In Hydrolyzed Cow's Milk *Helicobacter pylori* Becomes Nonculturable and the Growth of *Salmonella typhi* and *Escherichia coli* Is Inhibited

A. Orozco, T. Ogura, T. Hirosawa, R. Garduño, and I. Kubo

ABSTRACT: The colony forming unit (CFU) of *H. pylori* is reduced rapidly in lipase hydrolyzed cow's milk and a similar reduction was found in a physiological saline solution when it was supplemented with soluble C4 to C10 fatty acids of milk fat composition. Slight CFU decreases were observed for *E. coli* and *S. typhi* in hydrolyzed milk buffered to pH 3, while the counts in milk and physiological saline solution at pH 3 stayed almost unchanged for 24 h. *E. coli* proliferated in glucose-peptone medium, better at pH 4.7 than at pH 3. On the other hand, supplementation of the medium with soluble fatty acids of milk composition completely inhibited growth for 32 h. Supplementation of the medium with fatty acids reduced the growth of *S. typhi* to approximately 1/20 at pH 4.7. Therefore, milk hydrolyzed by gastric lipase may damage *H. pylori*, producing a nonculturable state. With *E. coli* and *S. typhi*, hydrolyzed milk does not induce inactivation to a nonculturable state but inhibits their proliferation potently. The latter is considered to be a state prior to VBNC (viable but nonculturable). However, the antibiotic effect will disappear when the fatty acids are absorbed by the intestine.

Keywords: antibiotic, *E. coli*, *H. pylori*, milk hydrolysate, *Salmonella*

Introduction

Weak acids have been widely used to preserve foods. Ray and Sandine (1992) reviewed the antibacterial action of short chain acids such as acetic, propionic, and lactic acids. Protonated weak acids are lipophilic; therefore, they pass easily through the lipid bilayer of a cell membrane into the cytoplasm. When the invading acids overcome the buffer action of the cytoplasm, the excess protons are removed by proton pumps located on the cell membrane using ATP. The bacteria are starved and die.

The antibacterial action of milk is well documented (Hakansson and others 1995; Isaacs and others 1995; Hamosh 1998; Dosogne and others 2001; Early and others 2001; Seifu and others 2004). Aqueous milk fatty acids show notable antibacterial effects (Wang and Johnson 1992; Petrone and others 1998; Sprong and others 1999, 2001, 2002; Sun and others 2002, 2003). Sun and others (2002) showed that partially hydrolyzed milk is bacteriostatic at pH 5 and 6 against *K. pneumoniae* and *E. faecalis*. However, little is known about the antibacterial effects of hydrolyzed milk.

Milk is a complex liquid food consisting of emulsified particles and aqueous constituents. On entering to the stomach, coagula are formed by the destruction of the emulsion by gastric HCl. Milk lipid may be one of the main constituents of the coagula.

The lipid is gradually hydrolyzed by the aid of gastric lipase to fatty acids. Milk fat is rich in soluble fatty acid constituents (C4 to C8), which mainly remain in the aqueous phase after the hydrolysis. Sparingly soluble fatty acids (C10 and larger) might pertain to the coagula. When bacteria in milk enter the stomach, they may be surrounded by the coagula or the liquid phase, which is similar to whey in cheese making. In this study, an acidic solution of soluble fatty acids was used as a model for the liquid phase and the whole milk hydrolysate as a model for milk in the stomach. The difference in the soluble fatty acid solution and the milk hydrolysate might be caused by the presence of coagula.

The prevention and eradication of *Helicobacter* and *Salmonella* by the use of foodstuffs is of continuing interest to us (Kubo and others 1999, 2004; Orozco and others 2003). *H. pylori* causes gastric and duodenal ulcers and gastritis. Salmonellosis is one of the most frequently occurring bacterial foodborne illnesses and is difficult to cure. This article focuses on the behavior of these pathogens in milk and milk hydrolysate and the possible application of milk products in the prevention and eradication of these pathogens.

Materials and Methods

Reagents

Reagents and ingredients for the culture media were purchased from Sigma-Aldrich Quimica (Toluca, Mexico), Merck & Co. (Whitehouse Station, N.J., U.S.A.) and Difco Laboratories (Detroit, Mich., U.S.A.). Antibiotics were purchased from Oxoid (Hampshire, U.K.). Horse serum samples were obtained from fresh horse blood samples by centrifugation and were preserved at −18 °C until they were used. Lipase (EC 3.1.1.3 from *Candida rugosa*) was purchased from Sigma-Aldrich Quimica (L-1754).

Bacteria, media, and culture conditions

E. coli (ATCC 11303) and *S. typhi* (ATCC 33495) were purchased from the American Type Culture Collection (Rockville, Md., U.S.A.). They were inoculated into a glucose-peptone minimum medium [MIN: 2.4% (w/v) glucose, 6.8% (w/v) gelatin peptone] or nutritious

MS 20070122 Submitted 2/16/2007, Accepted 7/5/2007. *Author Orozco is with the Scientific & Technological Development Div., Casa Herradura, Comercio 172-1, Col. Mexcaltzingo, Guadalajara, Jalisco, México. Author Ogura is with Univ. Autónoma de Guadalajara, Guadalajara, Jalisco, Mexico. Author Hirosawa was with Hospital Angel-Leaño, Univ. Autónoma de Guadalajara, Guadalajara, Jalisco, Mexico and is currently with Hospital Santa Fe, Querétaro nr 154-306, Col. Roma, Del. Cuauhtèmoc, C.P. 06700, México, Author Garduño was with Hospital Angel-Leaño, Univ. Autónoma de Guadalajara and is currently with Hospital San Javier, Av. Pablo Casals 640; Col. Prados Providencia, Guadalajara, Jalisco, Mexico. Author Kubo is with Univ. of California, Berkeley, CA 94720 U.S.A. Direct inquiries to author Ogura (E-mail: togura@uag.mx).*

doi: 10.1111/j.1750-3841.2007.00490.x

agar (NUT) (Merck & Co.) and incubated in an aerobic atmosphere at 36 °C for 24 h. *H. pylori* ATCC 43504 was used for the bioassay, which was inoculated into chocolate-gelose agar (Difco Laboratories) and incubated in a microaerobic atmosphere (N_2 85%, CO_2 10%, O_2 5%) at 37 °C for 72 h. The verification was made by the Gram stain technique with microscopy observation and an urease activity test.

Preparation of lipase-hydrolyzed milk (HYD) and nonhydrolyzed milk (MLK)

Milk cartons were purchased from a local market. The milk (95 mL) was hydrolyzed aseptically with a lipase (Sigma L-1754) at a concentration of 1 g lipase/L for 6 h at 37 °C. Then 5 mL of a pH 3 or pH 4.7 phosphate buffer (1 M) were added to the hydrolysate. Phosphate buffer solutions were prepared by mixing together (a omit a) 1 M H_3PO_4 (1 M) with 1M Na_2HPO_4 to attain pH 3 or pH 4.7. The resultant milk hydrolysate (HYD), 9.9 mL/tube, was placed in sterilized test tubes equipped with a screw cap. Asepsis was confirmed by incubating the tubes for 24 h at 36 °C. Milk was treated in the same manner, but without hydrolysis, to make MLK. An aseptic saline solution at pH 3 or 4.7 was obtained similarly as a blank (SAL).

Enumeration of bacteria in hydrolyzed milk (HYD)

Twenty microliters of a suspension of bacteria (*E. coli,* 2.7×10^5 CFU/mL; *S. typhi,* 2.6×10^5 CFU/mL; *H. pylori,* 4.0×10^4 CFU/mL) were added aseptically to 3 tubes containing 6.0 mL of HYD, MLK, or SAL adjusted to a pH of 3.0 or 4.7. The tubes were incubated at 36 °C for 4, 12, and 24 h in an aerobic atmosphere for *E. coli* and *S. typhi* and in a microaerobic atmosphere (N_2 85%, CO_2 10%, O_2 5%) for *H. pylori.* The bacteria were counted by the plate dilution method using NUT for *E. coli* and *S. typhi* and CGA for *H. pylori* in triplicate.

Enumeration of *H. pylori* in SAL in the absence and presence of a soluble fatty acid mixture

SAL at pH 4.7 was prepared by adding 5 mL of a phosphate buffer solution (1 M) at pH 4.7 to 95 mL of a saline solution (0.85%) and sterilized at 110 °C. For the preparation of SAL + FA, 94.6 mL of the saline solution (0.85%) were buffered with 5 mL of phosphate buffer solution (1 M). Butyric acid (110 μL, 1.2 mmol), hexanoic acid (100 μL, 0.8 mmol), octanoic acid (63 μL, 0.4 mmol), and decanoic acid (96 μL, 0.5 mmol) were added aseptically to the sterile buffered saline solution to replicate the composition of cow's milk, as described by Jensen (2002). The pH was decreased slightly by the fatty acid supplementation (0.1 to 0.2 unit) and was readjusted by the addition of 1 M NaOH.

One hundred microliters of a suspension of *H. pylori* (4.0×10^4 CFU/mL) were added aseptically to 6 tubes containing 6.0 mL of sterile saline solution at pH 4.7, with and without a mixture of soluble fatty acids (FA). They were incubated at 36 °C in a microaerobic atmosphere (N_2 85%, CO_2 10%, O_2 5%). The bacteria were counted by the plate dilution method using CGA for *H. pylori* in by triplicate.

Growth of *E. coli* and *S. typhi* in glucose-peptone media (MIN) in the absence and presence of a soluble fatty acid mixture

Twenty microliters of a suspension of *E. coli* (2.7×105 CFU/mL) or *S. typhi* (2.6×105 CFU/mL) were added aseptically to 3 tubes containing 6.0 mL of glucose-peptone media (2.4% glucose, 6.8% peptone from gelatin) with a phosphate buffer solution at pH 3.0 and 4.7, with and without a mixture of soluble fatty acids (FA) in a similar manner to that described above for SAL. The tubes were incubated at 36 °C for 32 h in an aerobic atmosphere. The growth was measured every 4 h by means of optical density (OD) at 750 nm. The bacterial purity of the cultures was verified microscopically by the Gram stain technique.

Results and Discussion

Antimicrobial effect of lipase-hydrolyzed milk (HYD)

Figure 1 compares the CFU of *H. pylori, E. coli,* and *S. typhi* in saline solution, in cow's milk and in hydrolyzed milk.

H. pylori. Interestingly, the *H. pylori* was completely deactivated in HYD within 24 h. It was confirmed that *H. pylori* was inactivated,

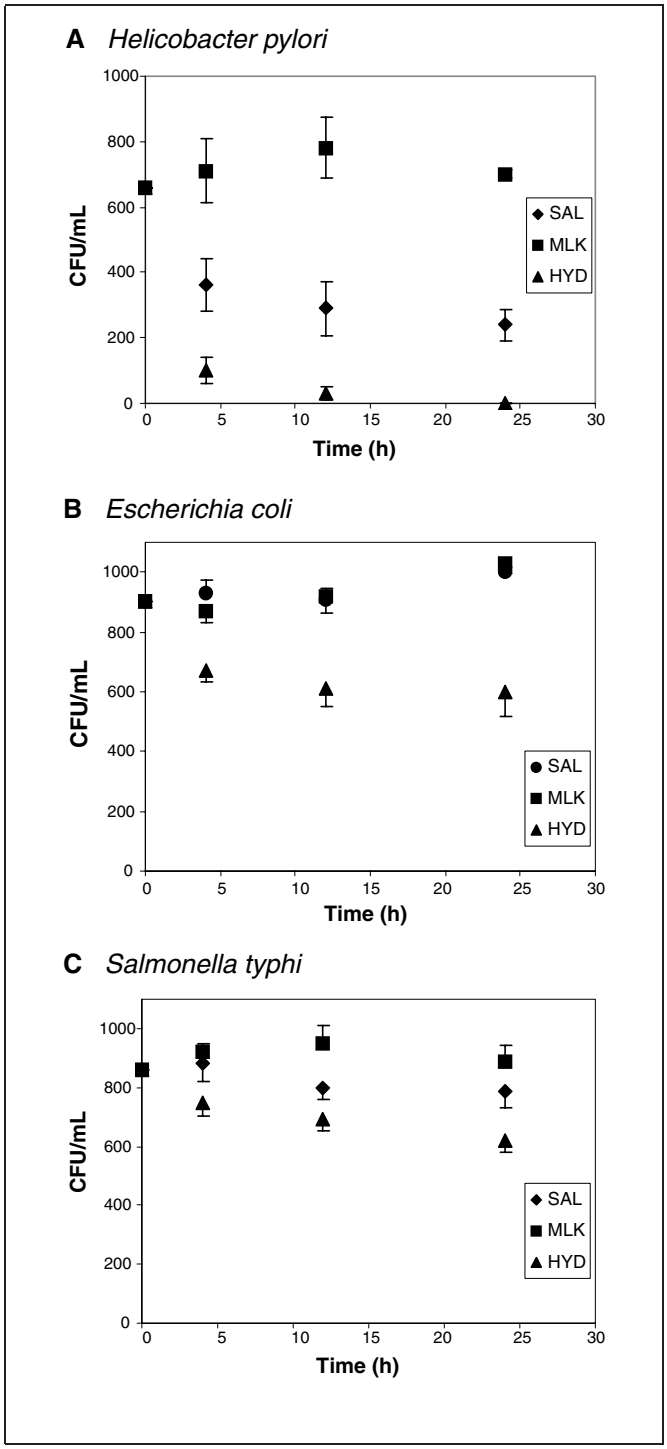

Figure 1—The change of CFU in saline solution, cow's milk, and hydrolyzed milk for *H. pylori, E. coli*, and *S. typhi* at pH 3. Vertical lines indicate standard deviation.

in a practically identical manner, when a mixture of soluble fatty acids having the composition of cow's milk (FA) was added to the saline solution. Therefore, it may be concluded that the inactivation of *H. pylori* in HYD is caused by the free soluble fatty acids.

H. pylori is found in more than 90% of duodenal and gastric ulcers and 70% to 80% of gastritis (Veldhyzen and Lee 1999). Marshall and others (1985) showed that drinking *H. pylori* culture caused gastritis. During the 1st half of the last century, the standard treatment for peptic ulcers was to drink a large amount of milk to which an alkali such as calcium carbonate or sodium carbonate had been added to neutralize gastric acid. This also supplied nutriment. The Sippy regimen, Doll's milk drip (Warner and McIsaac 1991), and Winkelstein's milk drip (Baron 2000) are well known. In these remedies, the bactericidal fatty acids produced by milk hydrolysis were forced to dissociate, resulting in nonbactericidal carboxylate ions as reported previously (Orozco and others 2003).

One of the authors (TO) is acquainted with a person who had frequently suffered from duodenal ulcers as a young man. On the recommendation of a medical practitioner, he took 50 to 100 mL of cow's milk, which he carried with him in a thermos, every 2 to 4 h for a month. His symptoms disappeared and have not returned in 50 y. This incidence is in accord with our finding that milk after being hydrolyzed in the stomach efficiently inactivates *H. pylori*.

We observed repeatedly the phenomenon that the count of *H. pylori* decreases in physiological saline solution but not in milk as seen in Figure 1. Therefore, the milk globules might provide a hydrophilic shelter for the bacteria from the unfavorable environment.

Since milk is the unique food for a neonate, the fact that hydrolyzed milk protects against *H. pylori* is important in understanding newborn defenses. When milk is fed to a baby, it serves also as a defense against *H. pylori* in the stomach, which is inactivated or at least weakened enough by the action of soluble and insoluble fatty acids from the fat to not cause infection. Finally, all of the mixture is drained to the intestine.

Enumeration of *E. coli* and *S. typhi* in hydrolyzed milk. Figure 1B and 1C show the CFU counts for *E. coli* and *S. typhi*, respectively, against duration time in HYD, MLK, and SAL at pH 3. For both bacteria, HYD reduced CFU to approximately 2/3 in 24 h.

As seen in Figure 1A, 1B, and 1C, the antibacterial action of the milk employed in this experiment is too weak to show any significant effect. However, the milk in the carton has been sterilized; therefore the important antibiotic peroxides (Min and others 2005) and living phagocytes (Dosogne and others 2001) have been completely destroyed.

Growth of *E. coli* and *S. typhi* in MIN with and without soluble fatty acids. The growth of *E. coli* and *S. typhi* was almost completely suppressed by the fatty acid mixture as seen in Figure 2, which is in high contrast to the slight decrease of CFU caused by hydrolyzed milk or the fatty acid mixture. The glucose-pepton medium (MIN) at pH 3 was invariably more toxic than that at pH 4.7, agreeing with the former observation that undissociated fatty acids are toxic to bacteria but not so deprotonated fatty acid ions (Orozco and others 2003).

The bacteria were suspended for a specific time in the saline solution with or without hydrolyzed cow's milk. The suspension was inoculated into the nutritious medium to determine CFU, and it was found that the CFU of the bacteria was practically unchanged by the addition of the fatty acids. On the other hand, *E. coli* and *S. typhi* did not grow in MIN supplemented by the fatty acid mixture. The bacteria kept in MIN with fatty acids for growth for 32 h were still viable when they were inoculated into the nutritious medium: the viability being greater for *S. typhi* than *E. coli*. These bacteria, exposed to the mixture for 32 h, grew poorly in the MIN medium.

It is known that many bacteria are physiologically active but are not able to be cultured. Colwell and Grimes (2000) named them "Viable but nonculturable" or "VBNC". Cases have been reported where VBNC bacteria have been resuscitated by means of nutritional stimulation and other methods (Keep and others 2006a, 2006b). Since bacteria are subject to environmental changes and diverse harmful conditions, they have to protect themselves by stopping physiological activity. Therefore, they may have many states of suspended physiological activity. In the case of the present article, we would like to consider that *E. coli* and *S. typhi* suspended growth in the presence of the fatty acid mixture and were resuscitated on removal of the acids and the enrichment of the medium.

S. typhi, when swallowed in a food containing fatty acids, may persist in the harmful environment suspending growth until resuscitated when the acids are absorbed by the intestine. It is well known that inulin and other soluble dietary fibers produce short chain fatty acids (SCFA) in the intestine (Rycroft and others 2001). Therefore, an intake of such soluble fibers may prevent further proliferation of pathogens in the intestine by the formation of SCFA. Moreover, since the growth of probiotic bacteria such as bifidobacteria is promoted by prebiotics (van de Wiele and others 2004), the growth of the pathogens might be suppressed. On the other hand, Ten Bruggencate and others (2004) reported that inulin administration decreases resistance to *Salmonella* in rats. However, they

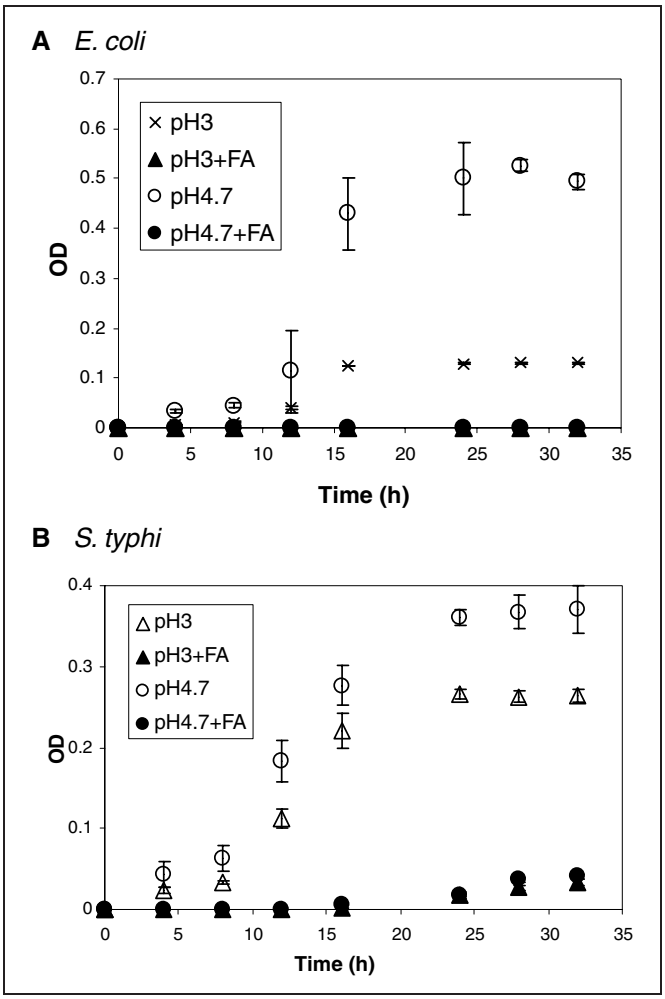

Figure 2—The growth of *E. coli* (A) and *S. typhi* (B) in glucose-peptone medium (MIN) with and without soluble fatty acids (+FA) at pH 3.0 and 4.7. Vertical lines indicate standard deviation.

found that calcium phosphate counteracted most of the adverse effects.

Kubo and others (1993, 2003) showed that the optimal carbon number of antimicrobial compounds is concentrated in the range of C10 to C12, and explained this observation on the basis that the length of these compounds is similar to the thickness of the lipid layer of a cell membrane. The finding of Sun and others (2003) that C10 and C12 fatty acids show stronger bactericidal action against *H. pylori* also supports Kubo's hypothesis that the facility of cell membrane penetration determines the bactericidal effect.

It is quite understandable that the depletion of ATP causes the bactericidal effect of weak acids (Ray and Sandine 1992). This hypothesis assumes that the toxic acids invade beyond the cell membrane as Kubo proposes. However, none of these proposals can explain the antimicrobial action of sorbic acid, which has been widely used in the food industry as a preservative.

Currently, we are developing methods for the classification of the depth of dormant states, including that introduced by exposure to organic acids. We would like to explain the action of fatty acids, sorbic acid, and other preservative acids such as benzoic acid, in the same context.

Conclusions

Milk is an excellent nutrient and is converted to fatty acids by enzymatic hydrolysis in the stomach. These show strong bactericidal or bacteriostatic effects, which are the legacy of mammalian neonate protection. It is worth remembering that fatty acid salts are protonated to fatty acids by the action of gastric acid. A 1.5-g tablet made of fatty acid salts is approximately equivalent to 50 mL milk. Such a tablet could be used to eradicate *H. pylori*. Supplementation of the tablet with inulin or other soluble fibers and a calcium salt might keep the inactivation effect of the acids in the intestine.

Acknowledgments

This study was supported by Consejo Nacional de Ciencia y Tecnologia (CONACYT 4110 P-N). We are grateful to Mr. Kunio Hiroishi, Guadalajara, Jalisco, Mexico, for providing the valuable information on the remediation of his gastric ulcer by drinking milk.

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