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Antagonistic Activity of *Lactobacillus* Sp. Against *Helicobacter pylori*

L. Medouakh, A. Ait Abdeslam and A. Bensoltane

Laboratory of Food and Industrial Microbiology,
Department of Biology, Faculty of Science, University Oran (Es-senia), 31000 Oran, Algeria

Abstract: The aim of this study was *in vitro* testing of the potential inhibitory effect of *Lactobacillus* strains isolated from goat's milk on *H. pylori* by using a standard antimicrobial plate well diffusion assay. Assays involving *H. pylori* cultures grown on solid agar as well as in liquid media were used for this purpose. All *Lactobacillus sp.* culture tested inhibited clinical *H. pylori* growth but this inhibitory effect was lost after neutralization of *Lactobacillus* supernate. The addition of supernatant of selected *Lb. rhamnosus 1* to *H. pylori* culture tested (HP1, HP2) decrease the viability of *H. pylori* strains (from 10^5 to 10^3 CFU/ml) after 24 h and no viable counts were found at 72 h for *H. pylori*1 (HP1) and at 60 h for *H. pylori* 2 (HP2). Also, dramatically decline in cell viability for *H. pylori* were observed in addition to lactic acid and complete inhibition was demonstrated at 48 h in HP1 and at 36 h in HP 2. It was concluded that this inhibition observed is related to lactic acid production by *Lactobacillus* strains and we suggest that lactic acid might play the major role in this antagonistic effect.

Key words: *Helicobacter pylori* · Goat's milk · *Lactobacillus* · Antagonism · Acid lactic and antimicrobial agent

INTRODUCTION

Helicobacter pylori is a spiral curved Gram-negative, microaerophilic human gastric pathogen that colonizes the mucous layer of gastric epithelium and survives in acidic environments [1]. *H. pylori* is a causative agent of a range of human diseases. It is the major aetiological agent in chronic gastritis, peptic ulcers [2] and also a risk factor for gastric adenocarcinoma and gastric lymphoma [3,4]. This infection is extremely common throughout the world [5] and affects over 50% of the population worldwide [6,7]. Triple therapy using a proton pump inhibitor combined with two antibiotics, is the most frequently recommended treatment for the eradication of *H. pylori* [8,9]. However, Antibiotic treatments are not always effective against *Helicobacter* infection, as antibiotic resistance is a growing problem worldwide and induces side-effects, especially antibiotic-associated diarrhea [8,10,11]. Therefore, the development of alternative methods is deemed necessary and a search for new antimicrobial agents is warranted [5,11].

Lactobacilli have been used since decades against infectious diseases [12] and have been extensively studied for their ability to protect against pathogens like *H. pylori* [13, 14]. These organisms have been widely used as probiotics [15,16]. Several authors have previously reported that *Lactobacillus* sp. exhibit inhibitory activity against *H. pylori in vitro* and *in vivo* [17,18]. Possible mechanisms of antimicrobial activity include the production of organic acid, hydrogen peroxide, as well as various antibiotics or bacteriocins [19,20] . It has been proposed that lactic acid production by these organisms, unrelated to pH, is responsible for inhibition of *H. pylori* [1,21]. Therefore, probiotic organisms could be exploited as potential therapeutic agents to eradicate *Helicobacter* infection and as adjuncts to current therapy strategies [20,22,23].

Therefore, the aim of the present study was to determine the antagonistic activity of *Lactobacillus* strains isolated from goat's milk against the strains of *H. pylori in vitro* using an agar well diffusion assay and the cause of any inhibitory activity.

Corresponding Author: L. Medouakh, Department of Laboratory of Food and Industrial Microbiology, Biology, Faculty of science, University Oran (Es-senia), 31000 Oran, Algeria Medouakh L.
E-mail: medouakhlinda@yahoo.fr

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions: *H.pylori* clinical strains were isolated from patients with gastroduodenal ulcers in the military hospital Oran, Algeria and stored at -70°C in brain heart infusion broth (BHIB; Oxoid) containing 10% (vol/vol) glycerol [5]. All *H. pylori* strains were cultured under microaerophilic conditions at 37 °C on Colombia base Agar (CBA; Oxoid) supplemented with 5% (vol/vol) defibrinated horse blood for 3 days. The strains were identified on the basis of the colony morphology, Gram negatif staining and positive reaction in biochemical tests (catalase, urease and oxidase) [24,25,26]. *H. pylori* liquid cultures were prepared in brain heart infusion broth (BHIB) supplemented with 10% horse serum under the same conditions in a shaking incubator [16].

Lactobacillus strains used in this study were isolated from the goat's milk and were stored in De Man-Rogosa-Sharpe (MRS) broth at -80°C. These strains were cultivated at 37°C in MRS agar acidified (5.4) and were grown in MRS broth overnight without shaking under a microaerophilic atmosphere. Identification tests of *Lactobacillus* strains have been described previously [27, 28]. Isolates were preliminary identified on the basis of Gram staining, morphology, catalase activity and spore formation. Catalase negative and Gram-positive rods were selected and screened for the production of CO2 from glucose, hydrolysis of arginine, aesculin and citrate and growth at different temperature 15 and 45°C. *Lactobacillus* isolates were further characterized by their carbohydrate fermentation pattern using the API 50 CHL (bio Mérieux, Marcy l'Etoile, France). Tests were performed according to the manufactures instructions. The APILAB PLUS database (bio Mérieux, Sa) was used to interpret the results. The results were recorded after 24h and 48h and interpreted using the APILAB Plus computer-aided identification program (bioMerieux). *Lb. delbrueckii bulgaricus* CNRZ 449 was used as reference strain.

Antagonist Activity: Antagonist activity was evaluated according to the method described by Geis *et al.* [29]. Cultures of *Lactobacillus* strains were grown overnight in MRS medium at the optimum growth conditions. The overnight cultures were spotted (5ul) onto MRS agar medium. The plates were incubated for 24-48 h at 37°C to allow producer colony to develop, then overlaid with 7 ml of soft agar inoculated with 0,1 ml of *H. pylori* at a concentration of 10^7 cfu/ml. the plates were incubated under microaerophilic conditions at 37 h for 72 h. and the diameters of inhibition zones around the wells were measured.

Well Diffusion Assay: *Lactobacillus* supernatants used in the experiments, were obtained by centrifugation twice (17000 g, 10 min) at 4°C of fresh overnight (o/n) *Lactobacillus* cultures and filtered with a 0.22-µm filter. Inhibition of the growth of *H. pylori* by *lactobacillar* culture supernatants as described above was screened as outlined by Wendakoon *et al.* [30]. Briefly, *H. pylori* cultures were spread on CBA agar plates without antibiotics (10^7CFU per plate). The wells were cut into the agar with a sterile Pasteur pipettes and were then filled with 70-µl aliquots of cell-free culture supernatants which were used at their native pH or were adjusted to pH 6.8 with 1 N NaOH. Plates were incubated for 72 h under microaerophilic conditions at 37°C and the diameters of inhibition zones around the wells were measured. MRS broth was used as negative control.

Liquid Culture Assay: In this experiment, culture supernate of *Lactobacillus* was used to examine its effect on the growth of *H.pylori. H. pylori* cells (10^8 CFU/ml) suspended in BHIB in the absence of antibiotics were incubated under microaerophilic conditions at 37°C with or without the addition of *Lactobacillus* supernatant at concentration of 50% [16]. In order to determine if lactic acid produced by *Lactobacillus* strains affects the viability of *H. pylori*, lactic acid (60 mM) was added to cultures of *H. pylori.* The viability of *H. pylori* as a function of time was evaluated by determination of viable CFU (colony counting) on CBA agar plates following incubation at 37°C under microaerophilic conditions.

RESULTS

Identification of Isolates: Clinical strains of *H. pylori* were isolated from antrum biopsy. All strains were Gram negative staining, curved rods, catalase positive, oxydase positive and urease positive. These characteristics confirmed the presence of *H. pylori.* In Parallel, fourteen strains (14 strains) of *Lactobacillus* were isolated from goat 's milk. All isolates were Gram positive rods, catalase negative, oxydase negative and non-spore-forming. The morphological, physiological and biochemical tests revealed that isolates correspond to *Lactobacillus* species (Table 1).

Table 1: Phenotypic characteristics of *Lactobacillus sp.* strains

Species Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
spore-forming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 15°C	-	+	+	-	+	-	+	-	+	+	+	+	+	+	-
Growth at 45°C	+	-	+	+	-	+	-	+	-	+	-	+	-	-	+
Production of CO2 from glucose	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis of															
Arginine	-	-	-	-	-	-		+	-	-	+	-	-	-	-
Aesculin	+	-	-	+	-	-	+	-	-	-	-	-	-	-	+
Citrate	+	-	+	+	-	+	-	-	+	-	-	+	-	-	-

(+): positive reaction; (-): negative reaction

1: *Lb. delbruekii subsp. bulgaricus*, 2: *Lb. pentosus,* 3: *Lb. brevis 1,* 4: *Lb. brevis 2,* 5: *Lb. rahmnosus1,* 6: *Lb. rahmnosus2,* 7: *Lb. rahmnosus3,* 8: *Lb. helveticus 1,* 9: *Lb. helveticus 2* , 10: *Lb. plantarum 1,* 11: *Lb. plantarum2,* 12: *Lb. casei subsp. casei* , 13: *Lb. casei subsp.* 14: *Lb. paracasei subsp. paracasei,* 15 : *Lb. delbruekii subsp. bulgaricus* CNRZ 449

Table 2: Growth inhibition of *H. pylori* by *Lactobacillus* cultures

Strains	HP 1	HP 2	HP 3*Lb.*
delbruekii subsp. bulgaricus CNRZ 449	+	+	+
Lb. delbruekii subsp.			
bulgaricus	+	+	+
Lb. pentosus	-	+	+
Lb. brevis 1	+	++	+
Lb. brevis 2	+	+	-
Lb. rhamnosus 1	+	++	+
Lb. rhamnosus 2	+	+	+
Lb. rhamnosus 3	+	+	+
Lb. helveticus 1	-	+	-
Lb. helveticus 2	+	+	+
Lb. plantarum 1	+	+	++
Lb. plantarum 2	-	+	+
Lb. casei subsp. casei	-	+	+
Lb. casei subsp. rahmnosus	+	+	+
Lb. paracasei subsp. paracasei	+	+	+

+: halo < 3 mm, ++: halo > 3 mm, -: no inhibition halo

Table 3: Growth inhibition of *H. pylori* by *Lactobacillus* supernatants

Strains	Final pH	HP 1	HP 2	HP 3
Lb. delbruekii subsp. bulgaricus CNRZ	4.75	+	+	+
Lb. delbruekii subsp. bulgaricus	3.90	+	++	+
Lb. pentosus	4.27	-	+	+
Lb. brevis 1	4.9	+	++	+
Lb. brevis 2	4.0	+	+	-
Lb. rhamnosus 1	3.9	++	++	+
Lb. rhamnosus 2	3.60	+	+	+
Lb. rhamnosus 3	3,73	+	+	+
Lb. helveticus 1	4,24	-	+	-
Lb. helveticus 2	4,03	+	++	+
Lb. plantarum 1	3,69	+	++	+
Lb. plantarum 2	3,27	-	+	+
Lb. casei subsp. casei	4,5	-	+	+
Lb. casei subsp. rahmnosus	4,02	+	+	+
Lb. paracasei subsp. paracasei	3,86	+	+	+

+: halo < 3 mm, ++: halo > 3 mm, -: no inhibition halo

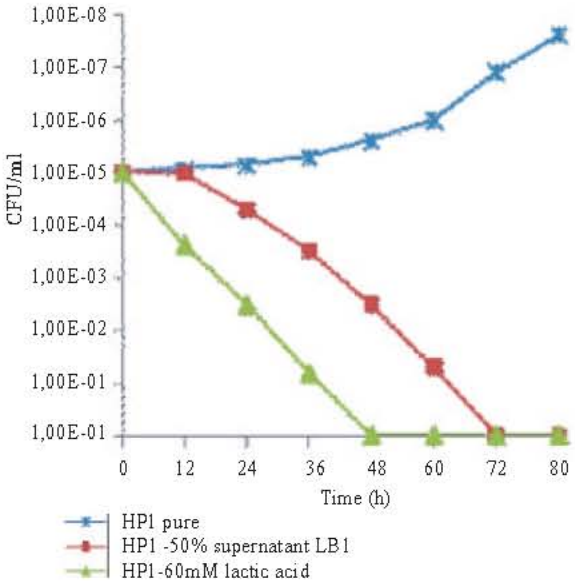

Fig. 1: Growth of H. Pylori (HP1) in pure culture; with supernatant of Lb. *rhomnous* 1 and with lacric acid

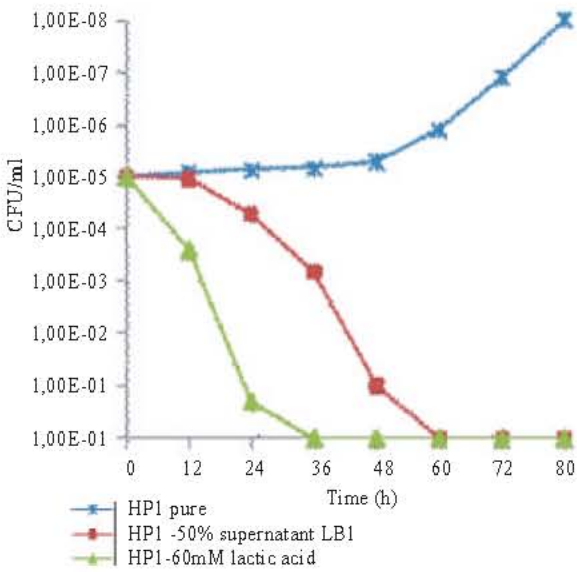

Fig. 2: Growth of H. Pylori (HP2) in pure culture; with supernatant of Lb. *rhomnous* 1 and with lacric acid

Inhibition of Growth of *H. Pylori* by *Lactobacillus* ultures: A well diffusion technique was used to evaluate 15 strains of *Lactobacillus* cultures against *H. pylori* by measuring the diameters of inhibitory zones on plate. All cultures of *Lactobacillus* strains tested were able to inhibit *H. pylori* (Table 2).

Inhibition of Growth of *H. Pylori* by *Lactobacillus* Supernatants: Cultures of *H. pylori* strains were tested against supernatants of *Lactobacillus* sp. which showed anti-Helicobacter activity (Table 3).

All supernants of *Lactobacillus* tested (natural pH) inhibited the growth of *H. pylori* , but the culture supernatant of *Lb. rhamnosus* 1 showed the clearest inhibition zone (average diameter of the zone 10 and 11 mm) against *H.pylori* 1 (HP1), *H. pylori* 2 (HP2) respectively. However, this inhibitory effect was lost when adjusting the pH of culture supernate to 6.8, indicating that it is mediated by the organic acid production. MRS medium used as control did not inhibit the growth of *H. pylori*. Among strain displaying the strongest growth inhibition of *H. pylori* (HP1, HP2) , *Lb. rhamnosus* 1 strain was chosen for further studies.

Viability of *H. Pylori:* A decrease in cell viability from 10^5 to 10^3 CFU/ml was observed after 24h of incubation when *H. pylori* was treated with 50% of the culture supernate of *Lb. rhamnosus*1 (LB1) supernatant (Fig. 1 and 2) and no viable counts were found at 72 h for *H.pylori*1 (HP1) and at 60 h for *H. pylori* 2 (HP2). Thus, in the addition of lactic acid to cultures of *H. pylori*, a dramatic decline in viable counts was showed for *H. pylori* cells and complete inhibition was demonstrated at 48 h in HP 1 and at 36 h in HP2. The strain HP 2 shows most sensitive than HP1against supernate of *Lb. rhamnosus* (LB1) and lactic acids.

DISCUSSION

Recently, attention had been paid to the interaction between *H. pylori* and probiotic *lactobacilli* [16]. *Lactobacillus sp.* strains, as representative of the normal microflora of intestine have been found to have an inhibitory effect on *H. pylori in vitro* and *in vivo* [18, 21]. Tow main substance have been implicated in the inhibitory by *Lactobacillus*: lactic acid and bacteriocins. In this present study, isolation and identification of *H. pylori* strains from gastric biopsy were confirmed according to [25,26,31,32]. In the other hand, the strains of *Lactobacillus sp.* were isolated from goat's milk and their species identification were confirmed by [27,33-35]. The isolation of *Lactobacillus sp.* from goat's milk was comparable with those of others workers [34, 35]. These strains of *Lactobacillus sp.* were selected as a potential probiotic because of its safety profile in humans and its antagonistic properties against different pathogenic

bacteria, including *H. pylori*. The results obtained in our study suggest that there is an antagonistic interaction *in vitro* between clinical *H. pylori* and *Lactobacillus* strains tested. So, cultures of *Lactobacillus* strains were able to inhibit strains of *H. pylori* using the agar diffusion assay. Bhatia *et al.* [36] were the first to observe an antagonistic effect of *Lactobacillus* strains against *H. pylori*. Since, several studies Regarding the antagonistic effect of *Lactobacillus* strains such as *Lb. acidophilus* [1]; *Lb. salivarius* [14]; *Lb. plantarum* [11], *Lb. casei* [16]; *Lb. GG* [37] and *Lb. gasseri* [22] against *H. pylori* have been reported , results that are compared to those presented in our study. However, the inhibitory effect of *Lactobacillus. s*p against *H. pylori* strains was lost after neutralization of supernatants *Lactobacillus* indicating pH-mediated effect. Similar trends were reported for *E. coli*, *Listeria* sp. and *S. aureus* [38]. Similarly, in our assays involving liquid *H. pylori* culture, the addition of *Lactobacillus* supernate (without pH adjustment) decreased the viability of *H. pylori* tested and dramatic decline in cell viability for *H. pylori* was observed when *H. pylori* cultures were treated with lactic acid. The finding presented suggests that lactic acid, major compound of *Lactobacillus* supernatant to be inhibitory for strains of *H. pylori*. Lactic acid is produced in largest amount during the metabolism of carbohydrate *Lactobacillus* and have a important role in decreasing pH, which is related with respect to H+ ions, important in the inhibition of *H. pylori in vitro* [20]. Similarly, several reports have related this inhibitory effect for the production of lactic acid [1,39,40]. These results concurred with those of our study. Also, Bhatia *et al.* [36] proposed that lactic acid product by *Lb. acidophilus* is responsible for the inhibition of *H. pylori*. Midolo *et al.* [20] reported growth inhibition of *H. pylori* by organic and inorganic acids in a concentration dependent manner and showed that lactic acid demonstrated the greatest inhibition. However, other mechanisms of antagonism, such as production of bacteriocins and H_2O_2 might play the role for *H. pylori* inhibition [18,,23]. So, *H. pylori* possed active catalase, which neutralize hydrogen peroxyde. Michetti *et al.* [41] who studied *Lb. acidophilus* concluded that another secreted product *not* determined in addition to lactic acid contributed to the inhibitory effect observed. In Parallel, they have demonstrated that *Lb. acidophilus* culture supernatant was effective *in vitro* and had a partial, long term suppressive effect on humans. The anti-Helicobacter activity of probiotic *Lactobacilli* used in dairy products has been reported in a number of studies *in vitro* and the results of clinical study have been encouraging.

So, *Lactobacillus* could be exploited and used an adjuvant therapeutic agent to eradicate *H. pylori* infection after antibiotic therapy failure.

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