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Suppressive Effect of Functional Drinking Yogurt Containing Specific Egg Yolk Immunoglobulin on *Helicobacter pylori* in Humans

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ABSTRACT

Helicobacter pylori is a human pathogen that infects over 50% of the population worldwide. It is the most important etiologic agent of gastroduodenal ulcers and malignancies. *Helicobacter pylori* urease enzyme is considered the main factor for the organism's colonization in the gastroduodenal mucosa. Hens immunized with the purified urease produce a highly specific anti-*H. pylori* urease immunoglobulin (IgY-urease) in their egg yolks. Immunoglobulin Y-urease was stable at 60 to 65°C for 30 min and at pH 4.0 for 7 h. Its activity was lost at 80°C for 20 min and at pH 2 for 4 h. Specially designed functional drinking yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium* spp. with 1% egg yolk IgY-urease was produced commercially. Immunoglobulin Y-urease activity showed stability in the product up to 7 d, and then decreased to 85% after 3 wk of storage. A clinical study was conducted to determine the effectiveness of IgY-urease yogurt to suppress infection in humans. Forty-two volunteers who tested positive for *H. pylori* using a ^{13}C-urea breath test were recruited. A total of 450 mL of IgY-urease (test group) or IgY-urease–free yogurt (control group) was consumed in 150-mL portions 3 times daily for 4 wk. Volunteers were tested after 2 and 4 wk; urea breath test values significantly decreased in the test group compared with the control group. The results indicate that suppression of *H. pylori* infection in humans could be achieved by consumption of drinking yogurt fortified with IgY-urease.

(**Key words:** *Helicobacter pylori* infection, anti-*H. pylori* urease egg yolk immunoglobulin, drinking yogurt, passive immunity)

Abbreviation key: IgY = egg yolk immunoglobulin, **IgY-urease** = anti-*Helicobacter pylori* urease IgY, **UBT** = urea breath test, **PBS-T** = PBS with 0.05% Tween 20.

INTRODUCTION

Helicobacter pylori, a spiral gram-negative microaerophilic pathogen, has been shown to be a common inhabitant of the gastric and duodenal mucosa (Offerhaus et al., 1990; Kimura et al., 1998). The organism is recognized as one of the most prevalent human pathogens. It infects over 50% of the population worldwide (Dunn et al., 1997), and is recognized as the etiologic agent of gastritis (McNutty and Watson, 1984; Blaser, 1990), peptic ulcer (Everhart, 2000), and has been linked to the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (Talley et al., 1991; Parsonnet et al., 1994). It was found that urease is the most abundant protein of *H. pylori*. Urease is recognized as an essential factor in *H. pylori* colonization of the gastric mucosa and the enzyme enables the organism to survive and persist in the stomach. The eradication of *H. pylori* by administration of oral antimicrobials is not always successful and may be associated with adverse effects (Marshall, 1994).

Passive immunization involving the delivery of pathogens' specific antibodies has been an attractive approach to establish protective immunity against a variety of microbial pathogens (Ebina et al., 1985; Tacket et al., 1992). The best source of passive antibodies (other than those derived from the mother) would be from hen egg yolk. Hyperimmunized hens could provide a convenient and economic source of immunoglobulin in their egg yolk (Hatta et al. 1990; Kim et al., 2000). The oral administration of specific yolk immunoglobulin (**IgY**) has been found to be effective in preventing viral or bacterial pathogens (Ebina et al., 1990; Hatta et al., 1997; Kim et al., 2000). Hen immunization with *H. pylori* whole-cell lysates was used to produce anti-*H. pylori* IgY. However, IgY produced by whole-cell im-

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munization could recognize the possibility of cross-reactivity with other bacteria and result in decrease of its efficiency (Shin et al., 2003). Interestingly, the organism expresses a high level of urease that ultimately occupies a strategic position on the bacterial surface that related to the essentiality of this enzyme in colonizing the mucus layer of the gastric mucosa. Accordingly, a novel approach in prevention and reduction of *H. pylori* infection has been reported based on production of urease-specific IgY that could suppress *H. pylori* colonization through urease-binding by anti-*H. pylori* urease IgY (**IgY-urease**).

Today, consumers prefer foods that promote good health and could reduce risk of diseases. Dairy products are excellent media to generate an array of products that fit into the current consumer demand for functional foods (Chandan, 1999). In general, worldwide consumption of fermented milk products, including yogurt, is increasing. Scientific and clinical evidence is mounting to corroborate the consumer perception of health from yogurt (Chandan and Shahani, 1993; Salminen et al., 1998). Recently, attention has been paid to the interaction between *H. pylori* and yogurt containing some specific strains of lactic acid bacteria. In vitro and in vivo inhibition of *H. pylori* growth by some *Lactobacillus* spp. and *Bifidobacterium* spp. has been reported (Coconnier et al., 1998; Sgouras et al., 2004). However, other clinical studies recorded a nonsignificant suppressive effect of that type of yogurt on the suppression of *H. pylori* in humans (Sakamoto et al., 2001; Wendakoon et al., 2002; Cats et al., 2003). On the other hand, other data suggest that yogurt containing live and active cultures may have an immunostimulatory effect and protect from pathogens (Perdigon et al., 1995; Matsuzaki et al., 1998).

Besides boosting host immunity against diseases, designing a yogurt fortified with IgY-urease could supply passive immunization with a natural and highly specific attempt to decrease the *H. pylori* infection. The aim of this study was to examine the efficacy of a specially designed functional yogurt containing IgY-urease on the suppression of *H. pylori* in humans.

MATERIALS AND METHODS

Immunization of Hens and Preparation of IgY-Urease

Aqueous extraction of the purified *H. pylori* urease enzyme (Icatlo et al., 1998) was used as an antigen. The protocol of Otake et al. (1991) was used to immunize hens, except that a booster injection was performed 8 wk after the first immunization. During the period of highest antibody titer in egg yolk, laid eggs were collected daily and stored at 4°C. Egg yolks collected every week were separated, pooled, and frozen (Hatta et al., 1990).

ELISA

Measurement of the IgY-urease activity of the separated egg yolks was determined by an indirect ELISA as previously described by Hatta et al. (1997), with some exceptions. Briefly, the purified *H. pylori* urease enzyme (5 μg/mL) was used at 50 μL/well to coat ELISA plates. Each well was washed 3 times with 150 μL of PBS containing 0.05% Tween 20 (**PBS-T**). Bovine serum albumin 3% solution (wt/vol; Sigma Chemical Co., St. Louis, MO) dissolved in PBS-T was used for blocking. Samples were diluted in PBS-T and added (50 μL/well) in triplicate to plates. Plates were incubated for 1 h at 37°C and then washed 3 times. Plates were incubated for 1 h at 37°C with alkaline phosphatase-conjugated rabbit IgG antichicken IgG (1:2000; Zymed Laboratories, Inc., San Francisco, CA) and then 50 μL of substrate (*p*-nitrophenyl phosphate, 1 mg/mL diethanolamine buffer, pH 9.8; Sigma Chemical Co.) was added to each well. The enzyme reaction was stopped after 15 to 30 min at 37°C by adding 2 *M* NaOH (50 μL/well). The amount of color development was read at 405 nm with a plate reader (model 680, Bio-Rad, Richmond, CA).

Heat and pH Stability of IgY-Urease

One-milliliter portions of egg yolk IgY-urease solution (0.1% in PBS, pH 7.2) were placed in test tubes and heated for up to 30 min at temperatures ranging from 60 to 80°C. The heated mixtures were cooled in an ice bath. To determine pH stability, an egg yolk IgY-urease solution (1% in PBS, pH 7.2) was diluted with 10 m*M* phosphate buffer containing 0.15 *M* NaCl, of various pH values. The pH of the solutions was adjusted with HCl or NaOH to final pH of 2 to 7. The solution was incubated at 37°C for 0 to 7 h, and then the pH of the solution was neutralized by 100-fold dilution with PBS-T. The remaining IgY-urease activity after heat and pH treatments was measured by ELISA.

Preparation of IgY-Urease Drinking Yogurt

Two series of drinking yogurt were prepared at a commercial facility and packed in 150-mL bottles. Both were inoculated with commercial yogurt starter cultures containing *Lactobacillus acidophilus* and *Bifidobacterium* spp. After the heat-treatment steps, egg yolk IgY-urease was pasteurized at 65°C for 30 min and then added at 1% to one series (IgY-urease yogurt: 1.5 g of egg yolk IgY-urease/bottle); the other series was

IgY-urease–free yogurt. Drinking yogurts were cooled and stored at 4°C for up to 3 wk (Maeil Dairy Industry Co., Ltd., Korea).

Stability of IgY-Urease in Drinking Yogurt

Measurements of pH and IgY-urease activity in drinking yogurt stored at 4°C were carried out weekly up to 3 wk. Drinking yogurt containing IgY-urease (5 mL) was mixed with an equal amount of carbonate buffer (pH 10) and mixed for 3 min with 10 mL of chloroform. After centrifugation at 3000 rpm for 30 min, the upper supernatant was examined for activity of IgY-urease by ELISA as described above, except that the enzyme reaction time was 45 to 60 min.

Clinical Test

One hundred sixteen volunteers, 21 to 60 yr of age, were recruited and screened using a Helikit ^{13}C-urea breath test (**UBT**) for the presence of *H. pylori* (Isotechnika Inc., Edmonton, Canada). The UBT was performed on the first day after an overnight fast (at least 8 h). A baseline breath sample was collected in a tube. An aliquot of 75 mg of ^{13}C-urea dissolved in 75 mL of citric acid solution was given orally. Another breath sample was collected after 30 min. Breath samples were analyzed to determine the ratio of $^{13}C/^{12}C$ by mass spectrometry. The $^{13}C/^{12}C$ ratio of each breath sample was expressed as a millipercentage (‰). Change in the ^{13}C value over baseline was expressed as delta ^{13}C. A positive result was defined as an increase greater than 4‰. Seventy-eight subjects were positive. To ensure *H. pylori* infection, the selection criteria required that volunteers had a UBT value over 30‰, had not taken antibiotics or medication in the previous month or during the study, had no history of gastric surgery, and were highly interested in participating. The selected 42 subjects included 36 males and 6 females, aged 38.85 ± 2.13 yr, without evidence of other gastroduodenal disease. Informed consent was obtained and the ethics committee of Pyungchon Sacred Heart Hospital, Hallym University, Korea, approved the study. The subjects were divided randomly into a test group (20 males and 2 females), and a control group (16 males and 4 females). The test group consumed one bottle (150 mL) of drinking yogurt containing 1.5 g of egg yolk IgY-urease 3 times daily (4.5 g of egg yolk IgY-urease containing 45 mg of IgY-urease/d) for 4 wk, and the control group received IgY-urease–free yogurt of the same volume. Volunteers had a second and third UBT after 2 and 4 wk to assess suppression of *H. pylori* infection.

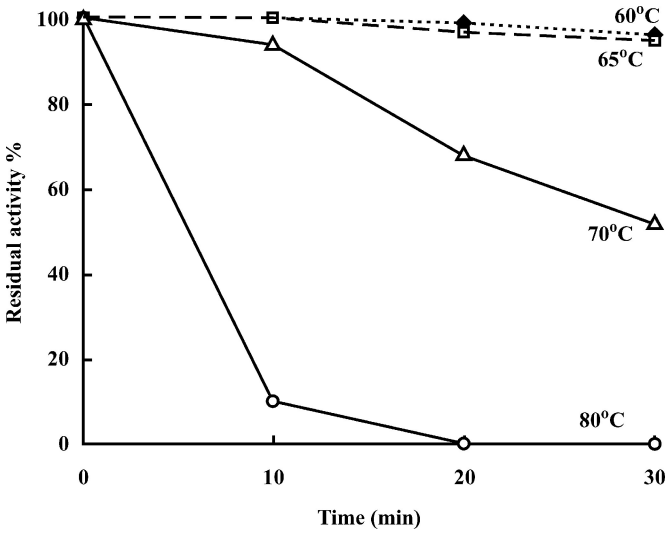

Figure 1. Changes in anti-*Helicobacter pylori* urease immunoglobulins (IgY-urease) activity upon heating at various temperatures. Residual IgY-urease activities after incubation at 60 to 80°C for periods up to 30 min were measured by ELISA and expressed as a percentage of the initial activity.

Statistical Analysis

The results were statistically analyzed by computing means and standard errors of the mean. Differences between means of the test and control groups were evaluated by Student's *t*-test and $P < 0.001$ was considered statistically significant.

RESULTS AND DISCUSSION

Eggs have been considered a convenient source for the production of polyclonal antibody specific to a variety of antigens such as bacteria, viruses, and enzymes (Burger et al., 1985; Shimizu et al., 1988; Yokoyama et al., 1992). Immunization of hens with purified *H. pylori* urease generated significant levels of antibodies in their egg yolk without any decrease of egg laying rate. The changes in antibody levels in egg yolks following immunization (data not shown) were similar to those reported previously (Otake et al., 1991), except that a maximum level of the antibody was recovered when a booster injection was conducted 8 wk after the first immunization.

Heat and pH Stability of IgY-Urease

Heat and pH stability studies were undertaken to ensure the possibility of fortification of drinking yogurt with egg yolk IgY-urease at a commercial facility. Figure 1 illustrates that IgY-urease activity significantly decreased at 80°C, showing only 10% residual activity after 10 min, and absence of activity after 20 min. At

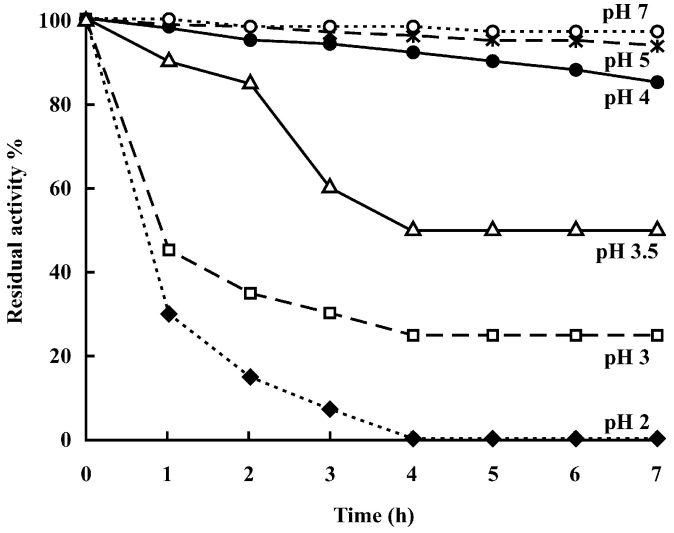

Figure 2. Anti-*Helicobacter pylori* urease immunoglobulins (IgY-urease) stability at different pH values ranged from 2 to 7. Residual IgY-urease activities after incubation at 37°C for periods up to 7 h were measured by ELISA and expressed as a percentage of the initial activity.

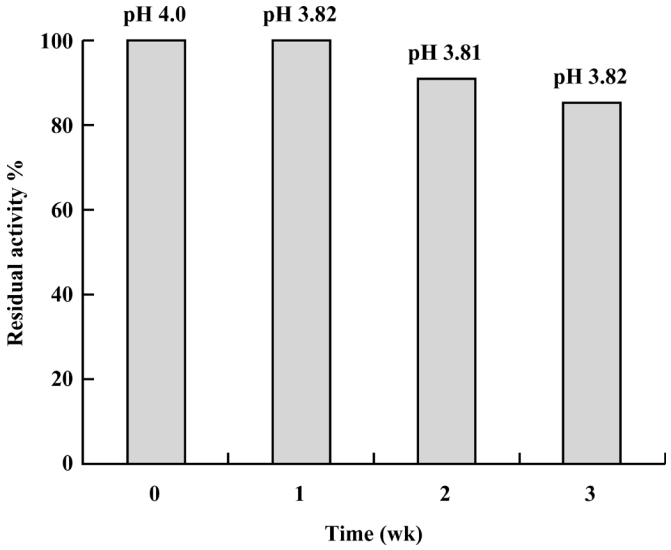

Figure 3. Anti-*Helicobacter pylori* urease immunoglobulins (IgY-urease) stability in drinking yogurt during storage at 4°C for 3 wk. Residual IgY-urease activities were measured by ELISA and expressed as a percentage of the initial activity. Changes in yogurt pH were indicated on each bar during 3 wk storage at 4°C.

60 and 65°C, about 95% of the activity remained after standing even for 30 min. The IgY-urease activity would be lost during yogurt manufacturing. However, its stability at 60 to 65°C for 30 min suggests that IgY-urease could undergo low-temperature pasteurization at 63°C for 30 min and then be added to the yogurt (after the yogurt underwent heat treatments). The pH stability of IgY-urease was examined. The activity of IgY-urease decreased after incubation at pH 3.5 or below, and was completely lost at pH 2 after 4 h incubation (Figure 2). The relatively high stability at pH 4 and instability at lower pH values are important considerations during manufacturing of drinking yogurt. Similar heat and pH stabilities of other specific IgY have been previously reported (Shimizu et al., 1992; Hatta et al., 1993; Shin et al., 2002).

Stability of IgY-Urease in Drinking Yogurt

The stability of IgY-urease in the final product was examined during storage at 4°C for 3 wk (Figure 3). The pH of the product changed from 4.0 to 3.82. ImmunoglobulinY-urease activity was reduced from 100 to 85% at the end of the first week to the end of the third week of storage, respectively. The reduction in IgY-urease activity may be attributed to the reduction of pH, or it may indicate that the lgY-urease is partially subjected to proteolysis.

Effect of lgY-Urease Drinking Yogurt on UBT Values

During the 4-wk study period, the ingestion regimen was well tolerated, and no adverse effects or any complications were observed. The urea breath test method was used to assess probable suppression of the *H. pylori* infection in test and control groups. This method, based on the detection of exhaled ^{13}C-labeled carbon dioxide resulting from *H. pylori* urease activity, has sensitivity and specificity values between 95 and 100% and is the most widely used noninvasive test. The advantages of UBT include its ability to detect the presence and activity of *H. pylori* in the stomach (Westblom and Bhatt, 1999; Chang et al., 2003). There was no significant difference in UBT values obtained from both groups at wk 0 (Table 1). The control group, which ingested IgY-urease–free yogurt, showed some decrease in UBT val-

Table 1. Comparison between control and test groups.

Item	Control group	Test group
Number of subjects (male: female)	20 (16:4)	22 (20:2)
Age (yr)	39.20 ± 2.42	38.50 ± 1.83
UBT value, 0 wk[1]	51.40 ± 4.48[a]	51.18 ± 3.40[a]
UBT value, 2 wk[1]	44.38 ± 5.17[a]	33.70 ± 3.50[b]
UBT value, 4 wk[1]	43.53 ± 5.48[a]	31.03 ± 3.54[c]

[a,b,c]Values with different superscript letters are significantly different ($P < 0.001$). Data are presented as the mean ± standard error of mean.

[1]UBT = Urea breath test for *Helicobacter pylori*.

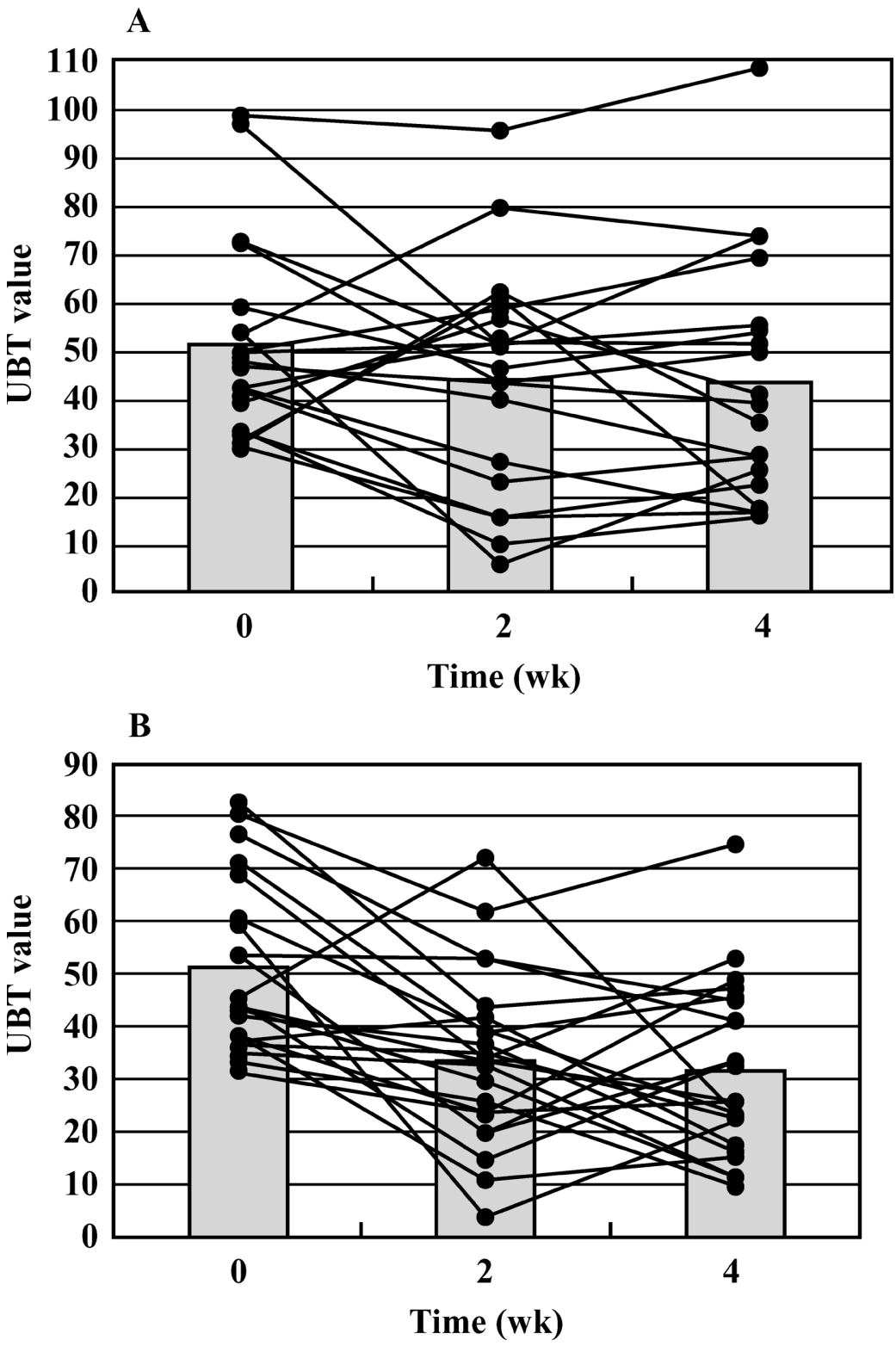

Figure 4. Effect of ingestion of (A) anti-*Helicobacter pylori* urease immunoglobulins (IgY-urease)–free drinking yogurt (control group), and (B) drinking yogurt containing egg yolk IgY-urease (test group) for 4 wk on the presence of *Helicobacter pylori* represented by urea breath test (UBT) values. Circles indicate the values for each individual. Values of the same individual are connected by lines. The bars represent the means at 0, 2, and 4 wk.

ues from 51.40 ± 4.48, to 44.38 ± 5.17, and 43.53 ± 5.48 at 0, 2, and 4 wk, respectively (Figure 4A and Table 1). There was no significant difference in the results obtained at wk 0 and wk 2 or 4 (Table 1). On the other hand, the test group, which ingested drinking yogurt fortified with egg yolk IgY-urease, showed a reduction in UBT values from 51.18 ± 3.40 at wk 0 to 33.70 ± 3.50, and 31.03 ± 3.54 at 2 and 4 wk, respectively (Figure 4B and Table 1). The UBT values obtained from the test group (Table 1) at wk 2 and 4 were significantly different from those obtained at wk 0 ($P < 0.001$), showing a 34.19 and 39.33% reduction in UBT values after 2 and 4 wk, respectively. The IgY-urease yogurt ingestion was very effective in suppression of *H. pylori* infection; however, some factors would interfere with its efficacy in humans, including pH condition of the stomach and the action of proteolytic digestive enzymes such as pepsin.

The use of probiotics for the suppression of *H. pylori* in humans has been studied by some investigators (Michetti et al., 1999; Wendakoon et al., 2002). However, none of these studies were able to show a significant suppression of *H. pylori* in humans, and others showed a slight but nonsignificant trend toward a suppressive effect of drinking yogurt containing specific lactic acid bacteria (Sakamoto et al., 2001; Cats et al., 2003).

On the other hand, passive immunization by supplementing food with specific antibodies from colostrums against some pathogens was previously reported (Ebina et al., 1985; Tacket et al., 1992). Meanwhile, the use of IgY has been documented in animal models as being effective in preventing gastrointestinal infections (Ebina et al., 1990; Peralta et al., 1994). Eggs from hyperimmunized hens could provide a convenient and economic source of antibodies for passive immunization (Hatta et al., 1990; Kim et al., 2000). It has been suggested that IgY could be applied for fortification of infant foods (Akita and Nakai, 1992), and in special cases of food (Kim et al., 2000). Therefore, the fortification of drinking yogurt with 1% egg yolk containing specific IgY-urease was conducted. As each gram of egg yolk contains about 10 mg of pure IgY (Rose et al., 1974; Deignan et al., 2000), the daily intake of 4.5 g of egg yolk (present in 450 mL of IgY-urease yogurt) provides about 45 mg of pure IgY. The recommended daily intake in this study (45 mg of pure IgY-urease/d per volunteer) was calculated based on our previous animal study (unpublished data).

From an immunological point of view, IgY specificity has a potential role in its efficacy. ImmunoglobulinY produced by *H. pylori* whole-cell lysate has been reported to prevent *H. pylori* infection; however, cross-reactivity with other bacteria, including the normal flora, could decrease IgY efficacy (Shin et al., 2003).

The use of IgY against a pathogenic factor of *H. pylori* would be a prudent way to suppress the infection. The bioadhesive function of *H. pylori* urease was recently described considering the surface localization of urease on the *H. pylori* outer membrane. Urease is a major, if not a decisive, factor in initial bacterium-mucus contact before colonization and during maintenance of colonization against physical constraints, such as the normal mucus turnover mechanism, peristalsis, and passage of luminal contents (Icatlo et al., 1998; 2000). It was demonstrated that IgY-urease was highly specific and had a significant effectiveness against *H. pylori* because of its ability to inhibit the organism from adhering to the gastric mucosa (Kim et al., 2000; Shin et al., 2002, 2004). Accordingly, the results herein support the finding that fortification of yogurt with egg yolk IgY-urease has a significant suppressive effect against *H. pylori* in humans. Because IgY-urease binds urease only, the functional efficacy observed in this study was presumably via capture of bacterium-associated urease within the gastric mucus layer, resulting in bacterial aggregation and clearance via the constant washing action of the gut. By such a mechanism, IgY-urease may play a dual role in suppression and prophylaxis against *H. pylori* in humans.

The present study demonstrated that administration of a specially designed drinking yogurt augmented with highly specific antibodies from egg yolk against urease enzyme, the pathogenic factor of *H. pylori*, could effectively suppress *H. pylori* infection in humans.

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