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In vitro and *in vivo* Activity of Lactococci Strains against *Helicobacter pylori*

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Abstract: Problem statement: Search for lactic acid bacteria that have *in vitro*, a significant inhibitory effect against the strains of *H. pylori* and to determine the inhibitory activity *in vivo*. **Approach:** The *in vitro* inhibitory activity of lactic acid bacteria isolated from milk against strains of *H. pylori* was determined by the agar diffusion method. Two groups of mice were inoculated for a week with TN2GF4. After three weeks, the infected group is treated for seven days with *E. faecium* (B13). *H pylori* was detected by a count after culture of gastric biopsy. The probiotic was determined by a count from fresh feces of mice treated. **Results:** Thirty strains of lactic acid bacteria were isolated and identified. *E. faecium* (B13) strain showed a highly significant inhibition. *H. pylori* was successfully detected in the gastric mucosa. *E. faecium* (B13) reduced the colonization in the stomach of *H. pylori* with a rate of 43% in a week. **Conclusion:** *E. faecium* (B13) has *in vitro* and *in vivo* an inhibitory effect against *H. pylori*.

Key words: *E. faecium*, *Lactococcus* spp, *H. pylori*, probiotics, gastric biopsy, agar diffusion method, *in vitro, in vivo*

INTRODUCTION

Infection with *H. pylori* is probably the most infection common worldwide. 20-90% of adults are infected in different countries. The infection is more common in disadvantaged, low socioeconomic status (Perez-Perez *et al*., 2004).

In 1994, the National Agency for Research on Cancer has determined that this is one of the pathogenic Gram-negative bacilli was recognized as a major carcinogen class (I and II) is indeed associated with gastric adenocarcinoma and gastric lymphoma of MALT type (Cavicchi and Lamarque, 2000).

Several treatment regimens have been proposed to treat *H. pylori*, including antibiotics and antacids, but treatment failure was observed in 20% of cases because of side effects or antibiotic resistance. Many patients after failure of initial treatment seeking other strategies in the treatment of *H. pylori*: alternative or addition of antibiotics (Lopez-Brea *et al*., 2008).

Lactic acid bacteria are considered as probiotics. Probiotics are defined as living microorganisms ingested able to exert beneficial effects on health (Ljungh and Wadstrom, 2006). Several probiotics have shown an antibacterial effect on *Helicobacter pylori* such as *Lactobacillus acidophilus* (Lin *et al*., 2011) and *Bifidobacterium bifidum* (Chenoll *et al*., 2011).

The aims of this study are to find lactic acid bacteria that have a significant inhibitory effect *in vitro* of *H. pylori* and to determine this antibacterial activity *in vivo.*

MATERIALS AND METHODS

Lactic acid bacteria: Samples of raw milk from cow, goat and sheep were taken from two regions: Miliana and Ain Defla. Other samples of camel milk were collected from Biskra and Tamanrasset.

Isolation of lactic acid bacteria was carried out on M17 medium (Pasteur Institute of Algeria) selective medium for lactococci. The Gram-positive bacteria have catalase and oxidase negative were selected for identification. They were then subjected to the pre-identification (type of fermentation and growth in hostile environments).

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Table 1: Characteristics of reference strains of *H. pylori*

Strains	Alias	pathologies	Infection	CagPAI	CagA3'	TPM	CagE	CagL	VirD4/VirB11	Vac	BabA	Ice	SabA	PMID
J99	ATCC700824	Ulcer	Non	+	+	P1+	+	+	+	s1m1i1	BabA2	A2	off	
26695	ATCC 700392	Gastritis	oui	+	+	P1+	+	+	+	s1m1i1	BabA1	A1	off	
TN2GF4		Ulcer	?	+	+	P1+	+	+	+	s1m1i1	BabA2	A1A2		
HpAG1		Gastritis atrophic chronic	?	+	+	P1+	+	+	+	s1m1i1	BabA2	A1		2,00E+07

Note: TN2GF4 resisted to: Amoxicillin, Clarithromycin and Levofloxacin J99 resisted to: Amoxicillin and Clarithromycin

Lactic acid bacteria were then identified at the species or subspecies by establishing their fermentation profiles using the micro-Api 20 strep identification.

***Helicobacter pylori*:** The bacterial strains used are from the National Reference Center for *Campylobacters* and *Helicobacters*, Bordeaux (reference strains: 26695, J99, HPAG1 and TN2GF4, clinical strain: HSA3068) (Table 1).

These strains were transported in the middle BIO-RAD.

These strains were inoculated on Brucella agar supplemented with 10% horse blood and incubated for three days at 37°C and under a microaerophilic atmosphere.

Interaction of lactic acid bacteria with the strains of *H. pylori*: Lactic acid bacteria inoculated into 05 ml of M17 broth and incubated at 37°C for 24 h were tested for antibacterial activity following the agar diffusion method (Tadesse *et al*., 2004). The petri dishes containing Muller Hinton medium, are inoculated with one ml of *H. pylori* strains preculture (inoculated into 5 ml of Brucella broth and incubated at 37°C for 24 h under a microaerophilic atmosphere), after drying for 30 minutes at 37°C, filter paper discs (6mm diameter) impregnated with 50 µL of the lactic acid bacteria preculture are deposited on the surface of the agar (three replicates for each strain of lactic acid bacteria). The petri dishes were prepared and preincubated under refrigerated conditions for 2-4 h at 4°C to allow diffusion of inhibitor to be followed by incubation for 24 h at 37°C. Inhibition is considered positive if the diameter of inhibition Zone (Zi) is greater than 2 mm (Thompson *et al*., 1996): Zi (mm) = diameter of the inhibition zone obtained (mm) - disc diameter (6mm).

Antagonism in vivo: The results of the antagonistic effects observed *in vitro* have been the subject of an *in vivo* study performed on 36 female mice, holoxenic and have two months old (from the Pasteur Institute of Algeria).

The antagonistic effect *in vivo* of *E. faecium* (B13) is studied on TN2GF4 strain.

These mice are divided into three lots to the number of 12 mice for each lot.

Stool analysis is done for half of lot 1 (control), one mice underwent a dissection which a biopsy specimen was taken and crushed. Culture of *H. pylori* was performed on Brucella agar supplemented with 10% horse blood and incubated at 37°C for 3-5 days and under a microaerophilic atmosphere.

Mice lots 2 and 3 were inoculated orally with 0.5 mL (10^9 CFU / mL ≈ 0.6DO) of *H. pylori* prepared for a fresh culture, incubated at 37°C for 24 h and under a microaerophilic atmosphere. The amount to be inoculated is, administered three times a week, with an interval of two days between inoculations (Sgouras *et al*., 2004).

After three weeks post infection, lot 3 was treated for seven days with 1 ml of milk inoculated with 10^7 cfu / ml ≈ 0.1 DO of *E. faecium* (B13) (Coconnier *et al*., 1998).

In the third and fourth weeks, six mice per group (Lot 2 and 3) and one mouse (control group) were killed and dissected aseptically. The stomach of each mouse was removed and *H. pylori* was detected by a count is made on the culture of gastric biopsy crushed, Gram stain, urease, catalase and oxidase.

Feces of treated mice were collected and analyzed for the presence of probiotic administration (Sgouras *et al*., 2004).

- Test with urea: A fragment of gastric biopsy is placed in a tube containing urea indole and incubated at 37°C for 18 h-24 h; the positive result is interpreted by the color change from orange to pink
- Gram stain: A fragment of gastric biopsy crashed with two blades by setting flame and then the smear is stained with Gram's method
- Culture: One biopsy specimen was crushed in 02 ml of medium BGT (glucose buffered broth). Decimal dilutions were performed; the Brucella medium supplemented with 10% horse blood was inoculated by the last dilution (10^{-4}). The petri dishes were incubated at 37°C for 3-5 days under a microaerophilic atmosphere

RESULTS

Thirty strains of lactic acid bacteria were isolated and identified. The bacterial genera found in samples of raw milk are represented by strains of *Lactococcus* and *Enterococcus* (Table 2).

Table 2: Biochemical and physiological characteristics of lactic acid bacteria

	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24	B25	B26	B27	B028	B29	B30
Type of milk	C	C	C	C	C	C	C	C	C	C	C	G	G	G	G	G	G	Ca	Ca	Ca	Ca	Ca	Ca	Ca	Ca	S	S	S	S	S
Original Collection	M	M	M	M	M	M	M	AD	AD	AD	AD	M	M	M	M	AD	AD	T	T	T	T	T	Bs	Bs	Bs	M	M	M	AD	AD
Growth:																														
- 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
- 45 °C	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-
- 2% NaCl	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	+	-	+	+	-	-	+	-	+
- 4% NaCl	+	+	+	-	-	+	+	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	+	-	-	+	-	-	+	-
- 6,5 % NaCl	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-
- pH 4,5	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
- pH 6,5	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
- 65 °C	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-
Vp	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HIP	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
ESC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PYRA	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
α GAL	-	+	-	+	+	-	-	-	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+
Bgur	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
βGAL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LAP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ADH	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
RIB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ARA	-	+	-	+	+	-	-	-	-	+	+	-	+	+	-	+	+	+	+	+	-	+	+	+	+	-	+	+	-	+
MAN	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SOR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LAC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TRE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
INU	-	-	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+
RAF	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-
AMD	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
Glyg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HEM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

C: Cow, G: Goat, Ca: Camel, S: Sheep, M: Miliana, AD: Ain Defla, T: Tamenrasset, Bs: BisKra

Table 3: Diameters of inhibition zones (mm) of lactic acid bacteria strains against *H. pylori*

	TN2GF4	J99	26695	HPAG1	HSA3068
B01	18	13	11	0	13
B07	10	6	5	0	4
B12	8	0	0	0	0
B13	20	15	15	0	16
B14	4	4	3	0	0
B17	6	6	5	0	3
B18	0	4	0	0	0
B19	0	6	0	7	0
B22	3	0	0	0	0
B23	0	0	0	0	0
B25	8	7	7	0	6
B26	5	0	0	5	0
B27	0	0	0	0	0
B29	3	0	0	4	0

Eleven strains of *Lactococcus lactis* subsp. *lactis* (B01, B03, B06, B07, B08, B09, B12, B15, B21, B26 and B29), nine strains of *Lactococcus lactis* subsp. *cremoris* (B04, B14, B16, B20, B22, B24, B25, B28 and B30), four strains of *Lactococcus garvieae* (B02, B11, B18 and B23) and six strains of *Enterococcus facium* (B05, B10, B13, B17, B19 and B27). The interaction *in vitro* of lactic acid bacteria and *H. pylori* showed that of 30 strains of lactic acid bacteria only 14 have an inhibitory effect (Table 3).

According to the results, it appears that the two strains *E. faecium* (B13) and *Lc. lactis* subsp. *lactis* (B01) have significant inhibition zones with diameters 20 and 18 mm are registered with the strain TN2GF4, 16 and 13 mm with the strain HSA3068, 15 and 11 mm with strain 26695, 15 and 13 mm with strain J99. For HPAG1 strain the best inhibition zone is found with *Enterococcus faecium* (B19) (diameter = 7 mm). Other inhibition zones were found with strains of lactic acid bacteria (Fig. 1).

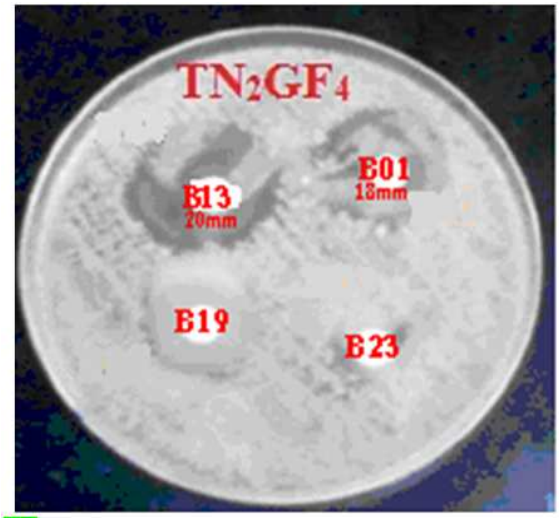

Fig. 1: Antibacterial activity of *Lactococcus* spp. and *Enterococcus faecium* strains against *H. pylori* (TN2GF4) by the agar diffusion method

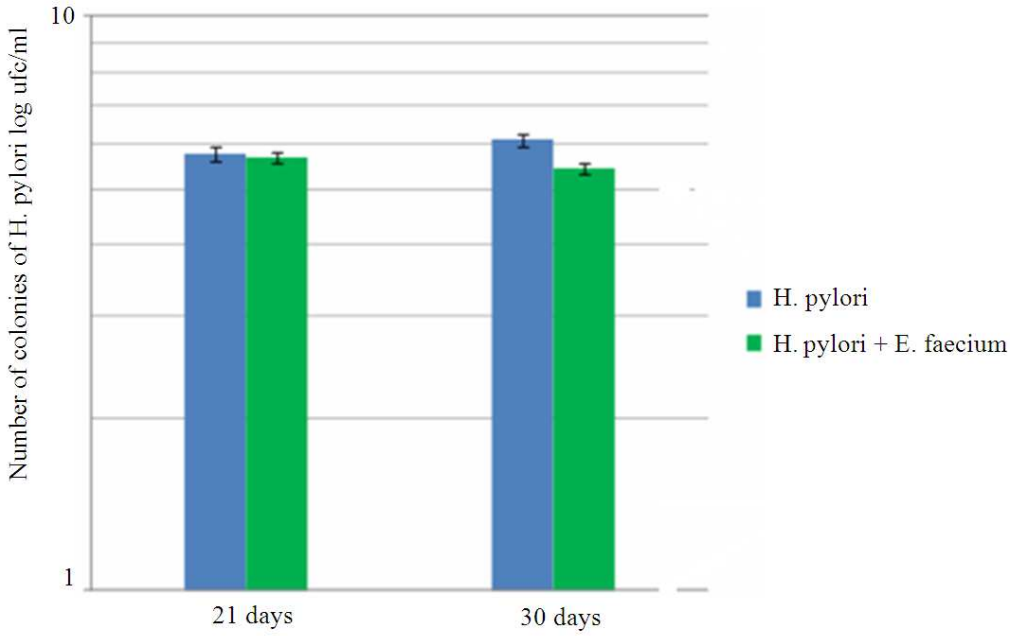

Fig. 2: Number of colonies of *H. pylori* after treatment with *Enterococcus faecium* (B13) (D21 and D30)

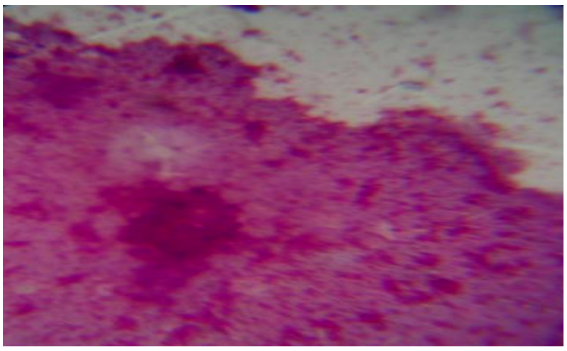

Fig. 3: Stomach of mice infected with *H pylori* by Gram stain (10×100)

The bacterial analysis of the stool and gastric biopsies of the mouse control group revealed no presence of *H. pylori* and *E. faecium*.

During the two phases of observation, *H. pylori* was successfully detected in the gastric mucosa of mice infected with strain TN2GF4. The population of the TN2GF4 isolated from fragments of mouse stomach in this group ranged from 1.5×10^5-1.2×10^6 cfu/mL.

From the 2nd phase (30 days) there is an increase of the population of *H. pylori* and peaked at 2.9×10^6cfu/mL. With a growth rate equal to 52.42% between the means of two phases of lot 2.

After 3 weeks post infection in the treated group, quantitative culture of *H. pylori* performed on fragments of gastric biopsies taken from 6 mice infected with TN2GF4 shows a number of bacteria ranging from 1.8×10^5 to 1.2×10^6 cfu/mL.

This number starts to decrease after administration of *E. faecium* (B13) from 1.2×10^6-1.6×10^5 cfu/mL on day 30, *E. faecium* (B13) reduces colonization of *H. pylori* in mice, in effect from 4.9×10^5-2.8×10^5 cfu/mL or a lowering of 42.86% between the means of two phases of lot 3.

Stool analysis of treated mice revealed the presence of *Enterococcus faecium* (B13) with concentrations ranged from 6×10^5-1.03×10^6 cfu/mL.

These results were confirmed by analysis of variance at the 5% applied to the evolution of *H. pylori* accounts at lot 2 and 3. This analysis shows that there is a highly significant difference between the infected group and the treated group. Indeed the burden of *H. pylori* in mice treated with *E. faecium* (B13) is decreased by nearly one log compared with mice receiving *H pylori* alone (Fig. 2).

A statistical analysis performed with a Student *t* test between the infected group and the group treated with *E. faecium* (B13) showed a highly significant difference ($\alpha = 0.05$).

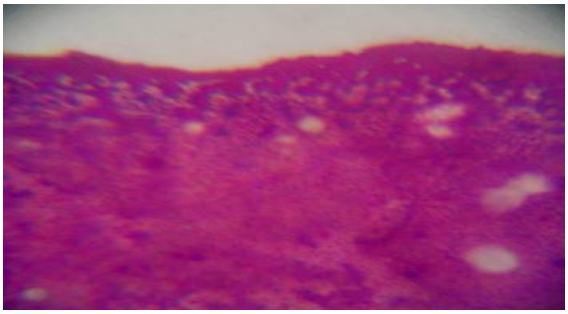

Fig. 4: Stomach of mice not infected with *H pylori* (control) after Gram strain (10×100).

For 3 weeks post infection, *H. pylori* readily colonize the stomachs of mice, with its flagella and its helical shape. It glides through the mucosa of the stomach and is anchored to the epithelial cells through adhesion. It secretes urease and in the presence of protein converts urea into ammonia and CO_2. The production of ammonia creates a buffered microenvironment around the bacteria and can survive in acidic medium.

Ammonia is toxic to epithelial cells and will damage the surface epithelial cells (Fig. 3). However, no effect was observed in mice control group (Fig. 4).

The results clearly indicate that *in vivo Enterococcus faecium* (B13) was present in the microflora during the consumption period. It is then removed in a few days without lasting settlement

DISCUSSION

The results from the inhibition of *H. Pylori* strains (TN2GF4, HPAG1, J99, 26695 and HSA3068) show that lactic acid bacteria do not have the same spectrum of action, they express more or less important variations depending firstly on the strain of *H. pylori* target and secondly to the indicator strain of lactic acid bacteria. The best inhibition zones were recorded by *E. faecium* (B13) isolated from goat's milk and *Lactococcus lactis* subsp. *lactis* (B01) isolated from cow's milk.

Strains of *Enterococcus faecium* isolated from Chungkukjang have proven resistance against gastrointestinal conditions such as the acidic environment and bile salts. These strains also showed a bile salt hydrolase activity, but neither hemolytic activity nor the virulence factor has been detected. For this reason, the strains could be used as input or selected cover crops in food production from fermented soybeans. This bacterium showed inhibitory activity *in vitro* of *Listeria monocytogenes* (Yoon *et al*., 2008) and *H. pylori* (Lopez-Brea *et al*., 2008).

Lactococcus lactis subsp. *lactis* (B01) have antibacterial activity against Gram-negative strains of *H. pylori* (J99, HSA3068, 26695, HPAG1 and TN2GF4). These results are in the same direction as those found by Joshi *et al*. (2006) who isolated to radish, a strain of *Lactococcus lactis*, which has antibacterial activity against many Gram-negative species.

The outcome after ingestion of a strain of *Enterococcus faecium* used in a probiotic product has been studied by Lund *et al*. (2002). These authors showed that in 8 out of 10 volunteers who consumed approximately 10^9 cfu daily for 10 days, the bacteria were recovered in rates between $10^{3.1}$ and $10^{6.6}$ cfu/ g. For three volunteers, exogenous strain was dominant among the population of *E. faecium*. The authors also showed that the presence of the strain was transient and did not persist after cessation of consumption.

This bacterium passes through the digestive system and is resistant to stomach acid and Biliopancreatic secretions by the rapid passage through the stomach. Tsai *et al*. (2004) found that the strain *Enterococcus faecium* TM 39 with the ability to tolerate acid and bile salts inhibit the growth of *H. pylori in vitro*.

Thus, certain strains of *Enterococcus faecium* are shown as probiotics that induce beneficial effects on consumer health. Market, these microorganisms are available as dietary supplements, such as *Enterococcus faecium* strain Cernelle 68 (SF 68) in a dehydrated form, marketed by Bioflorin (Sweden).

H. pylori urease activity and has a can produce ammonia by hydrolysis of urea. It can therefore contribute to hyperammonemia (Gubbins *et al*., 1993) but the introduction of *Enterococcus faecium* slowed the synthesis of NH3. The effective administration of *Enterococcus faecium* (SF68) was demonstrated by the work of Loguercio *et al*. (1995) confirms that this bacterium without urease decreases colonic ammonia synthesis.

Tsai *et al*. (2004) have described that treatment of *H. pylori* with cells of strain *Enterococcus faecium*TM39 significantly reduces it's binding to gastric carcinoma cells (TSGH 9201).

CONCLUSION

Lactic acid bacteria have been known for their inhibitory potency of pathogenic bacteria. *Enterococcus faecium* (B13) isolated from goat's milk of Miliana had inhibited *in vitro* the strains of *H. pylori*: TN2GF4, J99, 26695 and HSA3068.

Enterococcus faecium strain B13 reducing the colonization of *H pylori* in the stomach with a rate of 43% for a week. *Enterococcus faecium* B13 passes through the digestive system and is resistant to stomach acid and Biliopancreatic secretions by the rapid passage through the stomach.

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