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Research Paper

In vitro antagonistic activity of *Lactobacillus casei* against *Helicobacter pylori*

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Abstract

Helicobacter pylori is one of the most common causes of chronic infections in humans. Curing *H. pylori* infection is difficult because of the habitat of the organism below the mucus adherent layer of gastric mucosa. Lactobacilli are known as acid-resistant bacteria and can remain in stomach for a long time than any other organism, we aimed in this study to examine the efficacy of *Lactobacillus casei* as a probiotic against *H. pylori* in humans. Particularly, *L. casei* was opted as it is considered to be one of the widely used probiotics in dairy products. One hundred and seven strains of *H. pylori* were isolated from dyspeptic patients and were tested for their antibiotic susceptibility to metronidazole (MTZ), clarithromycin (CLR), tetracycline (TET), and amoxicillin (AMX) by the disc diffusion method. The strains were examined for their susceptibility toward *L. casei* - present in fermented milk products - by well diffusion method. It was found that 74.7% strains were resistant to MTZ; 1.8% to MTZ, TET, and CLR; 3.7% to MTZ and CLR; 4.6% to MTZ and TET; and 0.9% were resistant to MTZ, TET, and AMX. The antibacterial activity of *L. casei* against *H. pylori* was determined on all the tested *H. pylori* isolates including antibiotic resistant strains with different patterns. Our study proposed the use of probiotics for the treatment of *H. pylori* infection as an effective approach.

Key words: *Helicobacter pylori*, *Lactobacillus casei*, probiotics.

Introduction

Helicobacter pylori is a Gram-negative, microaerophilic, and spiral-shaped rod bacterium. It has infected over 50% of the population around the world and is considered to be the most important etiological agent for gastric cancer and mucosa-associated lymphoid tissue (Dubois and Boren, 2007; Wroblewski *et al.*, 2010). It is well known that early childhood is an important period where *H. pylori* infection is usually acquired. The colonization of this bacterium occurs very early in life, and is often persistent throughout the life without inducing any symptoms (Dunn *et al.*, 1997a). In developing countries, the percentage of population carrying *H. pylori* can reach 94%, while in developed countries, the prevalence of infection is lower, ranging from 1.2% to 30% (Hunt *et al.*, 2011). *H. pylori* infection is still a challenge for many researchers and physicians particularly when it is related to the treatment of this infection. The Maastricht IV Consensus Report recommended the choice of antibiotic combination for treatment according to local *H. pylori* antibiotic resistance patterns, and it recommended bismuth-containing quadruple therapies as the first-line empirical treatment in areas of high clarithromycin resistance (Malfertheiner *et al.*, 2012). If first-line clarithromycin-based regimen has already used, a second-line metronidazole-based quadruple therapy may be used, and then a levofloxacin-based combination would be a third-line option (Gisbert, 2009). However, eradication of these bacteria is still difficult for many reasons. First, these bacteria develop sophisticated strategies to colonize epithelial cells that line the antrum of the stomach and to survive in the acidic environment (Goodwin and Armstrong, 1990). The access of the antimicrobial agent from the lumen of stomach to this site is limited. Second, many antimicrobial agents are poorly secreted in gastric mucosa, and are inactivated in the stomach environment, or reach the stomach in a low concentration (Dunn *et al.*, 1997b). Third, in 10%-35% of the cases, the presence of resistance in *H. pylori* has been observed as in other species

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(Gotteland and Cruchet, 2003). Fourth, the clinical therapy is often accompanied by unwanted side effects of using multiantibiotics (Megraud and Lehours, 2007) as well as their high cost for families from the low socioeconomic level.

For these reasons, it is an essential demand to develop low-cost and large-scale alternative solutions to prevent *H. pylori* colonization and to search for new or additional treatment agents. Consequently, much attention has been paid to the health-promoting probiotics (Sgouras *et al.*, 2004). FAO-WHO has defined probiotics as nonpathogenic live microorganisms, which when administered in adequate amounts confer a health benefit on the host (WHO, 2001). Preoperative oral administration of symbiotics can enhance immune responses, attenuate systemic postoperative inflammatory responses, and improve the intestinal microbial environment (Sugawara *et al.*, 2006). Most commonly used probiotics are lactic acid-producing bacteria such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Bacillus* (Sullivan and Nord, 2005). Lactobacilli have been successfully used in prophylaxis and in the treatment of gastrointestinal disorders and infections (Servin, 2004). They are acid resistant and can persist in the stomach for a long time than any other bacteria (Bhatia *et al.*, 1989). Thus, *H. pylori* could be a good target for lactobacilli probiotic prophylaxis and therapy. Lactobacilli are noninvasive and induce various epithelial cell responses by competing with pathogenic bacteria for host adhesion-binding sites, thereby improving the epithelial cell barrier function and stimulating the host immune response (Forestier *et al.*, 2001). Several authors have reported the production of antimicrobial substances from lactobacilli (Bernet *et al.*, 1994; Bernet-Camard *et al.*, 1997; Silva *et al.*, 1987). Certain lactobacilli synthesize antimicrobial compounds that are related to the bacteriocin family (Jack *et al.*, 1995), while others are well-known metabolic end products of lactic acid fermentation such as lactic and acetic acids, and hydrogen peroxide (Vandenbergh, 1993). Recently, studies have reported that various *Lactobacillus* strains can inhibit the growth of *H. pylori* in vitro and in vivo such as *Lactobacillus gasseri* (Fujimura *et al.*, 2012), *Lactobacillus johnsonii* (Hsieh *et al.*, 2012), *Lactobacillus salivarius* (Lionetti *et al.*, 2010), and *Lactobacillus acidophilus* NAS (Lionetti *et al.*, 2010).

The aim of this study was to find out the potential, synergistic, and additive inhibitory activity developed by the spent culture supernatants of *L. casei* against *H. pylori* clinical isolates with mixed antibiotic resistance patterns in vitro by an agar-well diffusion method. The basis for the selection of *L. casei* was its known activity against Gram-negative pathogens, particularly, *H. pylori* and their probiotic properties. As reported in previous clinical studies, it has the ability to survive transit through stomach and resist the bile salts (Midolo *et al.*, 1995; Sykora *et al.*, 2005).

Materials and Methods

H. pylori strains

A total of 150 *H. pylori* suspected strains were isolated from antral biopsy specimens of patients with dyspeptic symptoms and clinically suggested for upper gastrointestinal endoscopy. All endoscopic examinations were carried out using a video endoscope, PENTAX EPM-3500. Three biopsy specimens (2-3 mm in diameter) were taken from the antrum region of the stomach along the greater curvature from each patient (Matsukura *et al.*, 2004). Consent was obtained from all patients to use their data in the current research work. The first biopsy specimen was pushed onto the reaction pad of the Pylori Tek test® (Serim, USA) to examine the urease production. The second one was examined by direct Gram's strain. The third was rapidly homogenized and streaked on fresh brain-heart infusion agar supplemented with 10% sterile horse serum and on Columbia blood agar with a selective supplement (Dent, Oxoid). The plates were incubated under microaerophilic conditions using the Gas Generating Kits Campylobacter system BR 056A (Oxoid, Hampshire, England) at 37 °C for 3-5 days. The suspected colonies of *H. pylori* were subjected to the following identification: morphological identification, microscopical identification, and biochemical reactions including testing for the presence of oxidase, catalase, and urease. A total of 107 isolates were confirmed as *H. pylori*. The antibiotic susceptibility test against metronidazole (MTZ), clarithromycin (CLR), tetracycline (TET), and amoxicillin (AMX) for the positive isolates of *H. pylori* was carried out using the disc diffusion method (Elviss *et al.*, 2004; McNulty *et al.*, 2002). Isolated colonies from 3-day culture were suspended in 1 mL sterile saline to a density equivalent to McFarland number 4 standard (12 108 colony forming units; Elviss *et al.*, 2004). One hundred microliter from this suspension was streaked on Müller-Hinton agar with 10% sheep blood using cotton swab (Duck *et al.*, 2004). Disks of MTZ (5 μg, Oxoid), CLR (15 μg, Oxoid), TET (30 μg, Oxoid), and AMX (25 μg, Oxoid) were kept on a plate (Duck *et al.*, 2004). The plates were incubated in a microaerophilic atmosphere at 37 °C for 3 days before examination. Plates used were of size as large as 16 cm in diameter. The strains were considered resistant to MTZ, CLR, TET, and AMX when the inhibition zone was < 15 mm, ≤ 40 mm, < 19 mm, and < 17 mm, respectively (Wolle *et al.*, 2002; Duck *et al.*, 2004). Polymerase chain reaction (PCR) for detecting the subunit of *H. pylori* urease gene (*ureA*) was carried out according to the procedure of He *et al.*, and Enany *et al.* (He *et al.*, 2002; Enany, 2005, 2011). All these tests were performed in duplicates.

Pure isolated *H. pylori* strains were subcultured at 37 °C for 72 h under microaerophilic conditions on Columbia agar enriched with 7% blood. A vial of *H. pylori*-selective supplement (Dent supplement; Oxoid, Hampshire,

England) was used in the subsequent agar diffusion method (Sgouras *et al.*, 2004).

L. casei Imunitass strains

L. casei Imunitass DN-114001 strains were isolated from a fermented milk product (ACTIMEL, DANONE, Spain)® and cultured in DeMan-Rogosa-Sharpe (MRS) broth (Oxoid, England) for 24 h at 30 °C and a pH around 5.5.

The growing *L. casei* colonies were subjected to the following identification: morphological identification, direct Gram strain, and biochemical reactions including the glucose fermentation test and oxidase test, and catalase production. It was stored in MRS broth at -80 °C till use. Isolated *L. casei* were subcultured twice in MRS broth for 24 h at 30 °C prior to the viable count. *Lactobacilli* were counted by serial dilutions and subcultured onto MRS agar plates under the same incubation conditions. The pH for each culture was measured after 0, 1, 2, 3, 4, 5, 6, and 24 h of incubation.

Agar-well diffusion assay

Subcultured *H. pylori* isolates were inoculated with McFarland turbidity equal to 2 in saline and were plated on MRS agar plates without antibiotics (Boyanova *et al.*, 2009). Wells were drilled into the agar using sterile Cork pourers of 7 mm diameter. Colonies of *L. casei* strain resuspended in fresh MRS broth were suspended in the agar wells at a concentration of 4 10^8 cfu/mL (Gotteland and Cruchet, 2003; Sgouras *et al.*, 2004). Plates were incubated for 48-72 h under microaerophilic conditions at 37 °C. The diameters of inhibition zones around the wells were measured in millimeters. The assay was performed in triplicates for each *H. pylori* isolate, and the result expressed as a mean diameter.

Results

Among 150 examined patients, 20.7% (31of 150) were normal, 20% (30 of 150) were suffering from gastric ulcer, 24% (36 of 150) from duodenal ulcer, 33.3% (50 of 150) from gastritis, and 2% (3 of 150) from gastric cancer. *H. pylori* was cultured, and the urease test was positive in 71.33% (107) of the total 150 gastric biopsy specimens. The confirmed *H. pylori* isolates were oxidase, catalase, and urease positive. Overall, the resistance rate for the 107 confirmed *H. pylori* was 86% (92 of 107) for MTZ, 7.4% (8 of 107) for TET, 5.6% (6 of 107) for CLR, and 0.9% (1 of 107) for AMX (Fig. 1A), and the antibiotic resistance pattern based on the combined resistance to antibiotics is shown in Figure 1B. Most strains (74.7%) were only resistant to MTZ, while 1.8% strains were resistant to MTZ, TET, and CLR. Four isolates (3.7%) were resistant to MTZ and CLR, and five isolates (4.6%) were resistant to MTZ and TET, while only one isolate (0.9%) was resistant to MTZ, TET, and AMX.

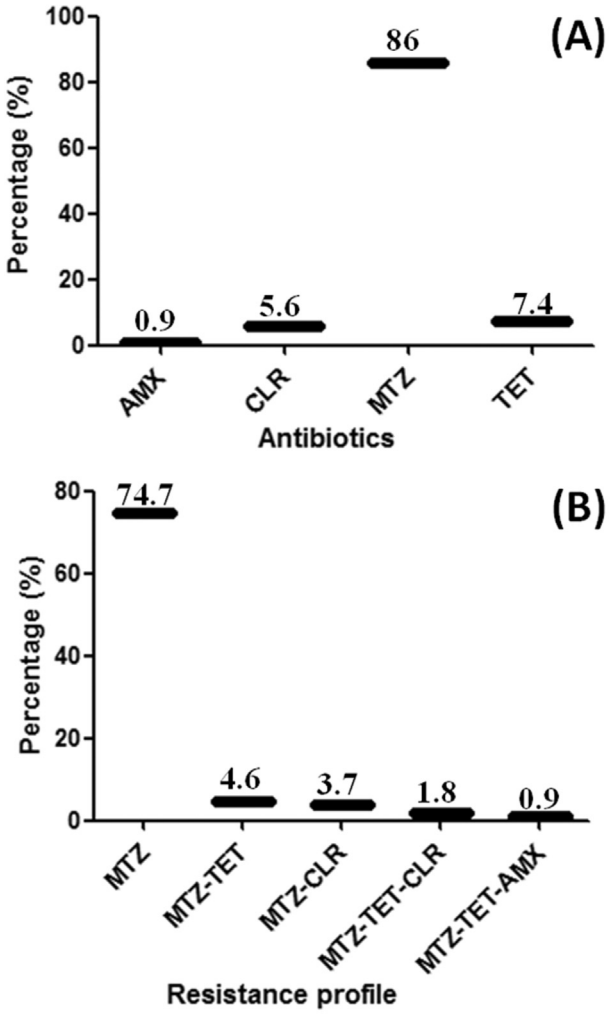

Figure 1 - Prevalence of antibiotic resistance among tested *H. pylori* isolates against AMX, amoxicillin; CLR, clarithromycin; MTZ, metronidazole; and TET, tetracycline (A) and their antibiotic resistance pattern (B).

The *lactobacilli* strains used in our study were catalase and oxidase negative and were able to ferment glucose. Apparent reduction in pH of the *Lactobacillus* culture was detected through time points. The antibacterial activity of *L. casei* strains against *H.pylori* was observed on the 107 tested *H.pylori* isolates, thereby showing the inhibition zones from 11.0 to 15.0 mm. All the clinical isolates of *H. pylori* were completely inhibited by *Lactobacillus* culture. Mean inhibition zones in millimeters are shown in Table 1.

Discussion

The results shown here confirmed and extended other studies indicating that *H. pylori* can be inhibited by lactobacilli (Gotteland and Cruchet, 2003; Sgouras *et al.*, 2004). This study is interesting, and suggests that it is possible to decrease the number of *H. pylori* in the subjected population through a regular ingestion of probiotic-containing dietary products, which are widely available in the local

Table 1 - Inhibition of clinical isolates of *H. pylori* by *L. casei* strain culture.

Mean diameter of inhibition zone (mm)	Number (%) of *H. pylori* strains inhibited by *L. casei*
11:12	9 (8.4%)
12:13	4 (3.7%)
13:14	66 (61.7%)
14:15	28 (26.2%)

market. Different lactobacilli could be used for inhibition of *H. pylori* colonization through different mechanisms. Oral intake of *L. johnsonii* La1 was reported in many clinical studies and confirmed by different bacteriological and pathological examinations to attenuate *H. pylori* by reducing pro-inflammatory chemotactic signals, which are responsible for the recruitment of lymphocytes and neutrophils in the lamina propria (Gotteland and Cruchet, 2003; Sgouras *et al.*, 2005). Moreover, *L. gasseri* OLL2716 was proved to follow the mechanism of induction. The coccoid conversion of *H. pylori* by lactic acid secretion (Canducci *et al.*, 2002) and *L. acidophilus* exert an antagonistic effect against *H. pylori* through the production of a heat stable protein that exerts the antimicrobial effect other than lactic acid, which is only sensitive to enzymatic treatments (Coconnier *et al.*, 1998). In this study, *L. casei* produces a large amount of lactic acid that has been confirmed by the reduction in pH; lactate has been implicated as an inhibitory factor for *H. pylori* due to its antimicrobial effect resulting from lowering the pH and its ability to inhibit the urease enzyme (Midolo *et al.*, 1995; Sgouras *et al.*, 2004). Although some studies have reported that only viable cells of *L. casei* can inhibit *H. pylori* growth (Coconnier *et al.*, 1998; Sgouras *et al.*, 2004), other studies have investigated the importance of other factors such as pH and lactic acid production (Bhatia *et al.*, 1989; Lorca *et al.*, 2001). In our study, we adjusted the pH to 5.5 as it is known that the low pH environment was found to increase the activity of lactobacilli (Boyanova *et al.*, 2009), while lactic acid production was found to inhibit urease enzyme secretion from *H. pylori*, which plays a role in counteracting the stomach acidity (Servin, 2004; Sgouras *et al.*, 2004). Recently, a striking mechanism for *L. casei* has been reported, which gleaned its power in making irreversible inhibition of the swimming motility of *H. pylori* through changes in cell morphology, replacing the helical cells by "c"-shaped and coccoid cells, and losing of FlaA and FlaB flagellins (Le Moal *et al.*, 2013). Another in vivo study showed that *L. casei* could prevent enteric infections and stimulate secretory IgA in malnourished animals by inducing the increased systemic immune response, and it is also proved in the same study that *L. casei* was the most effective among other lactobacilli (Perdigon *et al.*, 1995). Proteomic analysis of bacteria usually revealed plenty of information about its proteins and their role in the cell (Enany *et al.*, 2012; Enany *et al.*, 2013; Enany *et al.*, 2014). Two-dimensional gel electrophoresis, one of the gel-based proteomic methods, is used to fractionate, identify, and quantify proteins (Wu *et al.*, 2009; Magdeldin *et al.*, 2014). Proteomic analysis of *L. casei* using two-dimensional gel electrophoresis expressed different proteins, which act as stress response proteins and are also involved in central and intermediary metabolism. These proteins have a potential role for the adaptation to the surroundings, particularly the accumulation of lactic acid in the course of growth and the physiological processes in bacterial cells (Wu *et al.*, 2009).

It was interesting to find all antibiotic resistance patterns of our *H. pylori* isolates responded by 100% to the antagonistic effect of the *L. casei* strains. Human and animal clinical studies showed that the administration of *L. casei* probiotics alone decreases but does not clear *H. pylor*i infection, suggesting that it should be taken together with the antibiotic therapy to improve the eradication rates (Sgouras *et al.*, 2004; Cats *et al.*, 2003). Many studies have reported that the concurrent combination of yogurt probiotic products containing *L. casei* together with the antibiotic treatment could significantly reduce their side effects such as reduction in vomiting and nausea, decreasing the taste disturbance, and correcting antibiotic-induced intestinal dysbiosis and consequently reduction in diarrhea (Lesbros-Pantoflickova *et al.*, 2007). A recent meta analysis suggests that supplementation with probiotics compared with eradication therapy may be considered an option to increase eradication rates, particularly when antibiotic therapies are relatively ineffective (Dang *et al.*, 2014).

Our results in the present study can be used as a base for the additional assessment of the inhibitory effects of *L. casei* strains. More in vivo trials for *L. casei* and other lactobacilli are still under investigation.

In conclusion, the data presented here clearly show that *L. casei* strains isolated from probiotic preparation of dairy products inhibited the growth of *H. pylori* strains and even the antibiotic-resistant strains in vitro. Further, in vivo clinical studies are still required to assess *L. casei* ability to be utilized in the treatment of *H. pylori* infection.

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