Isolation of Helicobacter Pylori from Raw Milk and Study on Its Survival in Fermented Milk Products

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ABSTRACT
This research aimed to know if raw milk is a possible source of Helicobacter pylori infection to human and assessing the inhibitory effect of fermented milk products on H. pylori growth. Sixty samples of cows' milk and raw marketable milk (30 samples each) were tested to detect H. pylori and were collected from farms and supermarkets in Assiut City, Egypt. The pathogen could be isolated from 13.33 and 6.66 % of cows' milk and marketable milk, respectively, by using the conventional culture method. Confirmation of the isolated strains using the PCR technique revealed that, 50% of the isolated strains were positive for the 16S rRNA gene. The strong antibiogram of Lactobacillus acidophilus was evident, where the count of the tested two strains of H. pylori (S1 and S2) was sharply decreased to 2.6 and 2.17 log cfu/ g yoghurt and 2.47 and 3.04 log cfu/ml rayeb at the time of fermentation and could not be isolated at the first day. In case of Bifidobacterium bifidum, the count of S1 and S2 on the first day was 2.3 and 2.77 log cfu/ g yoghurt and 2.95 and 2.7 log cfu/ml rayeb. Regarding the viability, H. pylori strains have remained viable for two days in yoghurt that was not supplemented with probiotics and could survive for seven days in control milk samples. There was no significant difference between the growth patterns of S1 and S2 in all treatments. Finally, fermented milk products containing probiotics were more effective in the survival of H. pylori than fermented milk products with no probiotics with a significant difference (P-value < 0.05).

Keywords: Helicobacter pylori, Milk, Probiotics, Rayeb, Yoghurt.

Helicobacter pylori is a microaerophilic spiral-shaped, Gram-negative bacterium that colonizes the gastric mucosa of more than 50% of the worldwide population. It is considered the major cause of peptic ulcer disease, duodenal ulcer, type B gastritis, gastric adenocarcinoma, lymphoma of mucosa-associated lymphoid tissue and gastric B-cell and human is the main reservoir of H. pylori infection (Crowe, 2019; Parikh and Ahlawat, 2021).

The prevalence of H. pylori infection in developed countries is around 34.7%, while in developing countries is 50.8% (Zamani et al., 2018). The infection of this bacteria is acquired very early in life and is often persistent lifelong without inducing symptoms if not properly treated (Kalali et al., 2015).

The role of foods especially milk and milk products in the transmission of H. pylori is still unknown (Dixit et al., 2012), but the presence of H. pylori in the stomach of domestic animals without inducing any disease and its recovery from milk, meat and the stomach of ruminants suggests that, these animals and milk may act as reservoirs for H. pylori infection (Sambhaviah et al., 2011). In addition, milk and milk products are suitable for the growth and survival of H. pylori and consequently, its transmission to humans (Vale and Vitor, 2010).

Treatment of H. pylori is difficult due to its colonization of the epithelial cells that line the antrum of the stomach and the access of antibiotics in this place is limited (Goodwin and Armstrong, 1990). In addition to the inactivation of antimicrobial agents in the stomach, as well as, antibiotic therapy is accompanied by the growth of antibiotic resistance species and unwanted side effects (Gotteland and Gruchet, 2003; Megraud and Lehours, 2007). For these reasons, the development of alternative methods to prevent the colonization of H. pylori and searching for new better therapies have become an essential demand, so much attention has focused on probiotics in recent years.
Probiotics are defined as nonpathogenic life microorganisms that have a beneficial effect on the host (FAO/WHO, 2001). Lactic acid-producing bacteria and Bifidobacterium are the most commonly used probiotics (Sullivan and Nord, 2005). The antagonism of these groups of bacteria against pathogens is based on acids and inhibitory substances. These substances are oxygen peroxide, derivatives of oxygen metabolism, reuterin, aromatic compounds and bacteriocins (Georgalaki et al., 2013; Madureira et al., 2011). The resistance of lactobacilli to acid assists its persistence in the stomach; hence, they are used for *H. pylori* treatment and prophylaxis (Kaur, 2020).

Therefore, the main objective of this work was to detect the possibility of *H. pylori* transmission from milk and milk products and to determine its survival in fermented milk products.

**MATERIALS AND METHODS**

1. Detection of *H. pylori*:
   1.1. Collection of samples:
   A total of sixty random raw milk samples were examined for isolation of *H. pylori* including thirty samples of individual lactating cows were collected from the farms in Assiut City and thirty samples of raw milk were purchased from dairy shops. The dairy shops milk samples were tested for heat treatment using the Storch's test (Lampert, 1975). 100 ml of milk from each sample in a sterile glass container was transported to the laboratory with a minimum delay in an icebox.

   1.2. Isolation of *H. pylori* (Stevenson et al., 2000):
   One ml from each milk sample was inoculated in a test tube containing 9 ml brain heart infusion broth supplemented with *H. pylori* selective supplement (Oxoid, UK) and 4% iron supplemented calf serum and incubated at 37°C for 24-48 h under microaerophilic condition (5% O₂, 15% CO₂ and 80% Nitrogen) using gas generation kits (Oxoid, UK). One loopful from the inoculated tubes was streaked on plates of Columbia agar containing 5% defibrinated sheep blood and *H. pylori* selective supplement and incubated at 37°C under microaerophilic conditions for 3-5 days. The suspected colonies were identified based on their morphological characteristics and biochemical tests (Fox et al., 2000).

2. Molecular identification of the isolated strains (Chong et al., 1996):
   The previously identified six positives and four suspected strains using biochemical tests were sent to the Reference Laboratory for Veterinary Quality Control on Poultry Production in Animal Health Research Institute, Dokki, Giza, Egypt for molecular confirmation by PCR technique.

2.1. DNA extraction:
DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany) with modifications from the manufacturer's recommendations. Briefly, 200 μl of the sample suspension was incubated with 10 μl of protease K and 200 μl of lysis buffer at 56°C for 10 min. After incubation, 200 μl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 μl of elution buffer provided in the kit.

2.2. PCR amplification:
Primers were utilized in a 25-μl reaction containing 12.5 μl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 μl of each primer of 20 pmol concentrations, 4.5 μl of water, and 6 μl of DNA template. The reaction was performed in an applied biosystems 2720 thermal cycler. Primers supplied from Metabion (Germany) are listed in Table (1).

### Table 1: Primers used for identification of 16S rRNA of *H. pylori*

| Target gene | Primers sequences | Amplified segment (bp) | Amplification (35 cycles) | Final extension |
|-------------|-------------------|------------------------|----------------------------|----------------|
| 16S rRNA    | CTGGAGAGA CTAAGCCCTCC | 109                    | Primary denaturation 94°C for 5 min. | 72°C for 7 min. |
|             | GTCGATTGTGC GGCGGCGC |                       | Secondary denaturation 94°C for 30 sec. |                |
|             | GATTACTGAG GCCATGTC |                       | Annealing 60°C for 30 sec. |                |
|             | 109                |                       | Extension 72°C for 30 sec. |                |

2.3. Analysis of the PCR Products:
The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis,
15 μl of the products were loaded in each gel slot. Gelpilot 100 bp (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and all data were analyzed through computer software.

3. Survival of *H. pylori* in fermented milk products:

3.1. Culture preparation:

3.1.1. Indicator organism:

Two strains of *H. pylori* (S1 and S2) used in the experiment were the previously isolated and identified strains. They were propagated in brain heart infusion broth at 37°C for two days and tenfold serial dilution was made to detect the density of the organism/ml broth.

3.1.2. Strains of probiotics:

*Lactobacillus acidophilus* 20079 and *Bifidobacterium bifidum* ATCC 15696 strains were the used reference strains in the experiment. *L. acidophilus* strain was cultured on De Man, Rogosa and Sharpe broth (MRS broth), while *B. bifidum* strain was grown on MRS broth supplemented with cysteine and was incubated at 37°C for 48 h. anaerobically, then, tenfold serial dilution was made to detect their count/ml of the inoculated broth.

3.2. Experimental technique:

3.2.1. Preparation of yoghurt (Abo- Donia, 2008):

One liter of milk was purchased from Assiut City supermarkets and displaced to the laboratory. Milk was boiled for a few minutes and cooled to 45°C and 2% yoghurt culture (yoghurt sold in supermarket and was examined for *H. pylori* free by plating on Columbia agar plates) was added and mixed well. One portion was taken as control negative before inoculation with *H. pylori* strains and the remaining portion was divided into three groups of two bottles; the first bottle was inoculated with S1 and the second with S2 of *H. pylori* in a count of 5× 10^6 cfu/ml (6.69 log cfu/ml). The first group was inoculated with *L. acidophilus* and the second with *B. bifidum* to obtain a count of 5× 10^6 cfu/ml (9.69 cfu/ml). The third group was not inoculated with probiotics and then the content of each bottle was put in shallow or deep earthenware pots and was kept in warm dark place till the cream was raised. Milk was coagulated and this curd is named rayeb, then kept in the refrigerator. In addition, 100 ml of fresh milk was made rayeb and was not inoculated with *H. pylori* as a control negative.

3.2.2. Preparation of rayeb (Abo- Donia, 2008):

One liter of fresh milk was divided into three groups of two bottles one bottle was inoculated with S1 and the second with strain S2 of *H. pylori* at a count of 5× 10^6 cfu/ml (6.69 log cfu/ml). The first group was inoculated with *L. acidophilus*. The second with *B. bifidum* to obtain a count of 5× 10^6 cfu/ml (9.69 cfu/ml) and the third group was not inoculated with *H. pylori* and kept in the refrigerator at 4±2°C.

3.2.3. Preparation of control positive:

500 ml milk was boiled for 15 min and cooled to 37°C, then was put in two bottles. One bottle was inoculated with S1 and the second with S2 of *H. pylori* in a count of 5× 10^6 cfu/ml (6.69 log cfu/ml) as control positive.

3.2.4. Enumeration of *H. pylori* in the inoculated products:

Tenfold serial dilution of the inoculated groups, control positive and control negative of yoghurt and rayeb were carried out using 0.1% peptone water. The cells number of *H. pylori* was determined by plating on Columbia blood agar plates. Its count was detected at the time of fermentation of the products and on the first, second, third and seventh days.

4. Statistical analysis:

The Statistical analysis of the data was performed using Bartlett’s test for homogeneity and Prism 5 by Turkey’s Multiple Comparison Test to detect the significant difference between different treatments.

### RESULTS

As recorded in Table 2, *H. pylori* could be isolated from 4 out of the examined 30 cows’ milk samples in a percentage of 13.33. On the other hand, the incidence of *H. pylori* in the tested marketable milk samples was 6.66%, where it could be isolated from 2 out of the examined samples.

| Samples          | No. of examined | Positive samples |
|------------------|----------------|-----------------|
| Cows’            | 30             | 4               | 13.33%          |
| Marketable       | 30             | 2               | 6.6%            |

As observed in Figure1 and Table 3, confirmation of the isolated strains from Cows’ milk and Marketable milk by using PCR revealed that two and one out of the identified strains were confirmed positive for *H. pylori* with a percentage of 50%. Consequently, the incidence of *H. pylori* in the tested samples was 6.66 and 3.33%, respectively.
Table 3: Results of molecular conformation of the isolated *H. pylori* strains by using PCR technique:

| Samples       | Prevalence of *H. pylori* in the isolated strains No. | Incidence of *H. pylori* in the examined samples No. |
|---------------|-----------------------------------------------------|-----------------------------------------------------|
| Cows' milk    | 2/4                                                 | 2/30                                                 |
| Marketable    | 1/2                                                 | 1/30                                                 |

Fig. 1: Molecular identification of the isolated *H. pylori* strains by using PCR.

The data illustrated in Table 4 represented the effect of yoghurt on the survival of *H. pylori*, where the organism could be isolated from yoghurt supplemented with *L. acidophilus* in a count of 2.6 and 2.17 log cfu/g for S1 and S2, respectively, at zero time and could not be detected at the first day. While in case of *B. bifidum*, the initial count of *H. pylori* was 4.3 and 4.01 log cfu/g and could be isolated on the first day in a count of 2.3 and 2.77 logs cfu/g. On the contrary, *H. pylori* could survive in yoghurt with no probiotics for two days with a count of 4.77 and 4.3 logs cfu/g at the time of fermentation, 4.6 and 3.95 log cfu/g on 1<sup>st</sup> day, 2.0 and 2.7 at 2<sup>nd</sup> day and could not be isolated at the 3<sup>rd</sup> day.

Table 4: Survival of *H. pylori* in yoghurt supplemented with *L. acidophilus* and *B. bifidum*:

| Day       | L. acidophilus | B. bifidum | No probiotics |
|-----------|----------------|-------------|---------------|
|           | S1 count       | S2 count pH | S1 count      | S2 count pH | S1 count      | S2 count pH |
| Zero time | 2.6            | 4.3         | 2.17          | 4.4         | 4.3          | 4.5         | 4.01          | 4.4         | 4.77          | 4.8         | 4.3          | 4.9         |
| 1<sup>st</sup> day | ND            | ND          | ND            | ND          | ND           | ND          | ND            | ND          | ND           | ND          | ND           |
| 2<sup>nd</sup> day | ND            | ND          | ND            | ND          | ND           | ND          | ND            | ND          | ND           | ND          | ND           |
| 3<sup>rd</sup> day | ND            | ND          | ND            | ND          | ND           | ND          | ND            | ND          | ND           | ND          | ND           |

Zero time = time of fermentation, ND = not detected, S1 = strain 1 of *H. pylori* and S2 = strain 2 of *H. pylori*

According to the reported data in Table 5, the strains of *H. pylori* could survive in rayeb supplemented with *L. acidophilus* during fermentation of the product with a count of 2.47 and 3.04 log cfu/ml and failed to be detected on the first day. In case of *B. bifidum*, *H. pylori* strains could resist to the first day and their initial count was 4.6 and 4.5 log cfu/ml and on the 1<sup>st</sup> day was 2.95 and 2.7 log cfu/ml. On the other hand, the pathogen could withstand for two days in rayeb with no probiotics in a count of 5.95 and 5.32 at time zero and the microbial
population of S1 and S2 was 4.6 and 3.69 log cfu/ml on the 1\textsuperscript{st} day, 3.04 and 3.95 log cfu/ml at the 2\textsuperscript{nd} day, then, they could not be detected at the 3\textsuperscript{rd} day.

Table 5: Survival of \textit{H. pylori} in Rayeb supplemented with \textit{L. acidophilus} and \textit{B. bifidum}:

| Day    | S1  | S2  | S1  | S2  | S1  | S2  |
|--------|-----|-----|-----|-----|-----|-----|
|        | Count | pH  | count | pH  | Count | pH  | count | pH  | Count | pH  | count | pH  |
| Zero time | 2.47 | 5.9  | 3.04 | 5.7  | 4.60 | 5.8  | 4.50 | 6.0  | 5.95 | 6.0  | 5.32 | 6.1  |
| 1\textsuperscript{st} day | ND  | ND  | ND  | ND  | 2.95 | 5.4  | 2.70 | 5.6  | 3.6  | 5.8  | 3.69 | 5.9  |
| 2\textsuperscript{nd} day | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | 3.04 | 5.1  | 3.95 | 5.3  |
| 3\textsuperscript{rd} day | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  | ND  |

Zero time = time of fermentation, ND = not detected, S1 = strain 1 of \textit{H. pylori} and S2 = strain 2 of \textit{H. pylori}

Regarding Control samples, strains of \textit{H. pylori} could survive in control milk samples for seven days with a low decreasing percentage and their count was 6.6 and 6.3 log cfu/ml at zero time. While, the bacterial densities of S1 were 5.8, 5.2, 4.7 and 4.3 log cfu/ml at the 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd} and 7\textsuperscript{th} days and S2 was found in a population of 5.27, 4.95, 4.69, 4.2, respectively (Table 6).

Table 6: Results of control samples.

| Day    | Control +ve | Control –ve |
|--------|-------------|-------------|
|        | S1  | S2  | Yoghurt | Rayeb |
|        | Count | pH  | Count | pH  | ND  | ND  | ND  | ND  |
| Zero time | 6.6  | 6.7  | 6.3  | 6.8  | ND  | ND  |
| 1\textsuperscript{st} day | 5.8  | 6.7  | 5.27 | 6.7  | ND  | ND  |
| 2\textsuperscript{nd} day | 5.2  | 6.7  | 4.95 | 6.7  | ND  | ND  |
| 3\textsuperscript{rd} day | 4.7  | 6.6  | 4.69 | 6.7  | ND  | ND  |
| 7\textsuperscript{th} day | 4.3  | 6.6  | 4.2  | 6.6  | ND  | ND  |

Zero time = time of fermentation, ND = not detected, S1 = strain 1 of \textit{H. pylori} and S2 = strain 2 of \textit{H. pylori}

**DISCUSSION**

Although the internal contamination of milk with \textit{H. pylori} is not likely to be found, its presence in the environment leads to external milk contamination from the surrounding. In addition to milk properties that provide \textit{H. pylori} the opportunity for transition to human (Talaei \textit{et al.}, 2015).

Comparing the summarized results in Table 1 and 2 with the recorded findings by other authors revealed that, the highest incidence was reported by Elhariri \textit{et al.}, (2017), who found that, 30.0% of the tested cows’ milk were seropositive for \textit{H. pylori} by using different ELISA kits. Mousavi \textit{et al.}, (2014) and Yahaghi \textit{et al.}, (2014) could isolate the organism from cows’ milk in a nearly similar percentage to the present study (16.0 and 16.66%) and similar results for PCR detection of \textit{H. pylori} were recorded by Ranjbar \textit{et al.}, (2018), who could detect \textit{H. pylori} in 7.5% of the examined bovine milk samples. On the other hand, lower results were recorded by Talimakhani and Mashak (2017), who could isolate \textit{H. pylori} from 4% of the examined bovine milk samples. Guessoum \textit{et al.}, (2018) found that, 6.0 and 13.0% of the examined samples were positive for the organism by using ELISA assays and PCR technique and failed to isolate...
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*pylori* have the ability to survive low pH by the breakdown of urea found in foods to carbon dioxide and ammonia to neutralize the area around it, while in fermented milk products urea is exhausted at the time of ripening (Mobley, 2001).

*B. bifidum*, unlike lactobacillus, has an antagonistic effect on *H. pylori* but in addition to its antagonism, it improves the pathological lesions of the gastric mucosa and shifts the stages of peptic ulcer to a mild degree and decreases urease activity of *H. pylori*. In addition, *B. bifidum* declines the count of *E. coli* in the intestine and consequently decreases the molecular hydrogen in the stomach that is used as a source of energy for *H. pylori* growth (Olson and Maier, 2002; Wang et al., 2004).

It is obvious from the demonstrated data that probiotics have an inhibitory effect on *H. pylori* growth. The variations between the antagonistic effect of *L. acidophilus* and *B. bifidum* were clearly evident and *L. acidophilus* was more effective than *B. bifidum*. Moreover, the viability and activity of probiotics are the main consideration during the processing of fermented milk because they must survive during the shelf life to confer health benefits to humans and be found in an adequate count that can suppress the growth of pathogenic bacteria. So, over-acidification can be limited by good manufacturing practices and using a culture of reduced over-acidification behavior (Kneifel et al., 1993).

Fermented milk products containing probiotics especially *Lactobacilli* and *Bifidobacterium*, have beneficial effects on humans as a consequence of their obvious antagonism against *H. pylori* as reported in this literature. Therefore, we have attempted to develop a fermented dairy product containing an adequate amount of probiotics that strongly affect the in vitro viability of *H. pylori* to render fermented milk product *H. pylori* free and to be used for the treatment of *H. pylori*; on the other hand, the viability of probiotics must be taken in consideration.

**CONCLUSION**

The findings of this study declared that milk and milk products have an impact role in the transmission of *H. pylori* infection to humans and the antagonistic activity of fermented milk products against this organism, especially through probiotics, is clearly evident. In addition, complementary and extended studies in the future to elucidate the factors affecting the growth of *H. pylori* in fermented milk products must be carried out to establish a precise data of the probiotics densities to be used, supplementation of fermented milk products with more than one probiotic and consequently, using these products as an alternative method for treatment of this pathogen.

**Declaration of Conflicting Interests:**

The authors revealed that there is no potential conflicts of interest.

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How to cite this article:
Lamiaa M. Talaat AL Shrief and Salwa S. Thabet, 2022. Isolation of Helicobacter Pylori from Raw Milk and Study on Its Survival in Fermented Milk Products. Journal of Applied Veterinary Sciences, 7 (2): 73-81.
DOI: 10.21608/javs.2022.124671.1133

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