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Antibacterial Activity of Lactic Acid Bacteria against *Helicobacter pylori* Evidence by *in vivo* and *in vitro* Studies

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A B S T R A C T

Comparative studies on some probiotic potential of two selected strains of *Lactobacillus* were carried out during this work. Eight isolates of lactic acid bacteria were tested; the most effective isolates (LAB11 and LAB13) against *Helicobacter pylori* were selected for further probiotic properties and *in vivo*. The histological features of gastritis and the presence of *H. pylori* were also examined. The isolates LAB13 andLAB11 showed highly antibacterial activity which indicated by the largest zone diameter (50.0 and 49.0 mm, respectively). Based on tested probiotic properties the results of acid and bile salt tolerance showed a survival of both isolates with a significant ($p<0.001$) resistant for LAB13 more than LAB11. The gastric mucosa sections showed that rats which treated with probiotic isolate; LAB11 or LAB13 daily for a week before infection by *H. pylori*, and then infected rats treated with the same isolate (LAB11) for another 6 weeks (Group 3 and 5, respectively) and rats infected first by *H. pylori* and after 4 weeks of infection treated with LAB11 for 6 weeks (Group 4) showed negative *H. pylori* colonization. So it is recommended using dairy products containing probiotic bacteria as daily protective routine to eradicate growth or colonization of *H. pylori*.

Introduction

Helicobacter pylori is an important gastroduodenal pathogen of humans (Larsen *et al.*, 2013) and its infection is a growing public health problem with a prevalence rate of approximately 50%, especially in the developing countries (Eshraghian, 2014; Nagy *et al.*, 2016; Roberts, 2016). The infection by *H. pylori* when acquired in childhood and it will persist through life if a successful antimicrobial regimen is not performed (Mcnulty *et al.*, 2012; Arslan *et al.*, 2017). In spite of most infected individuals are asymptomatic; *H. pylori* infection is responsible for the development of chronic gastritis, functional dyspepsia, and gastric or duodenal ulcer (Smith *et al.*, 2014). Although eradication therapies with antibiotics are presently available; but side effects and an increase for antibiotic resistance of *H. pylori* have been reported (Gerrits *et al.*, 2006; Cheng *et al.*, 2015).

Probiotics include several microorganisms, mostly within the genus of *Lactobacillus*;

which can be defined as living microorganisms, which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition (FAO/WHO, 2001).

Lactic acid bacteria have the potential and ability to produce several important metabolites such as organic acids, probiotic properties and antimicrobial substances such bacteriocins, which attracted the attention of many researchers in recent years (Holzapfel *et al.,* 2001; Khan *et al.,* 2010). It is highly competitive largely due to their applications in the production of fermented food. Also they produce antimicrobial substances including bacteriocins that have ability to inhibit pathogenic and food spoilage bacteria. These compounds have shown to exert specific antagonistic properties against Gram-negative and Gram-positive pathogens.

Bacteriocins known as a kind of ribosomal synthesized and extra cellularly released antimicrobial peptides which become one of the weapons against microorganisms due to the specific characteristics of large diversity of structure and function, being stable to heat and it is natural resource (O'Sullivan *et al.,* 2002; Cotter *et al.,* 2005; Deegan *et al.,* 2006; Rattanachaikunsopon and Phumkhachorn, 2010; Yang *et al.,* 2014).

The beneficial effects of probiotics on gastrointestinal diseases have been widely described (Behnsen *et al.,* 2013; Sarowska *et al.,* 2013). Effects for the use of lactobacilli as additives in both human and animal diets were carried out (Hummel *et al.,* 2007). In recent years, the probiotic activity of lactic acid bacteria has been emphasized. A number of health benefits have been claimed for probiotic bacteria and using it as a preventive approach to maintain the balance of intestinal microflora is also being recommended (Shah, 2007). It has beneficial effects on humans stabilization of intestinal microflora through preventing colonization of entero-pathogenic bacteria by adhesion to the intestinal wall and competition for nutrients (Denev, 2006) and also it stimulate the immune system (Isolauri *et al.,* 2001).

Probiotic microorganisms should express high tolerance to acid and bile and ability to adhere to intestinal surfaces to survive in and colonise in gastro-intestinal tract. Thus, due to the gastric localization of *H. pylori* colonization and its relationships with gastric diseases; it is not surprising that several studies were carried out on the effects of probiotics on *H. pylori*. Numerous in vitro studies, demonstrating bacterial killing or inhibition, were followed by preclinical and clinical studies (Wilhelm *et al.,* 2011; Patel *et al.,* 2014). Moreover, probiotics have the potential to diminish side effects of antibiotics, increase the *H. pylori* eradication rate, and decrease host cell damage (Lesbros-Pantoflickova *et al.,* 2007). So, aims of this work were to study the antibacterial activity of some Lactic acid bacteria isolates; LAB11 and LAB13 against *H. pylori* (in vitro) and select the most promising isolates to antagonize *H. pylori* in vivo study on rats.

Materials and Methods

Activation of LAB isolates

Ten isolates were used in this study; the isolates were obtained from the culture collection of Agricultural microbiology department, Faculty of Agriculture, Fayoum University, Egypt. From these isolates; LAB11 and LAB13 were previously identified as *Lactobacillus casei* (Accession No. HQ177095) and *Lactobacillus paracasei* (Accession No.HQ177096.1), respectively (Elbanna *et al.,* 2010; Khider and Elbanna, 2017; Elbanna *et al.,* 2017). The isolates were activated in 10 % (w/v) sterilized skim milk at 37°C for 48h.

H. pylori and culture conditions

H. pylori was obtained from Naval American Medical Research Unit 3, NAMRU-3) and activated in Columbia blood broth medium, then incubated for 48 h at 37°C under microaerophilic conditions in jars with the AnaeroGen Gas Packs (Oxoid, Basingstoke, UK).

Preparation of cell-free culture supernatants (CFCSs)

The cell-free culture supernatants of all LAB isolates were concentrated till 5% by using vacuum rotary evaporator at 40°C then used for further experiment. It kept at 4°C till determination of the antibacterial activity (Eied, 2008).

Determination of the antibacterial activity of CFCSs

The antibacterial activity of CFCSs was qualitatively tested by agar well diffusion method (Wolf and Gibbons, 1996). The clear zone diameters around each well were measured in mm.

Some probiotic properties of selected lactic acid bacteria

Determination of acid and bile salt tolerance

The acid and bile salt tolerance of the strains was evaluated with the same method (Archer and Halami, 2015).To determine the acid tolerance; MRS broth was prepared at different pH values (pH 2, 3 and 4), then inoculated with 5% of fresh culture from both LAB11 and LAB13, in separate.

Aliquots from inoculated MRS broth for each isolate were taken at intervals of 0, 1, 2 and 4 h; serially diluted and plated onto MRS agar plates. After incubating anaerobically at 37 °C for 48 h, the number of viable colonies was counted.

Bile tolerance was determined using MRS broth supplemented with different concentrations of bile salts (0.3, 0.5 and 1% w/v), which then inoculated with freshly prepared LAB strains, samples taken at zero time were used as a control. Bacterial growth was monitored as described in acid tolerance experiment.

In vivo H. pylori inhibition by lactic acid bacteria

Experimental animal model and study design

Sixty four Albino male rats same age of Sprague Dawley strain weighted 150 ± 20 gm were purchased from Food Technology Research Institute, Agricultural Research Center, Giza and housed in well aerated cages under hygienic condition. The rats were fed on basal diet for one week in the animal house of the previous research center for adaptation. In this study the rats were inoculated by gavage with 1 mL *H. pylori* suspension (5×10^8 - 5×10^{10} CFU/mL) twice daily at an interval of 4 h for three consecutive days.

The lactic acid strains were used for preparinga fermented milk samples through inoculating heat treated skim milk withLAB11orLAB13 at 37 °C for 12 h until a viable count 10^9 - 10^{10} cfu/mL was achieved. The fermented milk was prepared every 7 days and stored at 4°C until use. A total of 64 rats were randomly assigned to the following eight groups ($n = 8$) as shown in Figure 1; Group 1: control negative (rats with noinfection) and Group 2: control positive (rats infected by *H. pylori*). Group 3: rats treated with probiotic isolate; LAB11 as a daily supplement in the animals' stomach for

a week before infection by *H. pylori*, and then infected rats treated with the same isolate (LAB11) for another 6 weeks, Group 4: rats infected first by *H. pylori* and after 4 weeks of infection treated with LAB11 for 6 weeks and Group 5: rats infected first by *H. pylori* and after 6 weeks of infection treated with LAB11 for 6 weeks. The same for the next groups, Group 6: Rats treated with the other probiotic isolate; LAB13 as a daily supplement in the animals' stomach for a week before infection by *H. pylori*, after infection the rats treated with the same strain for another 6 weeks, Group 7: rats infected first by *H. pylori* and after 4 weeks of infection; treated with LAB13 for 6 weeks and Group 8: rats infected first by *H. pylori* and after 6 weeks of infection treated with LAB13 for 6 weeks.

At the end of each experiment, rats will be anesthetized and killed according to committee on animal research and ethics. The entire stomach and duodenum of the rats in each group will be dissected and examined for histological features of gastritis and the presence of *H. pylori* by Haemotoxylin and Eosin (H&E) and Geimsa stain (Werawatganon, 2014).

Statistical analysis

Data were analyzed using General Linear Models (GLM) procedure of SPSS software (version 17.0.0). Duncan's multiple range tests was used to compare between the means (SPSS, 2008).

Results and Discussion

The antibacterial activity of CFCSs against *H. pylori*

All tested isolates against *H. pylori*showed obvious clear inhibition zone with a significant differences ($p<0.001$) between them (Fig. 2). Among these isolates, Both LAB13 and LAB11 showed the larger inhibition zone diameter (49.7 and 49.0 mm, respectively) followed by LAB105 (48.3 mm) with no significant differences in between. It is noticed that there is no significant differences in diameter of the clear zone between the further tested isolates; LAB5, LAB25 and LAB100, where the diameters of the clear zone were 45.7, 45.7 and 46.0 mm, respectively. Moreover, LAB107 showed the lowest inhibition zone diameter against *H. pylori*, which was 42.7 mm.

Probiotics had an in vitro inhibitory effect on *H. pylori* so, it could present a low-cost alternative solution to prevent or decrease *H. pylori* colonization (Lesbros-Pantoflickova *et al.,* 2007). Cats *et al.,* (2003) found that culture and supernatants from *L. casei* grown in MRS medium significantly induced clear inhibition zone of *H. pylori*. Also, another study found that use of *L. paracasei* strain 06TCa19 may prevent *H. pylori* associated gastric inflammation (Takeda *et al.,* 2017).

Some probiotic properties of selected LAB isolates

Determination of acid and bile salt tolerance

Based on the results obtained (Fig. 3); the survival of isolate LAB13 at zero time significantly ($p<0.001$) achieving higher viability with 2.71×10^6cfu/ml followed by the same isolate at 1 and 2 h. (2.31 and 2.27×10^6cfu/ml, respectively). The samples show less viable probiotic cells at pH 2 after exposure for 1-4 h, which was less than the minimum requirement of viable probiotic cell (10^6/ ml). From these results LAB13 isolate showed more resistant at low pH than isolate LAB 11 and the most suitable one for high growth was pH 4. The rate of bacterial survival as supplemented with bile salt was similar to the trend in acid tolerance test, with

higher inhibition of growth seen as the bile concentrations increased for both LAB11 and LAB13 isolates. Main effect of LAB isolates and different bile salt concentrations were shown in Figure 4. Media without bile salt acted as control for all experiments and it recorded the highest growth, significantly, ($p<0.001$).

In general for both tested isolates, there is a gradual decline in viable count as the bile concentrations increased. Moreover, LAB13 was significantly more resistant to bile salts than LAB11 isolate with a viability of 4.08 and 3.17×10^6cfu/ml, respectively.

Probiotic bacteria must be acid and bile tolerant to survive in the human gastrointestinal tract and survive in the acidic pH of gastric juice to reach the small intestine and colonize, thereby imparting their benefits. The pH of gastric acid is 1.5 to 3.5 so; acid tolerance is accepted as one of the desirable properties used to select potentially probiotic strains (Marieb and Hoehn, 2010). Aciduric members such as *L. acidophilus* generally could not survive in low pH environment as these cells were proven to be vulnerable at pH 2.0 or below. It is thought that environments with low pH inhibit the metabolism activity and growth of lactobacilli, thus reducing the probiotics' viability (Sultana *et al.*, 2000; Chan and Zhang, 2005; Sahadeva *et al.*, 2011). Another study confirmed that the viability count of the bacteria declined tremendously when exposed to simulated gastric juice of pH 1.5 after an incubation period of 3 hours (Mandal *et al.*, 2006). The threshold point to determine acid resistance was set at pH value of 3.0 and incubation period of 3 as it simulates the residence time in the stomach (Haddadin *et al.*, 2004). This is in accordance with findings from Liong and Shah (2005) which stated that resistance at pH 3 is set as standards for acid tolerance of probiotic culture. Therefore, result in Figure 3 indicates the strong inhibition on the viable bacteria numbers at pH 2 was well supported.

Fig.1 In vivo *H. pylori* inhibition by LAB (Animal model)

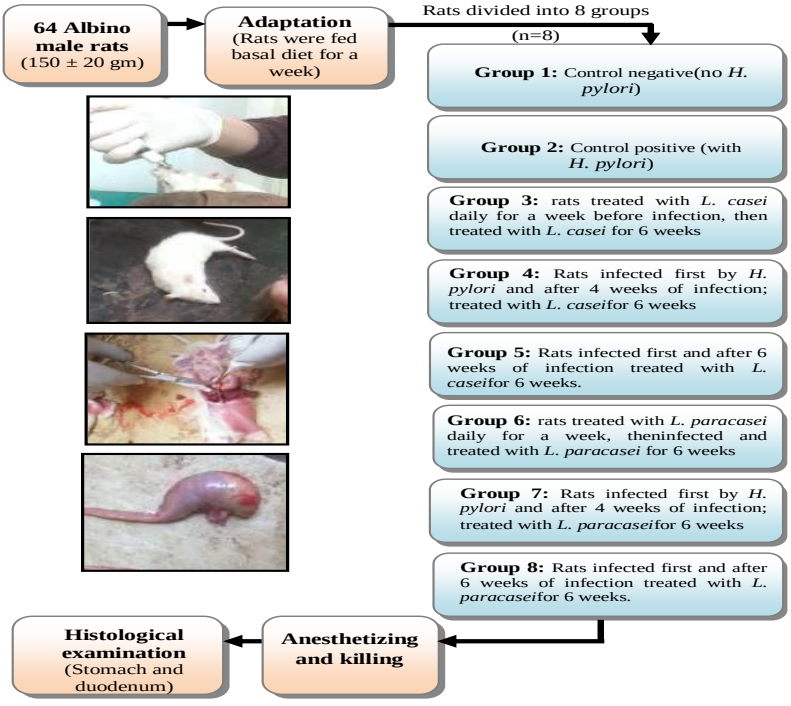


Fig.2 Effects of the cell-free culture supernatants (CFCSs) of 8 strains of LAB on inhibition zone diameter of *H. pylori*

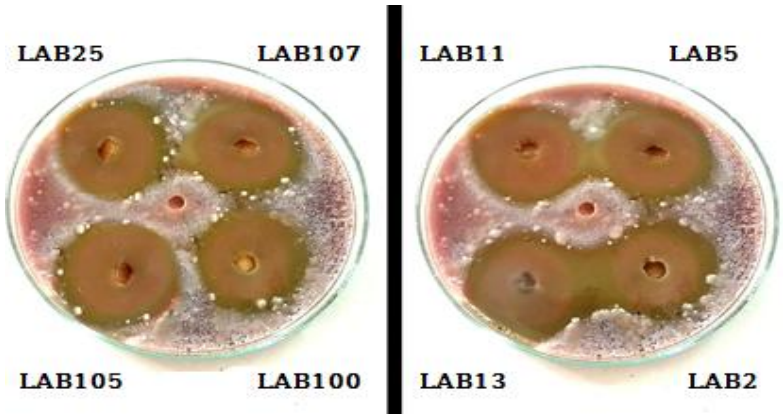
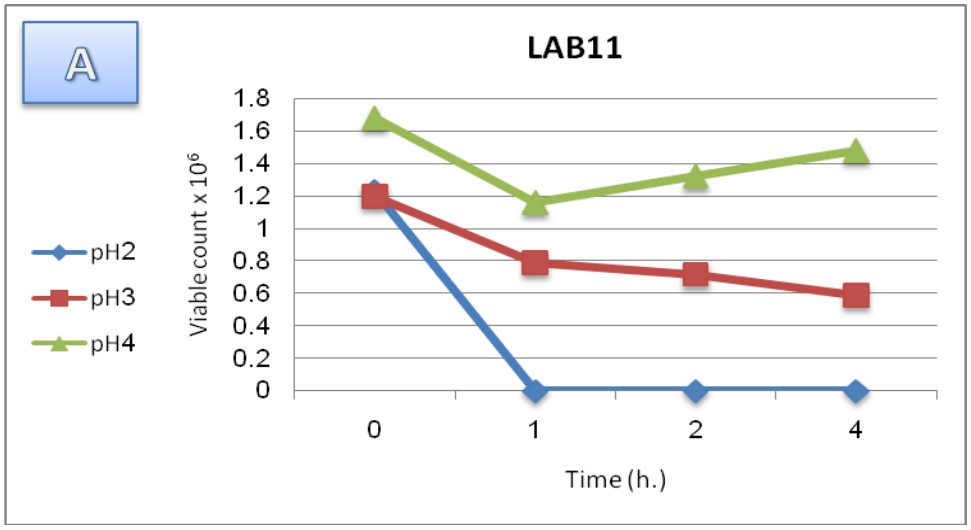


Fig.3 Variations of selected LAB isolates (CFU/mL) at different pH values

A: LAB 11 B: LAB 13



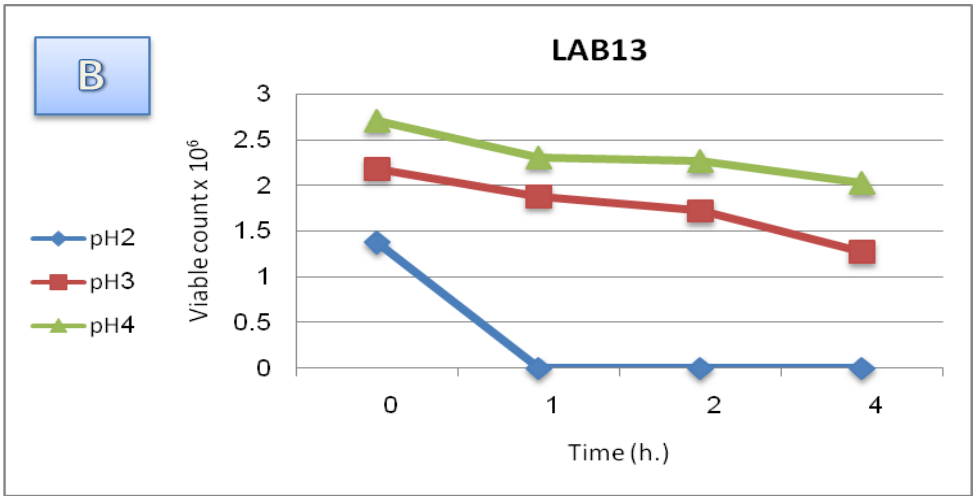


Fig.4 Variations of selected LAB isolates (CFU/mL) at different bile salts
A: LAB 11 B: LAB 13

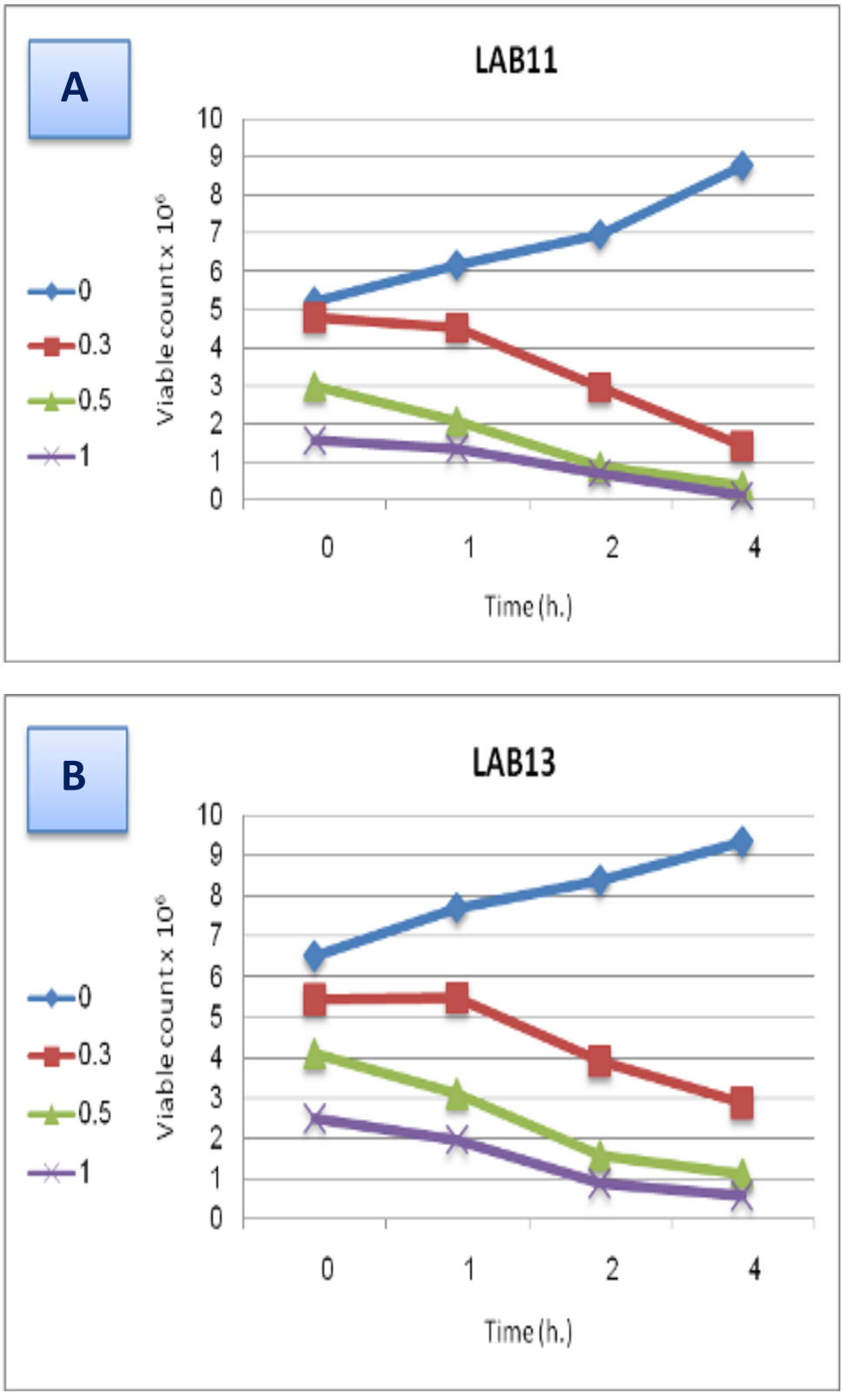
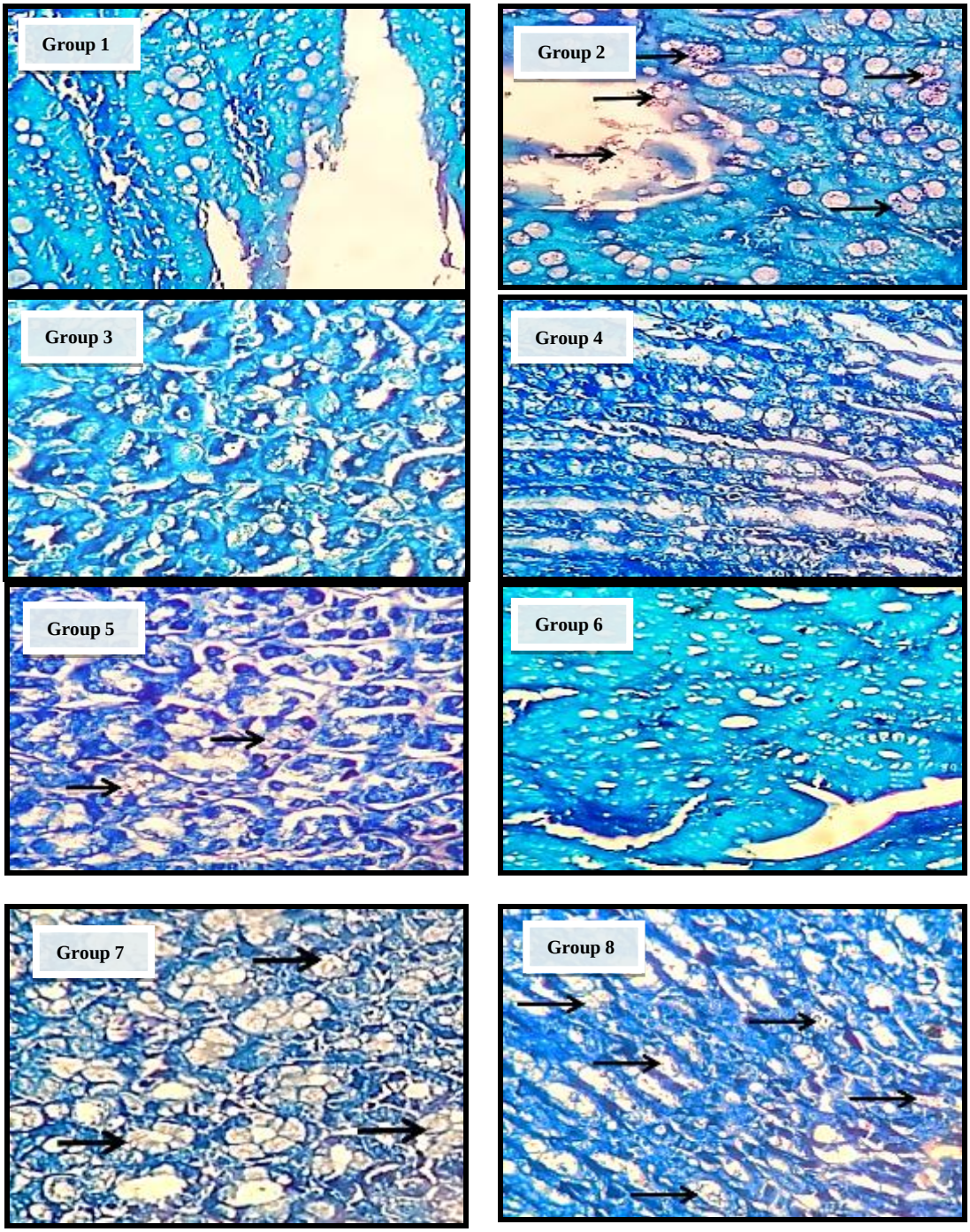


Fig.5 (x40): Histological examination in rats' gastric mucosa show the antibacterial effect of LAB isolates (LAB11 and LAB 13) on *H. pylori*. Group 1: Control negative. Sections show regular gastric glands with negative *H. Pylori* colonization. Group 2: Control positive. Sections show regular gastric glands with moderate *H. Pylori* colonization (arrows pointed). Group 3, 4 and 6: Sections show regular gastric glands with negative *H. Pylori* colonization. Group 7: Sections show regular gastric glands with low *H. Pylori* colonization (pointed with arrows). Groups 5 and 8: Sections show regular gastric glands with low to moderate *H. Pylori* colonization (arrows pointed).



On the other hand, there was a study reported that bile salt did not inhibit the growth of the bacteria completely as even when subjected to a concentration of 2% (Sahadeva *et al.,* 2011). The protective effect of food matrix also may prevent the bacteria from bile exposure and hence, giving rises to the increased bile resistance of the strains (Begley *et al.,* 2005). Suskovic *et al.,* (2001) examined the survival and morphological changes of a potential probiotic strain, *L. acidophilus* M92, in the presence of bile salts and proved that bile salt hydrolyzes was active when tested on lactobacilli and showed a relatively strong resistance to bile salts. According to the previous obtained results of acid and bile salt tolerance for both tested lactic acid bacterial isolates (LAB11 and LAB 13) it is concluded that these isolates have probiotic properties.

In vivo *H. pylori* inhibition by lactic acid bacteria as indicated by histological examination

Data in Figure 5, illustrates the histological examination of gastritis and the presence of *H. pylori* by H&E and Giemsa staining of stomach and duodenum samples of the rats in each group. Results showed that the examination of group 1 recorded no *H. pylori* colonization as shown with Giemsa stained sections while in group 2 (positive control) with no probiotic treatment; *H. pylori* were present in a moderate to severe infestation. On the other hand, groups 3, 4 and 6 showed no *H. pylori* colonization in the gastric lumen. The severity of *H. pylori* colonization in groups 5 and 8 ranged from low to moderate. Moreover, in all members of group 7; *H. pylori* were described with low colonization.

Many studies dealing with either animal models or humans found that the most frequently used strains to assay their effect on *H. pyroli* infection were *L. johnsonii* La1 and *L. rhamnosus* GG, either in a fermented milk preparation containing live bacteria or as a cell-free culture supernatant followed by other probiotics such as *L. casei, L. acidophilus, Lactobacillus brevis, L. gasseri OLL2716, L. reuteri, B. lactis, B. animalis* and *B. breve* (Felley *et al.,* 2001; Gotteland and Cruchet, 2003; Myllyluoma *et al.,* 2007). Various probiotics have shown favorable effects in animal models of *H. pylori* infection. In a mice model of infection, a probiotic combination containing *L. acidophilus* R0052 and *L. rhamnosus* R0011 was found to reduce the effects of *H. pylori* infection through reducing *H. pylori* colonization and alleviating *H. pylori*-induced inflammation of the stomach (Johnson-Henry *et al.,* 2004). Another studies in a mice model demonstrated that *L. casei* strain Shirota and *L. johnsonii* La1, both administered in drinking water, attenuated *H. pylori* infection-induced gastric mucosa inflammation. However, only *L. casei* strain Shirota was able to down regulate the colonization of *H. pylori* to gastric mucosa (Sgouras *et al.,* 2004 and 2005). Also, *L. gasseri* was found to decrease colonization of clarithromycin-resistant *H. pylori* (Ushiyama *et al.,* 2003).

In the gastric mucosa, *H. pylori* possibly interact with epithelial cells through secretory components or as a result of adherence. There are several possible mechanisms by which probiotic bacteria can inhibit the adhesion of *H. pylori* (Lesbros-Pantoflickova *et al.,* 2007). Certain lactobacilli such as *L. johnsonii* La1 (Michetti *et al.,* 1999) or *L. acidophilus* LB (Coconnier *et al.,* 1998) can exert their anti-adhesion activity by secreting antimicrobial substances. In addition, strain such as *L. reuteri* (Mukai *et al.,* 2002) can inhibit *H. pylori* growth by competing with adhesion sites. However, a nonspecific rather than a specific blockage of receptor sites is the most likely mechanism because lactobacilli can inhibit adhesion of large varieties of pathogenic bacteria, although each adheres to

its particular receptor on the cells (Bernet *et al.*, 1994). Animal studies demonstrated that previous colonization by probiotics prevented or reduced *H. pylori* infection in germfree mice (Johnson-Henry *et al.*, 2004). Thus, regardless of the mechanisms involved in the inhibition of the adherence of *H. pylori* to epithelial cells, probiotics could prevent *H. pylori* colonization of the gastric mucosa by inhibiting its adhesion to epithelial cells.

The ability of some probiotic strains to increase mucin production can protect the gastric mucosal barrier against the adherence of pathogenic bacteria such as *H. pylori* and also inhibiting its adhesion to epithelial cells. According to the obtained results; it can be concluded that both strains of LAB 11andLAB 13, exhibit antibacterial activity against *H. pylori*. Also, both strains are probiotics as they were resistant to acidic pH and bile salts concentrations. Moreover, both isolates were tested for in vivo (rat model) and the histological examination of gastric mucosa showed that groups which treated with each isolate daily for a week before the initial *H. pylori* infection and the group that treated with LAB 11 after 4 weeks of infection had regular gastric glands with negative *H. Pylori* colonization like negative control group. Sections of all other groups showed regular gastric glands with *H. Pylori* colonization less than positive control. So, we recommend using dairy products containing probiotic bacteria such lactic acid bacteria (*L. casei, L. paracasei*) as a daily protective routine to prevent infection with *H. pylori*.

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