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Lactobacillus acidophilus Autolysins Inhibit *Helicobacter pylori* In Vitro

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Abstract. Antibacterial activity of 17 strains of lactobacilli was tested against 10 strains of *H. pylori*. The inhibition observed was related to the acid production and the low pH attained. No relationship between CagA phenotype of *H. pylori* strains and tolerance to lactic acid was observed. In mixed cultures, *L. acidophilus* CRL 639 showed an autolytic behavior after 24 h of culture. At this moment, *H. pylori* CCUG17874 showed a decrease of 2 log-cycle, and no viable count was detected after 48 h. The bactericidal effect of *L. acidophilus* CRL 639 in mixed cultures is related to a proteinaceous compound released after cell lysis.

Helicobacter pylori is a spiral-shaped, Gram-negative rod that has developed sophisticated strategies to colonize epithelial cells lining the antrum of the stomach and to survive in acidic environments. *H. pylori* strains producing Vac A toxin (an important virulent factor) and CagA (the product of cytotoxin-associated gene A), which are generally co-expressed, induce more severe chronic gastritis, gastric atrophy, and also the precursor stages of gastric carcinomas and gastric MALT lymphomas [9, 31]. These strains have been designated as type I to differentiate them from the less virulent Vac- and Cag-negative strains, the so-called type II strains.

Lactobacilli have been used since decades against infectious diseases [2]. These bacteria are supposed to compete with other microorganisms on mucosal surfaces. Indeed, it is accepted that lactobacilli must present certain properties including adhesion, competitive exclusion capacity, and production of inhibitors in order to colonize the mucosal epithelial surface and to interfere with pathogens in vivo. Certain lactobacilli synthesize antimicrobial compounds that are related to the bacteriocin family [17]. Others are well-known metabolic end products of lactic acid fermentation, such as lactic and acetic acids, and hydrogen peroxide [30] or remain unidentified [3, 26]. It was recently reported that lactobacilli can inhibit growth of *H. pylori* in vitro and in vivo, but this antagonistic effect was due mainly to the production of lactic acid [19]. In contrast, other authors reported a protein-mediated effect [8].

Results presented here show a different antagonistic effect of lactobacilli-spent broths upon *H. pylori*; it was associated with acid production. In *L. acidophilus* CRL 639, this effect was also related to an intracellular proteinaceous component.

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Materials and Methods

Bacterial strains and growth conditions. Type strains and wild isolates of lactobacilli obtained from the culture collection of the Centro de Referencia para Lactobacilos (CERELA) are listed in Table 1. All strains were cultured at 37°C in MRS broth [11] for 16 h under a microaerophilic atmosphere. In total, 10 *H. pylori* strains were used in this study; type strains CCUG 17874, CCUG 17875 (Culture Collection of University of Gothenburg, Gothenburg, Sweden), and NCTC 11637 (National Collection of Type Cultures, London, UK), five clinical isolates HP1139, HP915, HP10/96, HP15/96, and HP66 from the University Hospital Lund; strain G33 obtained from N. Figura, Pediatric Hospital, Siena, Italy; and strain MO19 obtained from T. Borén, Department of Oral Biology, Umea University, Sweden. Strains were stored at −70°C in Tryptic Soy Broth containing 15% (vol/vol) glycerol, and were cultivated on GAB-CAMP agar [28]. Plates were incubated at 37°C, under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) for 3 days. Single colonies were inoculated into flasks containing 50 ml of gonococcal broth (GB), originally described for culturing *Neisseria gonorrhoeae* [27], supplemented with 5% horse serum (GIBCO BRL, Paisley, Scotland). Cultures were incubated at 37°C on a rotary shaker (200 r.p.m.) in anaerobic jars with a microaero-

Table 1. Growth inhibition of *Helicobacter pylori* by *Lactobacillus* supernatants[a]

Strain	Strain	Final pH	HP915	MO19	HP10/96	HP15/96	NCTC 11638	CCUG 17874	CCUG 17875	HP 1179	G33	HP66
L. acidophilus	4356[b]	3.98	−	−	−	−	+	±	−	−	−	−
L. acidophilus	639[f]	3.94	+	+	−	+	+	+	−	+	+	+
L. brevis	14869[b]	4,9	−	±	−	−	+	−	−	−	+	−
L. bulgaricus	449[e]	4.04	+	±	−	+	+	+	−	−	+	−
L. delbruekii subsp. *bulgaricus*	11842[b]	4,75	±	−	−	+	+	+	+	−	+	−
L. delbruekii supsp. *delbruekii*	9649[b]	3.97	−	+	−	+		−	−	−	+	−
L. delbrueki subsp *lactis*	12315[b]	4,27	−	−	−	+	+	+	−	−	+	−
L. casei subsp. *casei*	161[c]	3,76	−	+	−	+	+	±	−	−	−	−
L. casei subsp. *rhamnosus*	212[d]	3,73	−	±	−	+	+	±	−	−	−	−
L. fermentum	14931[b]	4,12	−	±	−	−	±	+	−	−	+	−
L. gasserii	20243[e]	3,82	+	+	−	−	+	±	−	−	−	−
L. helveticus	15807[b]	4,24	−	−	−	+		−	−	−	−	−
L. helveticus	15009[b]	4,03	+	−	−	+	+	±	−	−	+	+
L. plantarum	14917[b]	3,69	−	+	−	+	+	+	−	−	+	−
L. paracasei	F19[f]	3,86	−	−	+	+	+	±	−	−	−	−
L. reuteri	1098[f]	4,14	+	+	−	+	+	+	−	+	+	+
L. salivarius supsp. *salivarius*	11741[b]	3,71	−	−	−	+	+	+	−	−	−	−

[a] Inhibition was expressed as: +, halos < 4 mm; ±, halos > 4 mm; −, no inhibition halos.
[b] ATCC: American Type Culture Collection.
[c] NCDO: National Collection of Dairy Organisms.
[d] CNRZ: Centre National de la Recherche Zootechnique.
[e] DSM: Deutsche Sammlung für Mikroorganisms.
[f] CRL: Centro de Referencia para Lactobacilos. Wild type strains.

bic atmosphere generated by AnaerocultC envelopes (Merck, Darmstadt, Germany). Gas-generating envelopes were renewed every 24 h.

Determination of inhibitory activity. The plate diffusion technique was used to study the effect of spent broths (SB) of *Lactobacillus* cultures on *H. pylori* growth, according to standard methods [17]. *Lactobacillus* strains were subcultured in MRS broth, and the SB were assayed on *H. pylori* plates (GAB-CAMP agar) into which 4-mm holes had been punched and were filled with the samples to be tested, previously neutralized with 1 M NaOH. The plates were incubated for 72 h at 37°C. To test the inhibitory effect of intracellular extracts of *H. pylori* on *Lactobacillus* strains, cells were broken by grinding with glass beads; the crude extract was put into the wells (at a concentration of 50 μg of protein each) for diffusion test. *Lactobacillus* plates were prepared in MRS agar.

Mixed cultures of *L. acidophilus* and *H. pylori*. Cultures of *L. acidophilus* and *H. pylori* were mixed in GB broth supplemented with 5% horse serum in anaerobic jars on a rotary shaker (200 r.p.m.) at 37°C. The inocula were between 1×10^2 to 1×10^6 CFU ml^{-1} for *L. acidophilus*, and 1×10^4 to 3×10^6 CFU ml^{-1} for *H. pylori*. Growth of different bacteria was determined by the plate dilution method, with MRS agar for *L. acidophilus* and GAB-CAMP agar for *H. pylori*. Plates were incubated at 37°C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) for 72 h.

Determination of protease sensitivity. Protease sensitivity was tested according to De Klerk and Smith [10]. Two milliliters of 0.5% trypsin solution was incubated with an equal volume of the sterilized and neutralized SB at 37°C for 3 h. After incubation, the mixture was tested for inhibition of *H. pylori* CCUG 17874 (sensitive strain).

Antibacterial activity in broth culture. Two-day-old *H. pylori* CCUG 17874 cultures in GAB-CAMP agar were pelleted by centrifugation (3,000 *g*, 10 min), washed once with PBS 0.15 M pH 7.2, and cells were resuspended in the different SB at an initial $O.D._{540} = 1.0$. At several times, serial dilutions of each sample were plated in duplicate in GAB-CAMP agar by plate dilution methods. Plates were incubated at 37°C for 4 days. Results were expressed as CFU/ml; the survival rate of *H. pylori* CCUG 17874 was determined as N/No, where N is the CFU/ml after a given incubation time and No is the CFU/ml at zero time. Transitions between *H. pylori* coccoid and spiral shape were detected by Gram stain.

SDS-PAGE and renaturing SDS-PAGE. SDS-PAGE was carried out as described by Laemmli [20] with a Mini Protean II cell unit (Bio-Rad Laboratories). Renaturing SDS-PAGE was performed as described [24]; 0.2% (wt/vol) *Micrococcus lysodeikticus* ATCC 4698 (Sigma Chemical Co., St. Louis, MO) autoclaved cells were included in 12% polyacrylamide gels for detection of bacteriolytic activities. After electrophoresis, gels were washed for 30 min with distilled water at room

Table 2. Resistance of *Helicobacter pylori* to organic acids

Acid used	HP915	MO19	HP 10/96	HP 15/96	NCTC 11638	CCUG 17874	CCUG 17875	HP 1179	G33	HP66
Control[a]	−	−	−	−	−	−	−	−	−	−
DL-Lactic acid[b]										
100 mM	−	−	−	−	−	+	+	+	+	−
60 mM	−	−	−	−	−	−	−	−	−	−
20 mM	−	−	−	−	−	−	−	−	−	−
L-Lactic acid[b]										
100 mM	+	+	+	+	+	+	+	+	+	+
60 mM	−	+	−	+	+	+	+	+	+	−
20 mM	−	−	−	−	−	−	−	−	−	−
D-Lactic acid[b]										
100 mM	−	−	−	−	−	−	−	−	−	−
60 mM	−	−	−	−	−	−	−	−	−	−
20 mM	−	−	−	−	−	−	−	−	−	−
Acetic acid[c]										
100 mM	−	−	−	−	−	−	−	−	−	−
60 mM	−	−	−	−	−	−	−	−	−	−
20 mM	−	−	−	−	−	−	−	−	−	−

[a] Control: PBS adjusted to pH 3.5 with HCl.
[b] In PBS adjusted to pH 3.5.
[c] In PBS adjusted to pH 4.2.

temperature under gentle shaking, and transferred into renaturation buffer containing 50 mM Tris-HCl (pH 6.5) and 0.1% (vol/vol) Triton X-100 (Sigma). Gels were incubated at 37°C for 16 h under gentle shaking, rinsed with distilled water, stained with 0.1% (wt/vol) methylene blue in 0.01% KOH for 2 h at room temperature with gentle shaking [18], and destained with distilled water. The bacteriolytic activity appeared as clear bands on a blue background. The molecular masses were determined by comparison with molecular mass standards that were electrophoresed on the same gel and stained with Coomasie blue R-250. The standards were purchased from SIGMA in a range from 6.5 to 205 kDa.

Preparation of cell extracts. *L. acidophilus* CRL 639 cells grown in MRS broth at 37°C were recovered by centrifugation at 8,000 *g* for 15 min at 4°C and washed with 50 mM Tris-HCl (pH 7.0) buffer. For total cell extract preparation, cells were disrupted by grinding with glass beads. Autolysis induction was performed according to Fernandez Murga et al. [14] with the following modifications: A 16-h-old culture was used to inoculate MRS broth at an initial OD_{560} = 0.5 and tubes were incubated at 45°C until autolysis was observed. The SB were neutralized with 1 M NaOH and filtered through 0.22-μm pore, size Millipore membranes.

Statistical evaluation. All experiments were performed in triplicate; variations were less than 5%.

Results

Inhibition of *H. pylori* by *Lactobacillus* SB and lactic acid. A well diffusion technique was used to evaluate 17 strains of *Lactobacillus* against *H. pylori*. A question to be resolved was to know whether the CagA antigen is involved in the resistance of *H. pylori* to the lactic acid produced by lactobacilli strains. To this end, *H. pylori* strains CCUG 17874 and 17875, HP1179, G33, HP66, NCTC 11638 (phenotype cag^{+}) and strains HP915, MO19, HP10/96. HP15/96 (phenotype cag^{-}) were used as sensitive strains. Table 1 shows that the sensitivity of *H. pylori* is not associated with the CagA phenotype although it is dependent on the strain tested.

All *Lactobacillus* strains tested were able to inhibit *H. pylori*, but the effect was lost when adjusting the pH of the SB to 6.0, indicating that it is mediated by the organic acids produced.

To substantiate this hypothesis, well-diffusion tests were performed with different concentrations (20–100 mM of DL-lactic acid (synthetic mixture about 1:1), L-lactic acid, D-lactic acid, and acetic acid in PBS at pH 3.5, adjusted with HCl. Results are shown in Table 2. *H. pylori* strains CCUG17874, CCUG17875, HP1179, and G33 were inhibited by addition of 100 mM DL-lactic acid, while 60 mM L-lactic acid was enough to inhibit strains MO19, HP15/96, NCTC11638, CCUG17874, CCUG17875, HP1179 and HP33. In contrast, strains HP915, HP10/96, and HP66 were only sensitive to concentrations of 100 mM L-lactate. None of the strains was sensitvie to D-lactic acid isomer nor to acetic acid.

Recently, antibacterial activity of the crude lysate of *H. pylori* MO19 against Gram-negative (*Escherichia coli* D21) and Gram-positive (*Bacillus megaterium* Bm11) strains was reported [25]. Since *H. pylori* may also undergo "altruistic lysis" in vivo [23], the antibacterial activity of crude lysates of *H. pylori* strains against

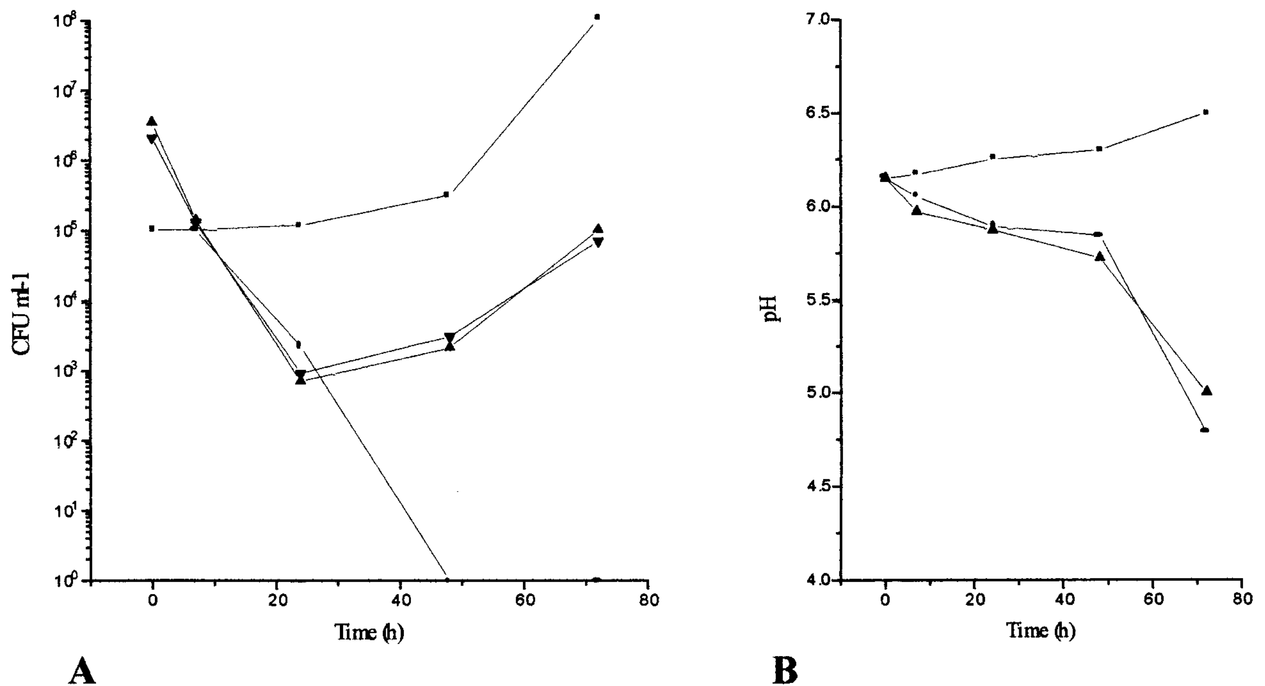

Fig. 1. Mixed cultures of *L. acidophilus* CRL 639 and *H. pylori* CCUG17874. (A) Viable counts; (B) pH. *L. acidophilus* in pure (▲), and mixed (▼) culture; *H. pylori* in pure (■), or in mixed (●) culture.

Lactobacillus was determined. None of the *H. pylori* strains (including MO19 used as control) was able to inhibit the *Lactobacillus* strains tested.

Inhibition of *H. pylori* by lactobacilli in mixed cultures. *L. acidophilus* CRL 639 and *H. pylori* CCUG 17874 were chosen for mixed culture experiments. The initial inoculum was 1×10^6 for CRL 639 and 1×10^5 CFU ml^{-1} for CCUG17874. *L. acidophilus* showed an autolytic behavior after 24 h of incubation in both pure and mixed culture, downloading its populations to 1×10^3 CFU ml^{-1} (Fig. 1A). At this moment, a decrease in cell viability (1×10^3 CFU ml^{-1}) was observed for *H. pylori*, and no viable counts were found after 48 h. This was not owing to the lactic acid produced by *Lactobacillus* strain since, at that moment, the pH of the mixed culture was 5.8 (Fig. 1B) and the concentration of DL-lactic acid was 20 mM.

To determine the effect of *L. acidophilus* CRL 639 on *H. pylori* morphology, samples of *H. pylori* CCUG 17874 from pure and mixed cultures were taken at several times and Gram stained. A shift from the bacillary to the coccoid form was observed in GB broth pure cultures after 72 h, while only bacillary forms were found in mixed cultures for the same period. These results would indicate that the inhibition observed in mixed cultures might be mediated by a bactericidal effect rather than the induction of the viable but non culturable coccoid form.

Table 3. Effect of MSB or PSB cultures of *L. acidophilus* CRL 639 on *H. pylori* CCUG17874 survival[a]

	Incubation time (h)		
Supernatant	0	4	9
GB pH 5.5[b]	6.69	6.30	6.00
GB pH 5.0[b]	6.77	6.47	6.07
GB pH 4.5[b]	6.74	6.01	5.69
PSB *L. acidophilus* CRL 639	6.79	4.60*	ND*[c]
MSB	6.81	4.69*	ND*[c]
PSB *H. pylori*	6.75	6.60	6.54

[a] Expressed as $\log_{10}$ of CFU/ml.
[b] GB broth adjusted with lactic acid (synthetic mixture).
[c] ND, not viable counts detected.
* Values significantly different ($P < 0.01$; Student's t test).

Characteristics of *L. acidophilus* CRL 639 antibacterial activity. For this experiment, the 24-h SB of mixed (MSB) and pure (PSB) cultures of *L. acidophilus* CRL 639 (Fig. 1A) were tested against a cell suspension of *H. pylori* CCUG 17874. As shown in Table 3, a 2-log cycle decrease in viable counts was observed for *H. pylori* after 5 h of incubation at 37°C, and no viable cells were recovered after 8 h in the presence of both spent broths. The same effect was observed when *H. pylori* was treated with autolytic supernatant (normally induced at 45°C) of *L. acidophilus* CRL 639 (data not shown). In

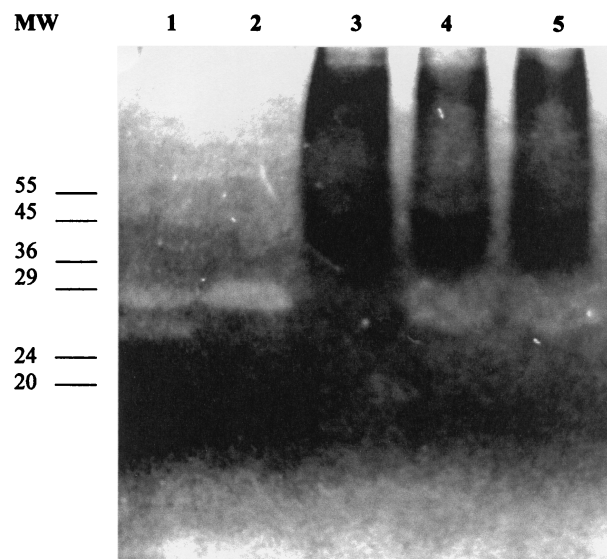

Fig. 2. Detection of bacteriolytic activities by renaturing SDS-PAGE in a gel containing autoclaved *M. lysodeikticus* cells as substrate. *L. acidophilus* CRL 639 crude extract (1); autolytic SB induced at 45°C (2); *H. pylori* SB from pure culture (3); SB from mixed culture (4); *L. acidophilus* CRL 639 SB from pure culture (5).

contrast, the PSB of *H. pylori* as well as the uninoculated GB broth adjusted to pH 4.5, 5.0, or 5.5 with DL-lactic acid showed no antibacterial activity (Table 3). The antibacterial activity of both PSB and MSB of *L. acidophilus* decreased by 40% after proteolytic treatment with trypsin, indicating that a proteinaceous compound might be involved.

Autolytic activity in SB of *L. acidophilus* CRL 639 by renaturing SDS-PAGE. The antibacterial activity of both *L. acidophilus* CRL 639 spent broths (PSB and MSB) was analyzed by the renaturing SDS-PAGE technique. Two different substrates were included into the gel: autoclaved cells of *Micrococcus lysodeikticus* (reported to allow more sensitive detection of lytic activities) [21] and *H. pylori* CCUG 17874 (sensitive strain used in this study for antibacterial activity). Samples from the autolytic SB (induced at 45°C) and the crude extract (from cells incubated at 37°C) of *L. acidophilus* were included in the gel as positive controls for autolysin activity.

The crude extract of *L. acidophilus* showed two bands of activity, of 28 kDa and 26 kDa (Fig. 2, line 1), while a unique band of 28 kDa was observed in the SB at 45°C (line 2, autolysin control). No activity band was detected with the PSB of *H. pylori* (lane 3), while an activity band of 28 kDa was observed in the PSB (lane 4) and the MSB (lane 5) of *L. acidophilus*. None of the extracts tested showed activity when *H. pylori* CCUG 17874 was used as substrate.

Discussion

The major groups of inhibitory compounds produced by probiotic lactobacilli are lactic and volatile acids, as well as bacteriocins [15, 29]. Bhatia *et al*. [5] proposed that lactic acid production by *L. acidophilus* is responsible for the inhibition of *H. pylori*. They reported 100 mM as being enough to inhibit growth of *H. pylori*, results that are comparable to those presented in our study.

The inhibitory effect of *Lactobacillus* SB is a strictly pH-mediated effect, since it was lost after neutralization. Similar trends were reported for *E. coli*, *Listeria*, and *S. aureus* [12]. On the contrary, the antibacterial activity of *L. acidophilus* CRL 639 in mixed cultures (MSB) was distinguishable from the acid produced, since a 3-log cycle decrease was observed even at pH 5.8 and with a DL-lactic acid concentration of 20 mM.

Many reports have related the inhibitory effect of lactic acid or the secreted proteinaceous substances [8, 26] on *H. pylori* to the induction of the viable but not culturable coccoid form. *H. pylori* has a helical bacillary appearance in favorable conditions, but undergoes transformation into the coccoid forms under unfavorable conditions as a mechanism to survive harsh environments [6, 7]. Our study showed that the inhibitory activity of *L. acidophilus* CRL 639 is not related to the induction of the coccoid form. These result would be interesting, since this morphological manifestation may be involved in the infection transmission of *H. pylori* and partially responsible for relapses of the infection after antibiotic therapy failures [1, 4].

The bactericidal effect of *L. acidophilus* CRL 639 is related to a proteinaceous compound that is released after cell lysis in mixed cultures (Fig. 1A). A number of bacterial species have been reported to contain autolytic enzyme systems which, under appropriate conditions, are able to cause bacteriolysis by hydrolysis of various susceptible bonds in the cell wall peptidoglycan [16]. Other authors described temperature-sensitive strains that exhibited thermo-inducible lysis when subjected to temperature shifts. They attributed this property to induction of a prophage [13, 22]. The autolysis observed in *L. acidophilus* CRL 639 is not related to induction of a prophage, since the induction with mitomycin C was negative (data not shown).

The different assays performed in our study suggest that autolysins in the MSB of *L. acidophilus* CRL 639 might be involved in the inhibition of *H. pylori*.

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