single literature was found that mentioned its bioactivity, which is modulation of the bitter taste receptors and was named β-casein 207–224 [23].

Antibacterial activity and minimum inhibitory concentration determination

Antibacterial assays of the peptides against *E. coli* were performed using naked-eye observations. All the result mixtures were assigned as blurred or turbidity
significantly reduced, which indicate that *E. coli* were successfully inhibited by each group of antimicrobial peptides. This proved that the extracted antimicrobial peptides in each group were active.

With regards to the *H. pylori* MIC assays, all eight concentrations of the antibacterial peptides showed anti-*H. pylori* activity. The MIC$_{90}$ of both pH 1.5 and pH 2.0 were 6.25 mg/mL, whereas, for both pH 2.5 and pH 3.0, the MIC$_{90}$ was 12.5 mg/mL.

Regression analysis showed that when the concentration of the peptide increased, the optical density (OD) values became smaller (p < 0.001) (Table 3), indicating that the higher the concentration of the peptide, the better the anti-*H. pylori* effect. The Pearson correlation analysis between pH and the MICs of the antibacterial peptides in different concentrations showed a positive correlation between the pH and the MIC value with a correlation coefficient of 0.82. This indicates that the greater the pH value, the larger the MIC$_{90}$ (p < 0.001). Hence, the anti-*H. pylori* effects of the peptides are higher in lower pH environment, such as in the human stomach.

**Discussion**

Due to an increase in *H. pylori* resistance to antibiotics, such as clarithromycin, the triple therapy treatment has become gradually less efficacious [8]. This study discovered that the major products of bovine milk casein digestion have promising anti-
*H. pylori* effects. The two major peptides identified in this study were casecidin 17 and β-casein 207–224. The anti-*H. pylori* effects of bovine milk has been suggested by Wang *et al.* [24] and the non-*H. pylori* specific antibacterial activities against both certain Gram-positive and Gram-negative bacteria of bovine casein has also been demonstration [19]. Casecidin 17 is well-documented in the literature and several bioactivities have been suggested. Rojas-Ronquillo *et al.* found the angiotensin-converting enzyme inhibitory and antithrombotic properties of casecidin 17 with inhibition efficiency ratio of 0.1%/peptide concentration (μg/mL) and 4.6%/peptide concentration (μg/mL), respectively [25]. Sandre *et al.* found that casecidin 17 has immunomodulatory activity in mice, probably by enhancing the antimicrobial activity of macrophages [26]. In terms of antibacterial properties, Birkemo *et al.* were the first to establish the inhibition effects of casecidin 17 of *E. coli*, indicated by *in vitro* MIC values of 0.4 mg/mL [27]. In this study, we found that the peptide extract mainly containing casecidin 17 and β-casein 207–224 had anti-*H. pylori* properties. Further studies will be focused on the anti-*H. pylori* activities of individual peptides.

As no antibacterial information on β-casein 207–224 can be found in various databases, we believe this study is the first to reveal its potential anti-*H. pylori* activities. β-casein 207–224 has only one extra amino acid residue than that of casecidin 17 and
the rest of their sequences are in the same order. Hence, their structures and sequences are highly similar, which suggests that they may have similar biological functions. The reason for the small amount of information in the literature about β-casein 207–224 means the hydrolysis of this peptide is not common in most experimental conditions. Here, the enzymolysis was performed in acidic environments, which could be an important factor for producing β-casein 207–224. As shown in Figures 1 and 2, the amount of β-casein 207–224 is much higher when the casein was enzymolysed at pH 2.0, compared to at pH 3.0. Hence, the amount of β-casein 207–224 could be one of the major antibacterial peptides found in a human empty stomach, where the pH value is approximately 1.5 to 2.5. Furthermore, the MIC90 values at pH 2.0 are lower than that at pH 3.0, indicating that better anti-\textit{H. pylori} activities were achieved at the pH 2.0 environment, where the concentration of β-casein 207–224 is higher (Figures 1 and 2).

Several studies have produced antibacterial peptides mixture with casecidin 17 through different methods [25-27]. Our study is the first to use a simple \textit{in vitro} pepsin enzymolysis method to successfully establish casecidin 17 in extract. According to the supplier product information sheet (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/c5890pis.pdf), the caseins used in this study were obtained from bovine milk and contain four main types of casein: α-s1
Casein, α-s2 Casein, β-Casein and κ-Casein. Pepsin is one of the major enzymes in the human gastrointestinal tract required for the digestion of ingested proteins, including milk. Hence, this *in vitro* study simulated the digestion process of bovine milk casein by pepsin, and we found that the major products of such a process are casecidin 17 and β-casein 207–224. A study showed that bovine milk contains about 32 g of protein per litre [28]. Casein has been considered to be the main constituent in milk and it makes up approximately 80% of the total protein in bovine milk [18].

The MIC₉₀ values in this study were obtained from *in vitro* experiments, hence, many other *in vivo* factors that may affect the activity of the peptides were excluded. For example, there are other enzymes that may digest casein and produce other types of antibacterial peptides, which may not be casecidin 17 and β-casein 207–224. Another excluded factor is that the human gastrointestinal tract is highly complex and contains food and many other bacteria, hence, casecidin 17 and β-casein 207–224 may bind to other bacteria or other substances and have less direct contact with *H. pylori*. Nevertheless, this study provides the basis for further investigation on casecidin 17 and β-casein 207–224 for novel anti-*H. pylori* peptide design. Further optimization of antibacterial peptide extraction could assist in developing therapeutic agents to modulate the effect of antibiotics on *H. pylori* infections.
Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.
References


Figure legends:

**Figure 1.** Mass spectrum of the antimicrobial peptide extraction with pH 2.0.

**Figure 2.** Mass spectrum of the antimicrobial peptide extraction with pH 3.0.
Table I. Antibacterial peptide extraction rate

<table>
<thead>
<tr>
<th>pH values</th>
<th>Weight of extraction (g)</th>
<th>Extraction rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>5.84</td>
<td>29.2</td>
</tr>
<tr>
<td>2.0</td>
<td>5.15</td>
<td>25.8</td>
</tr>
<tr>
<td>2.5</td>
<td>4.35</td>
<td>21.8</td>
</tr>
<tr>
<td>3.0</td>
<td>7.36</td>
<td>36.8</td>
</tr>
</tbody>
</table>

Table II. Peptide profiles of the enzymolysed casein at pH 2.0 and 3.0

<table>
<thead>
<tr>
<th>pH 2.0</th>
<th>Observed</th>
<th>Calculated</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2877.70</td>
<td>2876.56</td>
<td>179–204</td>
<td>SLSQSKVLVPQKAVYPQRPMDIIQA</td>
<td></td>
</tr>
<tr>
<td>2107.32</td>
<td>2106.22</td>
<td>206–224</td>
<td>LLYQEPVLGPVRGPQPIIV</td>
<td></td>
</tr>
<tr>
<td>1881.14</td>
<td>1880.66</td>
<td>208224</td>
<td>YQEPVLGPVRGPQPIIV</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>pH 3.0</th>
<th>Observed</th>
<th>Calculated</th>
<th>Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2877.65</td>
<td>2876.56</td>
<td>179–204</td>
<td>SLSQSKVLVPQKAVYPQRPMDIIQA</td>
<td></td>
</tr>
<tr>
<td>1881.10</td>
<td>1880.66</td>
<td>208–224</td>
<td>YQEPVLGPVRGPQPIIV</td>
<td></td>
</tr>
<tr>
<td>1460.94</td>
<td>1459.90</td>
<td>211–224</td>
<td>PVLGPVRGPQPIIV</td>
<td></td>
</tr>
</tbody>
</table>

Table III. Optical density values of the extract with different concentrations and at environments with different pHs

<table>
<thead>
<tr>
<th>Extract concentration (mg/mL)</th>
<th>pH 1.5</th>
<th>pH 2.0</th>
<th>pH 2.5</th>
<th>pH 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.0505±0.1247</td>
<td>0.0503±0.0024</td>
<td>0.0552±0.0022</td>
<td>0.0456±0.0013</td>
</tr>
<tr>
<td>12.5</td>
<td>0.0499±0.0082</td>
<td>0.0453±0.0014</td>
<td>0.0462±0.0013</td>
<td>0.0536±0.0070</td>
</tr>
<tr>
<td>6.25</td>
<td>0.0493±0.0082</td>
<td>0.0687±0.0179</td>
<td>0.2530±0.1178</td>
<td>0.3168±0.0885</td>
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<tr>
<td>3.125</td>
<td>0.2527±0.0477</td>
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<td>0.3556±0.0621</td>
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<td>1.5625</td>
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<tr>
<td>0.1953125</td>
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<td>0.3431±0.0484</td>
<td>0.3650±0.0198</td>
<td>0.3759±0.0488</td>
</tr>
</tbody>
</table>

Values after (±) indicate standard deviations.

Preprint
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Figure 2. Mass spectrum of the antimicrobial peptide extraction with pH 3.0.