



Original research

Production of functional fermented camel milk with Anti-*Helicobacter pylori* activity

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ABSTRACT

The aim of this study was to use two probiotic strains, *Lactobacillus rhamnosus* and *Lactobacillus casei*, in order to produce a functional fermented camel milk drink with anti-*Helicobacter pylori* activity. During a 35-day storage period, chemical (pH and acidity contents), sensory characteristics, and *Helicobacter pylori* inhibitory activity of fermented camel milk samples have been investigated; *Helicobacter pylori* activity was examined using the Berthelot Method. At the end of the cold storage period, the lactic acid bacteria count was acceptable for probiotic products. Camel skim milk and fermented milk samples showed promising *Helicobacter pylori* inhibitory activity. The IC₅₀ results from this study were IC₅₀ = 24.58 μM and IC₅₀ = 55/73 μM). Specific inhibition or reduction of urease enzyme activity would result in an increased sensitivity of the bacteria in an acidic medium, and therefore, it can be considered a new functional food for stomach problems.

Keywords: Fermentation; Camel milk; Probiotic; *Helicobacter pylori*; Inhibitory activity; Sensory evaluation

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1. Introduction

While most probiotic foods are functional, such foods are fortified or improved foods that provide health advantages in addition to essential nutrients when taken in adequate quantities as part of a daily diet (Khaneghah & Fakhri, 2019; Khaneghah et al., 2020). The market demands for probiotic functional foods are growing dramatically due to consumers' consciousness regarding the value-added properties of probiotic functional foods (Tripathi & Giri, 2014; Hashemi et al., 2017; Jafari et al., 2019).

Among the used bacteria in the production of probiotic functional foods, lactic acid bacteria (LAB) are amongst the most important ones (Mahmood Fashandi et al., 2018; Khaneghah et al., 2017). The fermentation of milk by lactic acid bacteria (LAB) is one of the most economical, promising, and practical techniques for the production of fermented dairy products wealthy in biologically

active peptides (Hayes et al., 2007). The advantages of LAB in terms of health and nutrition are nicely highlighted through the correlation between their metabolites and health-promoting attributes (Hayes et al., 2007). LAB is capable of releasing different substances such as organic acids, diacetyl, hydrogen peroxide, bacteriocins, and biologically active peptides (Moraes et al., 2010). The proteolytic activities of LAB contribute to the liberation of milk proteins-originated peptides in a variety of fermented functional dairy products such as yogurt, cheese, buttermilk, or cultured milk. These peptides can improve the beneficial health effects and sensory characteristics of the products (Fitzgerald & Murray, 2016).

According to the statistics published by the Food Organization in 2008, from the total camel in population the world, the largest camel population is in Africa (85%), and only 3% of camels were in the Persian Gulf and Oman sea countries and 0.6% of camel

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population are allocated to Iran (Faostat, 2010). Camel milk, as a well-balanced supply of nutrients and biological components, is an excellent source for fermented milk production (Chandan, 2004). It also contains more vitamin C and niacin than bovine milk and higher amounts of copper and iron. Camel milk, like human breast milk, has significant amounts of α -lactalbumin, making it a useful source for toddler formula growth (Salami et al., 2009). In this regard, the *in vivo* and *in vitro* investigations demonstrated that the consumption of fermented camel milk offers some health promotion properties like antimicrobial (López Expósito & Recio, 2006), cholesterol-lowering (Hartmann & Meisel, 2007), antihypertensive (Korhonen & Pihlanto, 2006), and antidiabetic (Shori, 2015). Also, the spent culture supernatants (SCS) produced by fermented camel milk can be accounted for as an efficient agent against *Helicobacter pylori* infection among the human gastric epithelial cells.

Lactobacilli are organisms that include lactobacilli, lactococci, *Bifidobacterium*, and yeasts classified as safe organisms (Suresh et al., 2013). A few studies are available regarding the viability of probiotic bacteria inside the human intestine as their habitat, but many strains, which are currently used as a probiotic, have considerable survival rates inside the human gut. Probiotic bacteria affect the intestinal system through the production of some metabolites (Bezkorovainy, 2001). Their mechanisms of action include: 1) direct interaction with the complex ecosystem in the gut lumen 2) interaction with the gut mucus and the epithelium 3) affecting other organs, including the liver, systemic immune response, and the brain (Mättö et al., 2006). Furthermore, probiotic bacteria such as *Lactobacillus plantarum*, *Lactobacillus fermentum*, and *Lactobacillus casei* and their metabolites have shown an inhibitory effect on preventing or delaying *Helicobacter pylori* colonization in the gastric mucosa (Sickarchi & Fozouni, 2018). Due to the production of lactic acid and other potentially inhibitory metabolites in fermented milk and culture supernatant fraction, direct and even synergetic effects against pathogens, particularly *Helicobacter pylori*, have been obtained (Lin et al., 2011).

Helicobacter pylori, a microaerophilic, gram-negative spiral bacterium, is one of the most common pathogens in humans (Owen, 1995). A significant percentage of people in the world are infected in different ways by *Helicobacter pylori*. The health issues associated with the affected person are noncompliance, which can be controlled by drugs such as antibiotics (clarithromycin and amoxicillin) and metronidazole. However, ingestion of these types of medicines is expected to have side effect., about 20% of the patients undergoing antibiotics therapy would experience therapeutic defeat (Wolle & Malfertheiner, 2007).

Research into finding natural treatments for *Helicobacter pylori* infection has gotten a lot of attention. Previous research has shown that two strains of LAB, *Lactobacillus casei* and *Lactobacillus rhamnosus*, exhibited direct anti-*Helicobacter pylori* properties (Lin et al., 2011; Chen et al., 2010). To this date, no study on the impact of functional foods on *Helicobacter pylori* has been done as far as our understanding. Therefore in this study, the inhibitory activity of fermented camel milk with *Lactobacillus rhamnosus* PTCC 1637 and *Lactobacillus casei* PTCC 1608 as a functional beverage on *Helicobacter pylori* was investigated for the first time.

2. Material and Methods

2.1. Milk collection

The camel milk samples were supplied from local producers (Turkman Sahara, Iran). 6 liters of fresh camel milk were collected in autumn from Varamin, Tehran province, Iran, and stored at the refrigerator at 4°C during before fermentation.

2.2. Milk analysis

Raw camel milk samples were analyzed for total nitrogen (TN) by the Kjeldahl method (AOAC, 2000), fat by Gerber method (Kleyn et al., 2001), lactose by Fehling method (Hutcheson, 1978), total bacterial count (TC) by Plate Count Agar (Merck, Darmstadt, Germany) at 30°C for 72h (ISO 4833-1, 2013), coliforms by Violet Red Bile Agar (Merck, Darmstadt, Germany), at 37°C for 24 h (ISO 4832, 2006), pH with a digital pH meter (Consort, C860), and titratable acidity (TA) according to the titrimetric method by AOAC NO.947.05 (AOAC, 2002).

2.3. Milk fermentation

Lactobacillus rhamnosus PTCC 1637, and *Lactobacillus casei* PTCC 1608 were obtained from Persian Type Culture Collection (PTCC, Tehran, Iran). They were stored at -70 °C in 15% skim milk and 30% glycerol (Merck, Darmstadt, Germany). Furthermore, they were reactivated in sterile 10 mL aliquots of the de Man Rogosa and Sharpe (MRS) broth (Hi-Media, India) at 37°C for 24 h (Moslehishad et al., 2013). The cultures were centrifuged (6000 ×g, 20 min, and 25°C) to separate bacteria, followed by washing them twice with sterile distilled water. Biomass was then inoculated into skim milk 12% (w/v) and incubated at 37°C for 24h as pre-culture to obtain approximately 10⁸ CFU mL⁻¹.

Fresh whole camel milk was centrifuged at 6500 ×g for 20 min at 4°C to produce skim camel milk and then was pasteurized at 80°C for 20 min in a water bath and then cooled to 43°C (Moslehishad et al., 2013). Afterward, the pre-cultures were inoculated (1, 5, 10% v/v) into skim camel milk and incubated at 37 °C for 24 h. Milk fermentation was continued until the pH reached 4.6. Samples were prepared as follows: A mixture of mint and spearmint flavors as a flavoring agent was added into 3 samples with 1, 5 and 10% inoculation, and 3 other samples with 1%, 5%, 10% inoculation were without flavorings. Eventually, all samples were kept at 4°C for 35 days. All analyses were carried out on days of 1, 7, 14, 21, 28 and 35 of storage. The production procedure of fermented camel milk is demonstrated in Fig. 1.

2.4. Fermented camel milk analysis

The fermented camel milk samples were assessed for enumeration of lactic acid bacteria in MRS Agar (Hi Media, India) at 37°C for 24h (Matalon & Sandine, 1986), coliforms by Violet Red Bile Agar (Merck, Darmstadt, Germany), at 37°C for 24h (ISO 4832, 2006), pH with a digital pH meter (Consort, C860), and titratable acidity (TA) according to the titrimetric method by AOAC NO.947.05 (AOAC, 2002).

2.5. *Helicobacter pylori* inhibition assay

The ability of skim camel milk and fermented camel milk samples to prevent *Helicobacter pylori* was investigated utilizing the urease inhibition method based on released ammonia using a modified Berthelot reaction and measuring the absorbance at 625

nm (Mahernia et al., 2015). The reaction mixture consisted of 850 μ L urea, 15 μ L of Jack bean urease (Sigma, St. Louis, MO, USA), and the fermented camel milk samples of various concentrations (in the range of 0 to 100 μ L). After that, phosphate buffer (100 mM, pH 7.6) was added to reach the total volume of the mixture to 985 μ L. After pre-incubation for 30 min at 37°C, the reaction was terminated by consecutive addition of 500 μ L of solution A (containing 4.47 g salicylic acid, 2.5 g NaOH, and 20 mg sodium nitroprusside in 50 mL of distilled water) and solution B (containing 1.5 mL chlorine water and 0.5 g NaOH in 70 mL of distilled water). The mixture was then kept at 37°C for 30 min for developing color. Urease activity of control was taken 100%, and the enzyme inhibition was calculated according to the following equation:

$$I (\%) = \left(1 - \frac{T}{C}\right) \times 100 \quad (1)$$

where I (%) is the percentages of inhibition of the enzyme, T (test) is the absorbance of the sample (skim camel milk and fermented camel milk samples) in the presence of an enzyme, C (control) is the absorbance of the solvent in the presence of the enzyme.

The results were compared with the inhibition activity of hydroxyurea (IC_{50} = 100 μ g/mL) as standard.

2.6. Sensory evaluation

11 semi-trained panelist assessed the sensory characteristics of the fermented camel milk samples. The samples were distributed randomly to the panelists. On a 5-point hedonic scale, the samples were evaluated for flavor, appearance, and overall acceptability (Moslehishad et al., 2013).

2.7. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) with the help of SPSS software version 22.0 (SPSS Inc., Chicago, IL: USA, 2013). Effective treatment means were separated by Duncan's new multiple range tests. Correlation analysis also was performed based on a two-tailed Pearson correlation. Data are reported as mean \pm standard deviation (SD).

Table 1. Chemical and microbiological analyses of raw camel milk.

pH	Lactose (g/100)	TA ($^{\circ}$ D)	Protein (g/100g)	Fat (g/100g)	Coliform (CFU/mL)	Total microorganism count (CFU/mL)
6.2	3.6	9	2.9	2.6	4.1×10^4	7.57×10^4

Table 2. Sensory scores of flavored and unflavored fermented camel milk.

	Sample	Storage (day)		
		1 d	14 d	35 d
Aroma	FCM1%	3.37 ± 0.55^a	2.50 ± 0.28^a	2.37 ± 0.55^a
	FCM5%	3.00 ± 0.57^a	3.37 ± 0.23^{ab}	3.37 ± 0.55^{ab}
	FCM10%	3.25 ± 0.25^a	3.62 ± 0.62^{ab}	2.75 ± 0.72^{ab}
	FFCM1%	3.37 ± 0.23^a	3.75 ± 0.47^{ab}	3.75 ± 0.47^{ab}
	FFCM5%	3.75 ± 0.62^a	4.00 ± 0.40^b	4.12 ± 0.12^b
	FFCM10%	4.37 ± 0.23^a	4.25 ± 0.25^b	4.25 ± 0.25^b
Taste	FCM1%	3.12 ± 0.96^a	2.50 ± 0.50^a	3.00 ± 0.40^a
	FCM5%	3.00 ± 0.57^a	2.50 ± 0.64^a	2.37 ± 0.85^a
	FCM10%	2.75 ± 0.25^a	3.00 ± 0.91^a	2.37 ± 0.85^a
	FFCM1%	2.87 ± 0.71^a	3.25 ± 0.25^a	3.00 ± 0.40^a
	FFCM5%	3.25 ± 0.47^a	3.50 ± 0.50^a	3.25 ± 0.82^a
	FFCM10%	4.00 ± 0.40^a	3.87 ± 0.12^a	3.25 ± 0.28^a
Overall acceptability	FCM1%	3.12 ± 0.65^a	3.00 ± 0.40^a	2.62 ± 0.68^a
	FCM5%	2.87 ± 0.31^a	3.12 ± 0.42^a	2.62 ± 0.74^a
	FCM10%	3.00 ± 0.40^a	3.12 ± 0.71^a	2.56 ± 0.79^a
	FFCM1%	3.37 ± 0.37^a	3.50 ± 0.28^a	3.37 ± 0.28^a
	FFCM5%	3.00 ± 0.40^a	3.87 ± 0.12^a	3.62 ± 0.62^a
	FFCM10%	4.12 ± 0.42^a	4.00 ± 0.25^a	3.75 ± 0.42^a

^{a-b} Means in the same row with different superscript letters are significantly different ($p < 0.05$).

*FCM 1%, FCM 5% and FCM 10% are fermented camel milk produced by 1%, 5% and 10% LAB inoculation, respectively. FFCM 1%, FFCM 5% and FFCM 10% are flavored fermented camel milk produced by 1%, 5% and 10% LAB inoculation.

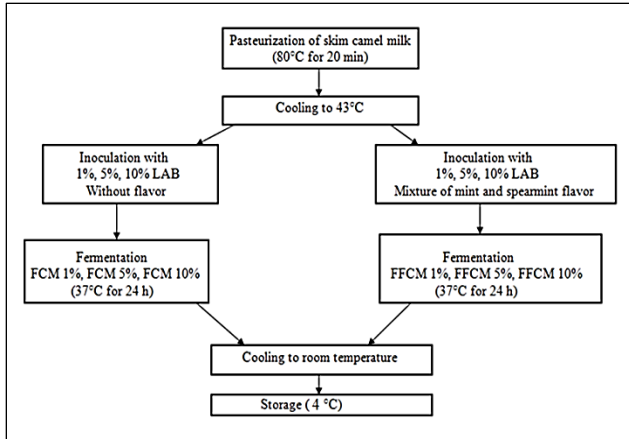


Fig. 1. Flow scheme of fermented camel milk production. FCM (Fermented Camel Milk) 1%, FCM (Fermented Camel Milk) 5% and FCM (Fermented Camel Milk) 10% are fermented camel milk produced by 1%, 5% and 10% LAB inoculation, respectively. FFCM (Flavored Fermented Camel Milk) 1%, FFCM (Flavored Fermented Camel Milk) 5% and FFCM (Flavored Fermented Camel Milk) 10% are flavored fermented camel milk produced by 1%, 5% and 10% LAB inoculation (Kök-Taş et al., 2012).

3. Results and Discussion

3.1. Raw camel milk analysis

Table 1 lists the chemical (pH, titratable acidity (TA), total nitrogen (TN), fat and lactose content, as well as microbiological (coliforms and total count (TC)) characteristics of the raw camel milk used in this study. According to Kenya's raw camel milk standard (2007), the total microbial count of raw camel milk should not exceed 10^6 CFU mL⁻¹, whereas coliforms count should be less than 10^3 CFU mL⁻¹. Kenya is one of the world's leading producers of camel milk. Raw camel milk had a coliform level of 4.1×10^4 CFU mL⁻¹ in this research. As a consequence, comparing the findings of this investigation to this criterion reveals the quality of raw milk utilized in the experiment. The mean values for pH, lactose, fat, and TA content in raw camel milk are lower than those reported by Moslehishad et al. (2013). In contrast, mean values for crude protein content were higher than that reported by Moslehishad et al. (2013). In this regard, the variations in camel milk composition can be associated with factors such as the stage of lactation, age, calving number, breeding, seasonal variations, geographical origin, and feeding conditions (Al haj & Al Kanhal, 2010).

3.2. Microbiological content of fermented camel milk samples during cold storage

LAB counts of fermented camel milk samples after fermentation and during a 35-day cold storage period were shown in Fig. 2. After 24 hours of fermentation, the bacterial count for each treatment (1, 5, and 10% inoculation of LAB) was 9.3, 9.44, and 9.57 log CFU mL⁻¹, respectively. After 14 days of cold storage, the corresponding values were reduced to 7.07, 7.36, and 7.61 logs CFU mL⁻¹, respectively. Similarly, the flavored counterparts' LAB counts reached 9.30 to 6.47 logs CFU mL⁻¹, 9.44 to 7.00 logs CFU mL⁻¹ and 9.57 to 7.11 log CFU mL⁻¹, respectively. The slow growth

rate of bacterial cells during cold storage could be correlated with the mesophilic or slightly thermophilic nature of LAB (Hammes & Hertel, 2009). Moreover, the release of peptides and amino acids as the nutrient resources due to the activity of the proteolytic *Lactobacillus rhamnosus* can be associated with the viability and minimal decline in the cell population (Pastar et al., 2003). Also, reduction in probiotic bacteria counts caused a further increase in acidity and decreased pH. While an unexpected increase in LAB counts after decreasing probiotic bacteria counts could result in serum proteins' cumulative behavior in fermented milk. These changes offer a protective effect on probiotic bacteria and the buffer capacity of the bacteria environment around that as a key factor responsible for cellular protection by milk proteins (Picot & Lacroix, 2004). For reaching the maximal therapeutic properties, the minimum number of probiotic bacteria in fermented milk should be 7 logs CFU mL⁻¹ (Jafari et al., 2017)., in the present study, the LAB counts for both flavored or unflavored fermented skim camel milk samples during cold storage were higher than the recommended level for probiotic foods. Therefore fermented camel milk can be considered a natural probiotic product due to its high contents of beneficial microorganisms. Even at the end of the 35 days of cold storage, despite the reduction in the number of LAB, their levels were not less than the acceptable limit, while a further decrease was noticed around day 35. However, at the end of 35 d of cold storage, the microflora of fermented camel milk (with or without flavoring agent) was stable.

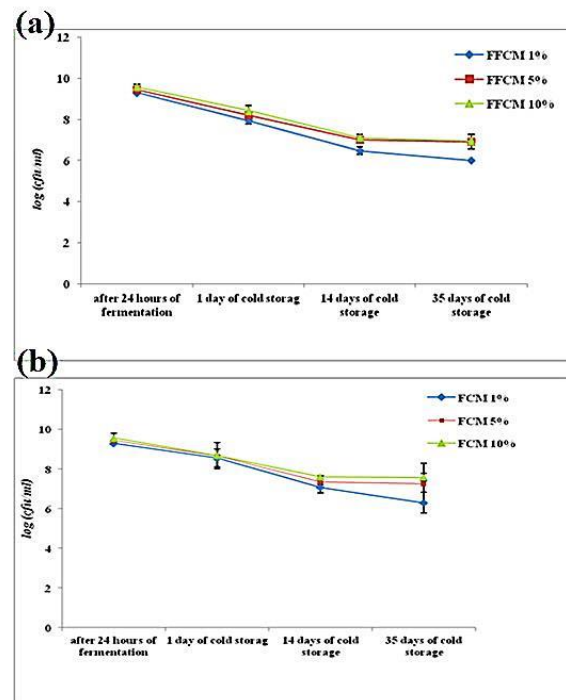


Fig.2. Changes in LAB of fermented camel milk with (a) or without (b) flavorings during cold storage.

Other investigations demonstrated that until day 2 of storage, lactobacilli count in the fermented milk (kefir) was around 10^8 CFU mL⁻¹ (Irigoyen et al., 2005; Kök-Taş et al., 2013). In the current study, after fermentation during storage, a higher level of Lactobacilli was noted among the samples prepared using 10%

inoculation, while lactobacilli levels were lower in the samples prepared using 1 and 5% of inoculation. According to Koroleva (1998), the number of lactic acid bacteria tended to increase in line with their inoculation dose into the source milk.

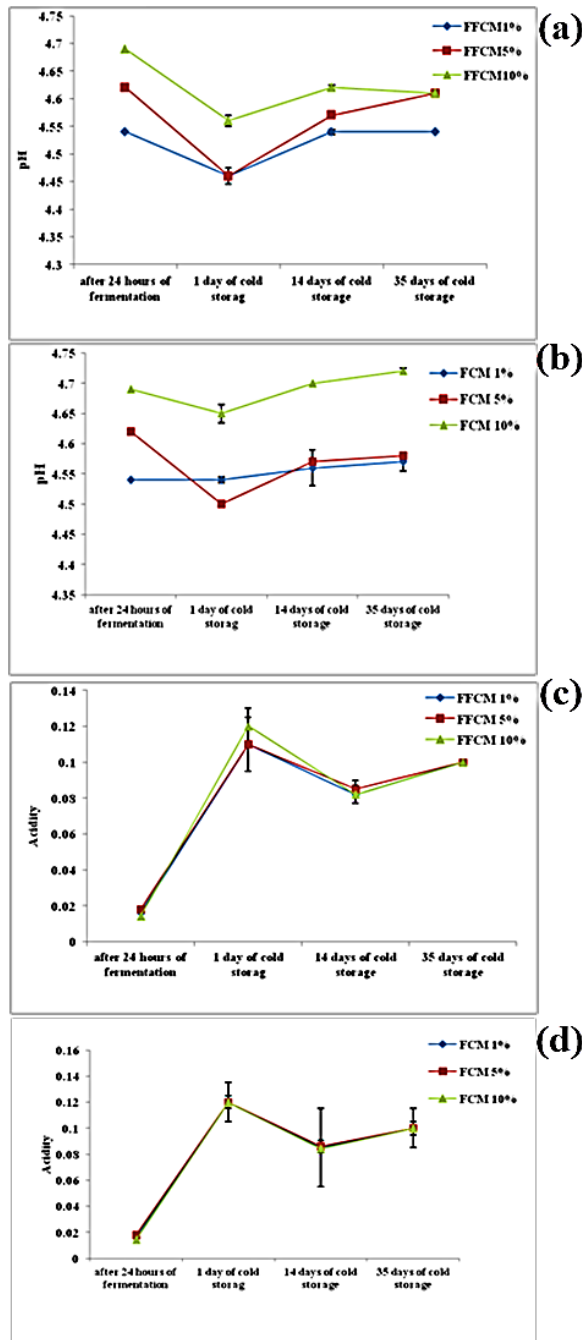


Fig. 3. Changes in pH of fermented camel milk with (a) or without (b) flavorings and acidity of fermented camel milk with (c) or without (d) flavorings during cold storage.

3.3. Chemical analysis of fermented camel milk samples

Fig. 3 represents the changes in pH and titratable acidity of fermented camel milk samples during storage. After 24 h of

fermentation, the significant increase and decrease in acidity and pH values can be correlated with the production of lactic acid by *Lactobacillus rhamnosus* and *Lactobacillus casei*. Although the final pH in some fermented camel milk was lower than 4.6 (isoelectric pH value for bovine milk caseins), no coagulation was observed our data were in a good agreement by findings of Moslehishad et al. (2013). After 14 days of cold storage, there was a rise in pH and a reduction in acidity, which might be linked to a decline in the population of probiotic bacteria. However, it should be noted that the changes in pH and acidity were not meaningful after day 14 of storage. No significant difference regarding pH among fermented cow's milk with *Lactobacillus fermentum* at 37°C for 24 was noted during cold storage (Zhang et al., 2013). In addition to generating lactic acid, additional generation and release of amino compounds (short peptides) can be detected during fermentation, which could be the consequence of a rise in pH after a few days of cold storage.

3.4. Sensory evaluation of fermented camel milk samples

The results of the sensory evaluation for fermented camel milk samples on cold storage days 1, 14, and 35 are summarized in Table 2. Each treatment had good acceptability in the initial days of storage. However, the flavored fermented camel milk sample received higher overall acceptability scores while compared with the fermented camel milk counterparts on day 1 ($p > 0.05$). While the fermented camel milk samples did not substantially vary in terms of appearance, aftertaste, or texture after cold storage, the mean values for taste, appearance, aroma, and overall acceptability analyses were higher than those reported by Moslehishad et al. (2013). According to El-Agamy (2009), the favorable color and appearance of fermented camel milk can be correlated with a relatively broad size distribution of casein micelles and the small size of fat globules in camel milk. Among the fermented camel milk samples, the FFCM (flavored fermented camel milk) with 10% had the best aroma and taste with mean scores of 4.37 and 4.00, respectively, on day 1 of storage (Table 2). Finally, the scores of all the sensory characteristics decreased significantly with an increase in storage time (35 d). The sensory analysis results revealed that the FFCM samples (especially the FFCM 10% sample) were more acceptable than FCM (fermented camel milk) samples ($p < 0.05$). This superiority confirmed the positive effect of flavoring agents on the sensory quality of the fermented camel milk. In previous studies, some of the proteolytic LAB may lead to the formation of bitter peptides and flavor defects in dairy products (Arvanitoyannis et al., 2009).

3.5. Anti-*H. pylori* activity of fermented camel milk samples

The literature has recently emphasized the possible role of probiotics in the prophylaxis and treatment of *H. pylori* infection. Probiotics can inhibit *H. pylori* by producing and secreting a variety of compounds such as short-chain fatty acids and bacteriocins, including nisin A, pediocin PO2, leucocin K, and reuterin (Fuller & Gibson, 1998; Rolfe., 2000). Lactic acid could inhibit the activity of *H. pylori* urease by lowering the stomach pH. However, anti-*Helicobacter pylori* effects of *Lactobacillus* are variable among its different strains (Aiba et al., 1998; Sgouras, 2004). Different mechanisms have been suggested for anti-*Helicobacter pylori* effects of probiotics. One study suggests that they could compete with and limit *H. pylori* infection in humans.

On the other hand, utilization of probiotics alone does not inhibit *H. pylori* activity (Ruggiero, 2014; Cats et al., 2003).

Several studies have reported that camel milk exhibits the antimicrobial effect against Gram-positive and Gram-negative bacteria (Benkerroum et al., 2004). This might be to the presence of natural antibacterial substances in camel milk, excluding lysozyme, lactoferrin, lactoperoxidase, and immunoglobulins (El-Agmay et al., 1992). Furthermore, some studies have showed that LAB isolated from Tunisian camel raw milk had antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium* (Mahmoudi et al., 2016). In addition, *Mycobacterium tuberculosis* was impressively inhibited by camel milk (Sharma et al., 2014). In another similar investigation, among the ten strains of lactic acid bacteria, the best anti-*Helicobacter pylori* activity was associated with *Lactobacillus rahmnosus*, *Lactobacillus bulgaricus*, and *Lactobacillus casei* (Lin et al., 2011). Our findings indicated that all the fermented camel milk samples had inhibitory effects against *H. pylori*, related to the concentration of organic acids and low pH value (Lesbros-Pantoflickova et al., 2007). While the IC₅₀ value of fermented camel milk with 10% inoculation was 24.58 µg/mL, the IC₅₀ value of camel milk counterpart was 55.73 µg/mL. The IC₅₀ value of FCM10% was lower than some plants known for this effect, such as *Ginkgo biloba* (IC₅₀= 36.17 µM) (Mahernia et al., 2015). Likewise, IC₅₀ of skim camel milk was lower than the corresponding values for extracts of *Rhus coriaria* and *Matricaria inodora* (Mahernia et al., 2015). Therefore, fermented camel milk can be considered a functional food with health-promoting properties.

4. Conclusion

One of the significant challenges for the production of fermented camel products is its taste. This research studied fermented camel milk with the best sensory characteristics to inhibit *Helicobacter pylori* growth. The present study results indicated that fermented camel milk can be considered a natural probiotic product due to its high contents of beneficial components. Furthermore, specific inhibition or reduction of urease activity results in an increased sensitivity of the bacteria in an acidic medium, leading to its elimination by stomach acidic condition or the body's immune system. Since prevention is better than treatment and synthetic drugs have many side effects, the best way to prevent *H. pylori* infection is to use functional foods regularly.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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