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Low co-existence rates of *Lactobacillus* spp. and *Helicobacter pylori* detected in gastric biopsies from patients with gastrointestinal symptoms

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ABSTRACT

Background: bacterial diversity of the stomach includes various species. Among them, *Helicobacter pylori,* a microorganism which has been associated to gastric diseases, is frequently isolated in this habitat. In addition, *Lactobacillus* spp., a genus including probiotic strains, has also been documented in this habitat. The co-existence of these two species in the stomach of symptomatic patient needs to be elucidated.

Aims: our goal was to establish if *Lactobacillus* spp. and *H. pylori* co-exist in the stomach mucosa of symptomatic patients.

Methods: gastric biopsies (antrum and/or the body) from 427 Chilean patients with gastrointestinal discomfort were analyzed. The *H. pylori* infection and/or *Lactobacillus* spp. colonization status was determined for each patient by standard culture techniques, and statistical correlations between the presence of those species and the age, gender, or the severity of the gastric disease were also established.

Results: only 6.1% of the samples presented co-existence of *Lactobacillus* spp. and *H. pylori*. This former species was isolated in 42.6% of the patients as unique species, while *Lactobacillus* spp. was isolated as single species in 19.4% of the individuals. Chronic non-atrophic gastritis was prevalent in *Lactobacillus* spp. non colonized individuals, while chronic non-atrophic and chronic atrophic gastritis diagnosis was similar in *Lactobacillus* spp. harbouring individuals ($p < 0.001$). The presence of *Lactobacillus* spp. significantly increased with age ($p = 0.005$), independently of gender.

Conclusion: the negative Pearson correlation between *Lactobacillus* spp. and *H. pylori* ($r = -0.112$, $p = 0.020$) indicates that the co-existence of both species is low in human gastric mucosa of symptomatic patients.

Key words: *Lactobacillus. Helicobacter.* Co-existence. Gastric mucosa.

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INTRODUCTION

Human microbiota in both healthy and non-healthy individuals has greatly incited the interest of the medical science research community. Molecular techniques have demonstrated that the bacterial biodiversity of various sites, including stomach, were underestimated by classic microbiological techniques (1,2). Gastric microbiota has been primarily characterized by the culture of gastric biopsies and/or gastric secretion (3,4). Molecular techniques have enabled a more detailed study of this ecosystem, resulting in the detection of 128 different phylotypes, and the identification of species belonging to the *Firmicutes, Proteobacteria, Actynobacteria* and *Fusobacteria phyla* and yeast. Among these, approximately 10% were newly described bacterial species in the human stomach (1,5,6). One member of this microbiota is *H. pylori*, a species considered to be a promoting agent of severe gastric diseases, such as peptic ulcer, mucosa associated lymph tissue (MALT) lymphoma and gastric cancer (7,8).

The genus *Lactobacillus* is one of the main genus containing probiotic strains (9-11) which are acid-resistant (12). Probiotics are live organisms that are orally administrated, usually in addition to conventional antibiotic therapy. They may modulate the human microbiota and promote health, prevent antibiotic side effects, stimulate the immune response and directly compete with pathogenic bacteria (13). They could be present in this acidic gastric environment (14). However, the ability of these species to either

co-exist or to compete in the gastric habitat remains to be established. Possible mechanisms include inhibition of *H. pylori* urease enzyme, disruption of bacterial cell membrane, and modulation of the host immune system (13).

We hypothesize that these two species are, in general, mutually exclusive in the human gastric stomach mucosa based on the antagonism showed *in vitro* by several strains of *Lactobacillus* spp. against *H. pylori*.

Our goal was to determine the presence of both *Lactobacillus* spp. and *H. pylori* in the stomach of Chilean patients that exhibited gastrointestinal symptoms, in order to establish the ability of these two species to co-exist or compete. The correlation between gastric damage, age, gender and the prevalence of these two species in the stomach were also investigated.

METHODS

Samples

This study is part of a larger analysis designed to determine the prevalence of *H. pylori* infection in Chile, the resistance phenotype of the clinical isolates toward first line and second line antibiotics used in eradication therapy, and the search for new anti *H. pylori* active compounds (probiotics and phytopharms).

Patients

Four hundred and twenty-seven patients, children and adults with gastric disorders, recruited between years 2005-2007 were included. Patients were 60% women (256 individuals) and 40% men (171 individuals), and were distributed exclusively according to age in four groups, as follows: group A, patients under age 10 (11 individuals); group B, patients between ages 10-19 (42 individuals); group C, patients between ages 20-64 (237 individuals); and group D: over age 64 (54 individuals). Eighty three patients were not considered in any group because none age data was available. All patients included in this study signed a consent form for tissue organ donor, previously approved by the Ethics Committee of the School of Medicine at the University of Concepción, Chile. Individuals were fasted for at least 12 hours prior to the gastric endoscopic procedure. Vaira et al. (15) criteria for patients exclusion was used which considers at least four months without H2 antagonists or proton pump inhibitors intake to be eligible when serological analysis are included.

Isolation and identification of the species

Two gastric biopsies per patient that were obtained from the antrum or the body of the stomach were homogenized in a mortar and inoculated in Columbia agar supplemented with 5% of horse blood and DENT (Oxoid), at 37 °C under microaerobic atmosphere for 5 days to isolate *H. pylori*. Identification was made using Gram stain, urease and catalase tests. *Lactobacillus* spp. was isolated by pre-incubating 0.1 ml of the above homogenate in 1 ml of MRS broth at 37 °C under 10% CO_2 atmosphere for 2 days, followed by subculture in MRS agar. The isolates were further identified by using Gram stain, catalase and oxidase tests.

Molecular identification of *H. pylori* was carried out using conventional PCR with primer-specific for Hpy-F (5'-TGCGAAGTGGAGCCAATCTT-3') and Hpy-R (5'-GGCCCGTATTCACCGCAACA-3'), that renders a 199 bp PCR product from the 16S rRNA gene (16). *Lactobacillus* spp. was identified using conventional PCR and API 50 CH kit (Biomerieux) with specific primers for Lac-F (5'-GAATCGCTAGTAATCG-3') and Lac-R (5'-GGGTTCCCCCATTCGGA-3'), that renders a 186 bp PCR product from the hypervariable 16S - 23S rRNA region. BLAST sequencing analysis was used to identify the group of *L. rhamnosus, L. casei, L paracasei, L. zeae* and *L. curvatuns* (17). Primers LbG-F (5'-AGAAGAGGACAGTGGAAC-3') and LbG-R (5'-TTACAAACTCTCATGGTGTG-3') that generate a 750 bp PCR product were used to identify *L. brevis, L. casei, L. curvatus, L. fermentum, L. harbinensis, L. hilgardii, L. kefiri, L. kunkeei, L. parabuchneri, L. paracasei, L. plantarum, L. pentosus, L. sakei, L. salivarius, L. sanfranciscensis* as another bacterial group (10).

Histopathological analysis

Samples for histopathology were processed, as described elsewhere, using the Sydney classification (18). Only those samples with consensus for the histopathological diagnosis were considered in this study.

Statistical analysis

The results were analyzed by Chi-square test, with confidence intervals of 95%, using the Statistical Package for Social Science software (SPSS), version 10.0.

RESULTS

The lactobacilli strains isolated from the gastric biopsies were presumptively identified as belonging to the genus *Lactobacillus* by classical microbiological methods, and were further confirmed as belonging to one of the two lactobacilli groups described in *Material and methods*, by using conventional PCR. In addition, the first 21 isolated lactobacilli strains were also identified at the species level by using the API 50 CHL kit. Ten strains were identified as *L. fermentum*, three strains were identified as *L. rhamnosus*, three strains were identified as *L. salivarius*, one strain was identified as *L. paracasei*, and one strain was

identified as *L. plantarum*. On the other hand, three strains were not possible to be typified with this kit.

Among the 427 patients analysed, *H. pylori* was isolated from 42.6% while *Lactobacillus* spp. was isolated in 19.4% of individuals. Thirty six and a half percent of the total individuals presented only *H. pylori* in their gastric mucosa meanwhile 13.4% showed *Lactobacillus* spp. as single species. Interestingly, both species were found simultaneously in 6.1% of the patients (26 individuals), and 188 individuals were free of both *H. pylori* and *Lactobacillus* spp. (44% of patients). A negative correlation (r = -0.112) between these two microorganisms was detected, because 156 patients out of 182 were infected with *H. pylori* but were free of *Lactobacillus* spp. (85.7% of individuals). On the other hand, 57 of 83 patients colonized by *Lactobacillus* spp. were free of *H. pylori* (68.7% of individuals).

Histological diagnosis showed that 2.8% patients (12/427 individuals) had a normal gastric mucosa, 72.8% of patients presented chronic non-atrophic gastritis (Cnon-AG) (311/427 individuals), 23.7% had chronic atrophic gastritis (CAG) (101/427 individuals) and 0.7% had gastric carcinoma (3/427 individuals). *H pylori* infected and non infected individuals ratio was similar in both Cnon-AG patients –ratio 0.77 (135/176 individuals)– and CAG –ratio 0.8 (45/56 individuals)– (Fig. 1A). None of the individuals with normal mucosa were infected by *H. pylori* while this species was isolated from the gastric mucosa of two cancer patients (2/3 individuals). Similar analysis considering *Lactobacillus* spp. colonized patients as reference, showed that the colonized and non-colonized patients ratio was 0.17 (46/265 individuals) in Cnon-AG patients and 0.51 (34/67 individuals) in CAG patients (Fig. 1B). On the other hand, two of 12 patients with normal mucosa were colonized by *Lactobacillus* spp. and this species was also isolated from the mucosa of one of the tree cancer patients.

A statistically significant correlation was observed between Cnon-AG or CAG patients and the presence of *H. pylori* ($p = 0.02$) or *Lactobacillus* spp. ($p < 0.001$) in their gastric mucosa. Thus, *H. pylori* was isolated in similar proportion from the gastric mucosa of individuals with diagnosis of both Cnon-AG and CAG, but *Lactobacillus* spp. was isolated mainly from CAG patients ($p < 0.001$) (Fig. 1C).

Age of individuals also showed a correlation with the severity of gastric lesion and the status of *Lactobacillus* spp. colonization (Fig. 2). None of the individuals under age 10 presented CAG or gastric cancer, but an increasing number of CAG patients was observed in group B (2.4% of individuals), group C (26.2% of individuals), and group D (38.9% of individuals). On the other hand, gastric cancer patients were only detected in 2/237 patients of group C (0.84% of individuals) and 1/54 patients of group D (1.9% of individuals). Patients under 10 years old were not colonized by *Lactobacillus* spp. (Fig. 2A), but a statistically significant increase in *Lactobacillus* spp. colonization among individuals included in all the other groups was observed ($p = 0.005$), with prevalence of 4.8% in group B (Fig. 2B), 19.4% in group C (Fig. 2C), and 31.5% in group D (Fig. 2D) was observed. Prevalence of infection with *H. pylori* also showed positive correlation with age, from 18.2% in patients under 10 years old to 48.5% in patients belonging to group C ($p = 0.017$), but this correlation was lost in elderly patients (over 64 years old) were a prevalence of 29.6% was detected.

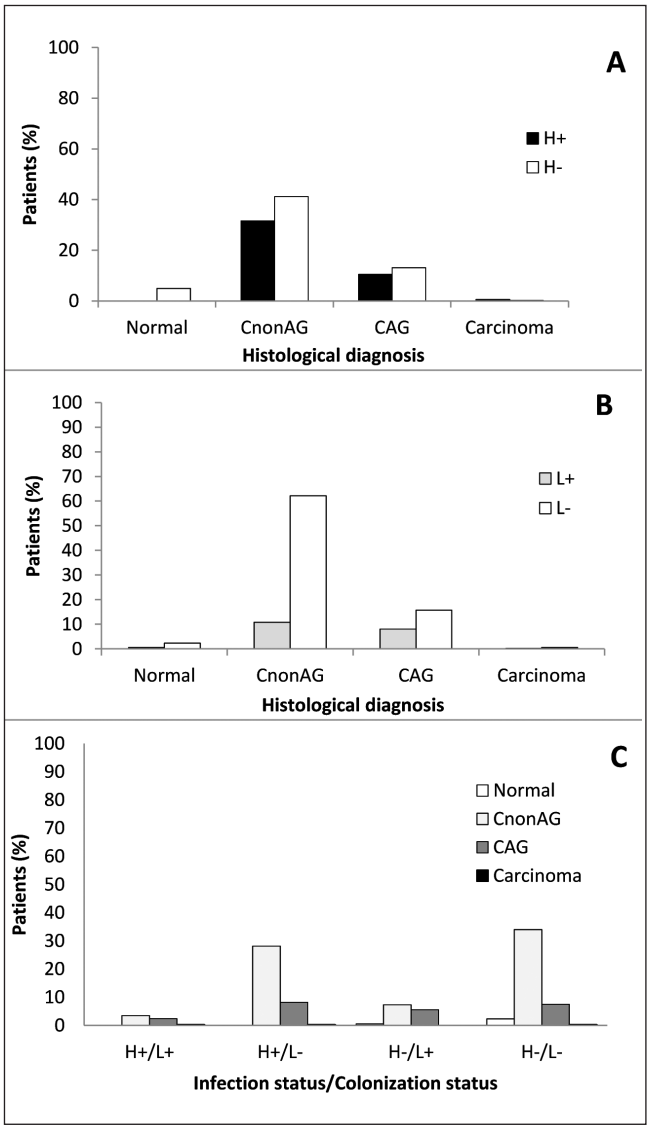

Fig. 1. *Relationship between Helicobacter pylori or Lactobacillus* spp. *status and histological diagnosis.* A. *Helicobacter pylori* infection status and histological diagnosis. B. *Lactobacillus* spp. colonization status and histological diagnosis. C. Presence of both *Helicobacter pylori* and/or *Lactobacillus* spp. and histological diagnosis (H: *H. pylori,* L: *Lactobacillus* spp.; CAG: chronic atrophic gastritis; and Cnon-AG: chronic non-atrophic gastritis).

Finally, *H. pylori* infection or *Lactobacillus* spp. colonization status showed no significant differences with gender because *H. pylori* was isolated from 41% of women (105/256 individuals) and 45% of men (77/171 individuals), meanwhile *Lactobacillus* spp. was isolated from 20.3% of women (52/256 individuals) and 18.1% of men (31/171 individuals).

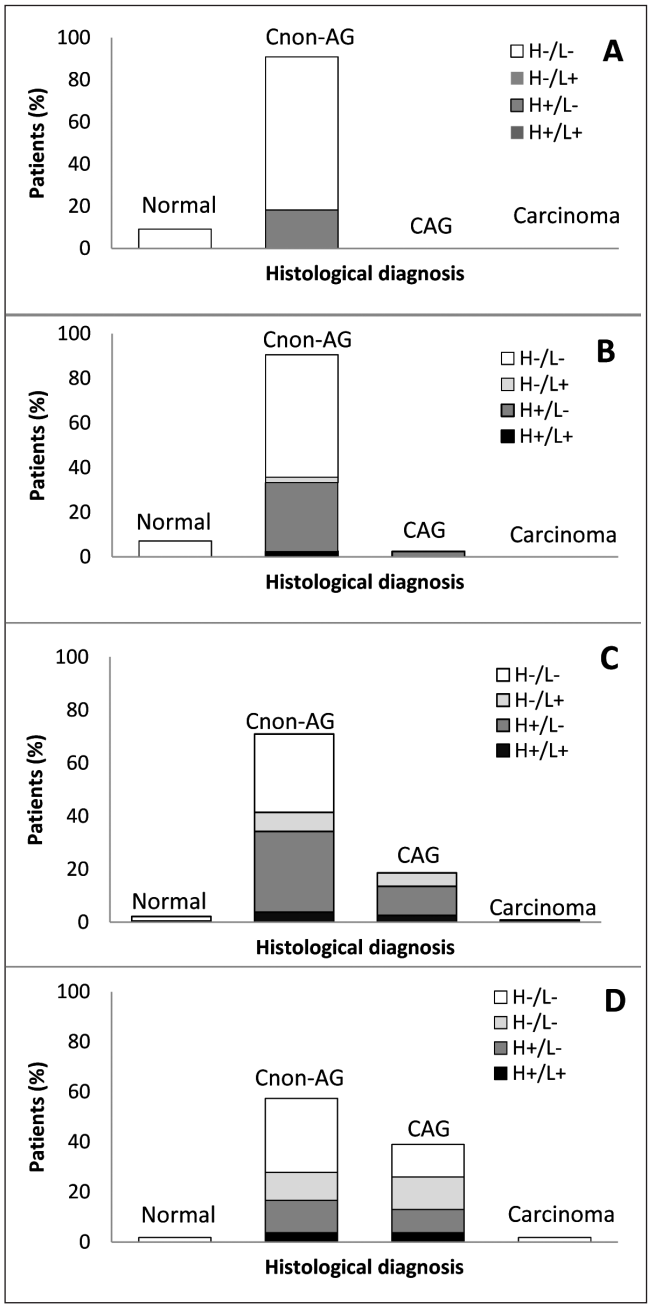

Fig. 2. *Relationship between Helicobacter pylori or Lactobacillus* spp. *status and age.* Patients were distributed in four age groups (A-D), and investigated for the presence of both bacterial species: A. Patients under age 10. B. Patients between ages 10-19; C. Patients between ages 20-64; D. Patients over age 64 (H: *H pylori;* L: *Lactobacillus* spp.; CAG: chronic atrophic gastritis; CnonAG: chronic non-atrophic gastritis).

DISCUSSION

Risk factors allowing the infection of humans by *H. pylori* and persistence are still under investigation, because new evidence is needed to better understand the pathogenesis of disease's development due to this microorganism. One of the factors that have attracted the attention to date in this theme is the role of co-colonization of the stomach by other bacterial species in gastric mucosa damage progress or in injury avoidance. Specifically, the role of *Lactobacillus* in the stomach of symptomatic individuals was addressed in this work to understand if this bacterial genus affects the evolution of *H. pylori* associated diseases.

Ten strains out of the first 21 strains analyzed by API50 CHL kit were identified as *L. fermentum*, and this species has been isolated with increased frequency from adenocarcinoma (5/9 patients) and benign gastric ulcer (5/8 patients) with an overall frequency of 44.8% (13/29 patients) (19). Our results are consistent with this finding because 47.6% (10/21 individuals) of patients were colonized by *L. fermentum* as the main species.

Lactobacillus spp. was isolated as single bacterial species in 10% of the patients with Cnon-AG (31/311 patients), and in 23.8% of CAG patients (24/101 patients). These results suggest that *Lactobacillus* spp. colonise the gastric mucosa of individuals previously injured by various conditions, including gastric cancer (2). The alkaline pH of the stomach in these patients or an increased exposure of the epithelial cells to the microorganisms due to mucosa damage could favour the growth of *Lactobacillus* spp. Although several studies indicate that *Lactobacillus* spp. colonisation diminish gastric inflammation, promote mucin regeneration and down-regulate several genes of the *cag* pathogenicity island (20,21), one study (19) speculates that *Lactobacillus* spp. might play a role in malignancy as they can be isolated from the gastric mucosa of patients with adenocarcinoma.

Medical records detailing the gastric history of patients participating in our study were not available. However, participants were considered to be long term infected by *H. pylori* because Chilean population acquire bacterial infections during early childhood (22), and over 70% of the adult population with gastric disorders harbors *H. pylori* as detected by serological analysis in a large population study (2,615 individuals) (23). Our results are based on culturing recoverable bacteria, which is the recommended test when antibiotic resistant pattern of clinical isolates should be determined, even though this method is less sensitive for detecting infected individuals than serology (24). The 42.6% of prevalence informed by us is similar with the results published by Otth et al. (25) for a Chilean cohort (41.3%). Thus, short-term infection among Chilean patients is rather scarce. Furthermore, *H. pylori* infection may persist for many decades prior to the outbreak of gastric disturbance (26), and subjects with decreased acid output usually show also atrophy in their gastric epithelium, with progression to multifocal metaplasia (27). Whilst these patients fail to present specific symptoms, their risk for gastric cancer development is increased 5- to 90-fold, depending upon the extension and severity of atrophy (27).

Moderate to severe inflammation found by histopathological studies of gastric biopsies suggests infection by type I strains of *H. pylori* (28). Nonetheless, previous results published by our research group (29) indicate that

some strains of type II *H. pylori* (genotype *s2m2*) may harbour specific type of LPS that induce pro-inflammatory cytokines in mice model, suggesting also a potential role of these strains in human gastric inflammation. Chilean clinical isolates of *H. pylori* are usually *cagA* positive, *vacAs1* and *vacAm1* (30), therefore, type I strains of *H pylori* may be partially responsible for the histopathological finding among Chilean patients. However, we cannot rule out the possibility that simultaneous infection with more than one genotype of *H. pylori* could play a role in damage severity, because it has been seen that a fraction of Chilean population is infected by two or more *H. pylori* genotype (31).

Interestingly, our results indicate that *H. pylori* and *Lactobacillus* spp. do not co-exist in the gastric mucosa ($p = 0.02$), suggesting a mutually exclusive behaviour of these two bacterial species for colonizing the stomach mucosa. Moreover, colonization may be attributed to chronic inflammation of the stomach due to *H. pylori* infection because a more suitable environment for *Lactobacillus* spp. development became available after long term infection as proposed by others (2,32). However, Ryan et al. (33), searching for the probiotic activity of *Lactobacillus* spp., did not find correlation between *Lactobacillus* spp. colonization and gender, age, *H. pylori* infection, or pathology. However, their result can be attributed to the reduced study group which was analysed (12 patients). Our results suggest that age is important for *Lactobacillus* spp. colonization of the stomach, with a concomitant decrease of *H. pylori* infection prevalence in those individuals over age 65. The results presented are based in larger group of individuals (427 patients), thus, the 19.4% of prevalence detected should be used as the reference value for *Lactobacillus* colonization of Chilean symptomatic individuals.

The newly described species of *Lactobacillus*, *L. gastricus* and *L. antri* in the subgroup *L. reuteri*, defined as members of the stomach resident microbiota, and which do not grow in MRS agar (14), increase the uncertainty of culture methods as searching tool for this gender in clinical samples. Thus, combined methods like PCR and conventional microbiological techniques should be used in future analysis to establish with more certainty an exclusive model of stomach colonisation for these two species. Nonetheless, the negative correlation observed should be maintained because cultures usually resemble the relative abundance of viable cells for *Lactobacillus* spp. and *H. pylori* in biological samples, especially if more than one biopsy samples per individual are used for searching of *H. pylori* and *Lactobacillus* spp. (34). More data are needed to confirm that long-term *H. pylori* infection is one of the main factors allowing *Lactobacillus* spp. colonization.

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