



ANALYSIS OF LACTIC ACID RESPONSIBLE FOR INHIBITION IN-VITRO OF *Helicobacter pylori* BY HIGH PERFORMANCE CHROMATOGRAPHY (HPLC)

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Abstract- Eradication of *Helicobacter pylori* by antibiotics has installed many problems for the organism, for that the aim of this work is to seek other antibacterial agents which can inhibit this bacteria *in vitro*. Bacterial associations were carried out between *H. pylori* strain isolated to gastric biopsy from a patient having ulcer and of two strains of lactic acid bacteria: *Streptococcus thermophilus* and *Lactobacillus acidophilus*. Supernatants obtained by centrifugation (12400 rpm/mn during 10 min) of the bacterial cultures treated with various temperatures: 100°C/60mn, 100°C/30mn and 121°C/15mn, are analyzed by HPLC. Qualitative analyze showed the presence of lactic acid and absence of acetic acid in both supernatants obtained, of which we recorded a quantity of 0.019 g / l secreted by the *Streptococcus thermophilus* and a quantity of 0.058 g / l secreted by *Lactobacillus acidophilus*.

Keywords- *Helicobacter pylori*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, inhibition, lactic acid, HPLC

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Introduction

The colonization of the gastric mucosa by *Helicobacter pylori* is common and often associated with gastritis, ulcers, cancer or lymphoma [1]. The rate of infection by this pathogen is different in the adult population and in the pediatric population. *Helicobacter pylori* is acquired in childhood, especially early as the socio-economic level is low [2]. To eradicate *Helicobacter pylori* several antibiotics have been proposed, but the use of antibiotics has created resistance strains of *H. pylori* and other side effects on health have emerged as esophageal reflux, which due to an increase in the gastric acidity [3]. The search for new antimicrobial agents showed that certain probiotic bacteria may inhibit the activity of *H. pylori* [4]. These probiotics have the potential to inhibit the growth of *H. pylori* *in vitro*, improve mucosal inflammation in animals infected with *H. pylori* and suppress the secretion of IL-8 in gastric cells infected by *H. pylori* [3].

Probiotics are living microorganisms ingested in adequate amounts can exert beneficial effects on the health of the host [5,6]. Most of probiotic is lactic acid bacteria [7]. These microorganisms are heterogeneous groups gathering a number of Gram-positive bacteria possess metabolic and physiological characteristics common. Their main characteristic is an exclusively fermentative metabolism that leads from glucose significant amounts of lactic acid, accompanied

in some cases other metabolites (ethanol, CO₂, other organic acids) [8]. These microorganisms are marketed as products fermented or unfermented, food supplements or pharmaceutical preparations [9].

The aim of this study is to determine the type of organic acid responsible for the inhibition of *Helicobacter pylori*, then quantify the organic acid secreted into the culture medium by the HPLC method.

Materials and Methods

Lactic Acid Bacteria and Culture Media

Two strains of lactic acid bacteria were used:

Streptococcus thermophilus (*Sc.tr*) isolated from cow milk.

Lactobacillus acidophilus (*Lb.ac*) isolated from feces of children (under 2 years ages).

Both strains are kept frozen at -18°C in reconstituted milk [10]. Transplanting bacteria is to sow a few microliters of thawed stem in 10 ml of milk. The incubation is done for *Sc.tr* 45°C and 37°C for *Lb.ac*. After 72Hrs. incubation, 1ml of each tube was raised aseptically in 9ml of MRS broth for *Lb.ac* 9ml of broth and M17 for *Sc.tr* then incubated for 3Hrs. [11]. Bacterial precultures are prepared by inoculation of 5ml *Lb.ac* in MRS broth and 5ml *Sc.tr* in M17 broth and then incubated at 37°C for 18Hrs.

Helicobacter pylori and Culture Media

A strain of *H.pylori* isolated from a biopsy of a gastric ulcer patient. This strain was kept frozen in the agar base supplemented with 10% horse blood at -18°C. It is subcultured in buffered glucose broth and Chocolat agar. The Petri dishes is incubated for 3 days at 37°C and under microaerophilic conditions [12].

Preparation of the Association Lactic Acid Bacteria Pathogenic Bacteria

From the isolates inoculated [13]
 A tube of 5ml of MRS broth with 250 µl of *Lb.ac* and 250 µl of Hp.
 A tube of 5ml of M17 broth with 250 µl of *Sc.tr* and 250 µl of Hp.
 After incubation for 18Hrs. at 37°C, inhibition of *H. pylori* was tested by culture of agar based supplemented with 10% of horse blood. The cultures centrifuged at 12400 rpm for 10 min to remove bacterial cells from the middle. The supernatant obtained was heat treated to 100°C/60min, 100°C/30min and 121°C/15min to eliminate the effect of bacteriocins [14].

High Performance Liquid Chromatography (HPLC)

Analysis of samples was performed at 25°C using Waters 1525 binary HPLC pump, Waters 2487 detector for UV and Waters for the degasser AF. The elution time is short, it is obtained by applying high pressure of about 300 bars, with an equal volume of 20.00 µl injection and retention time (RT) equals 40, 00 minutes. A cartridge filled with silica bonded with octadecyl was previously washed with 10 ml of methanol and then with 10 ml of water. The degassed sample was filtered through a cellulose membrane (pore diameter: 0.45 mm). A syringe previously rinsed with the sample is used to collect 8 ml. It eliminates the first three ml are collected following 5ml, always avoiding to bring the cartridge to dry. In the chromatograph is injected successively 10µl of reference solution (lactic acid, acetic acid) and 10µl of the sample prepared by repeating three times the injections in the same order [15].

Qualitative Analysis

The qualitative analysis is to determine the retention time of each respective compound eluted. The references organic acids are separated in order of elution following: lactic acid, acetic acid.

Quantitative Analysis

The quantitative analysis is to measure the peak areas (reference solution and the sample) and determining the concentration of organic acid in the sample according to the equation:

$$[\text{Sample}] = \frac{\text{Area sample}}{\text{Standard area}} \times [\text{standard}] \times \text{dilution}$$

Results and Discussion

Qualitative Analysis

The detection of two standards injected by spectrophotometer gave two peaks [Fig-1], one for lactic acid (RT = 4.887 min) and one for acetic acid (RT = 5.344 min). The results of the qualitative analysis of organic acids, lactic and acetic acid by HPLC in samples prepared are shown in [Fig-2] and [Fig-3].

A comparison is made by calculating the difference between the retention time of standard peak and the peak very close (in terms of value of RT) [Table-1] and [Table-2] showed the presence of lactic acid bacterial cultures and that lactic acid is responsible for

the inhibition of *H. pylori* [16]. The absence of acetic acid justifies that the two strains of lactic acid bacteria *Lactobacillus acidophilus* and *Streptococcus thermophilus* are homofermentaires [8]. *L.acidophilus* may secrete an agent antigestive and / or antiulcer in their culture medium. Such a float and such an agent extracted from this supernatant may thus themselves be of very great interest in the treatment of gastritis or ulcers of the stomach or pylorus [17]. The inhibitory effect of *Lb.ac* by lactic acid production has been demonstrated *in vitro* by the work-Lesbros Pantiflickova and al., [18].

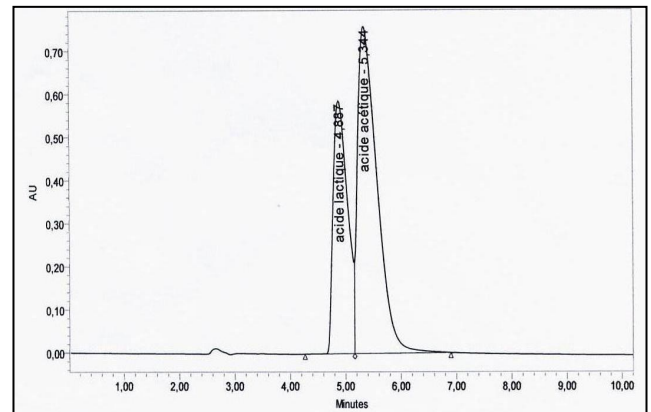


Fig. 1- Separation of organic acids of the reference acetic acid / lactic acid

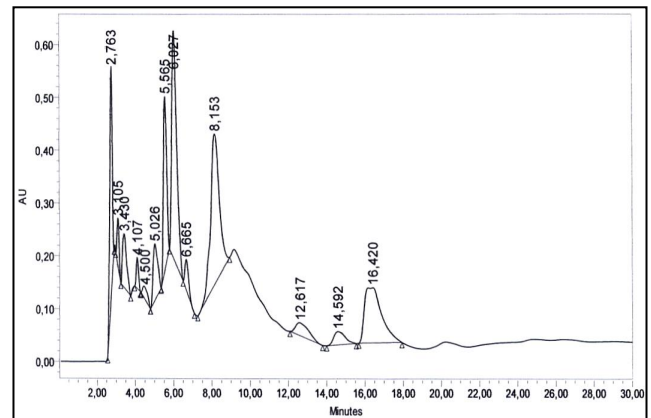


Fig. 2- Separation of organic acids in the sample *H. pylori* + *Sc.tr*

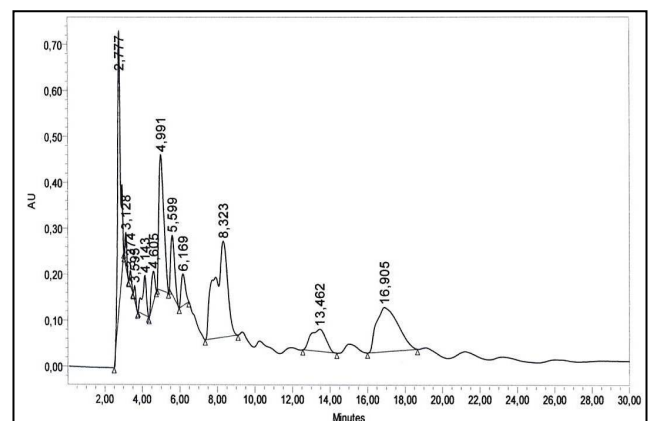


Fig. 3- Separation of organic acids in the sample *H. pylori* + *Lb.ac*

Table 1- Qualitative Analysis of lactic acid

Sample	RT (min)	D (difference in min)
<i>H. pylori</i> + <i>Sc.tr</i>	5,026	0,139
<i>H. pylori</i> + <i>Lb.ac</i>	4,991	0,104

Table 2- Qualitative analysis of acetic acid

Sample	RT (min)	D (difference in min)
<i>H. pylori</i> + <i>Sc.tr</i>	5,565	0,221
<i>H. pylori</i> + <i>Lb.ac</i>	5,599	0,255

Quantitative Analysis

The quantitative analysis showed the secretion of a quantity of 0.019 g / l of lactic acid in combination *Sc.tr* + *Hp* and a quantity of 0.058 g / l of lactic acid in combination *Lb.ac* + *Hp* [Table-3]. According to FAO, [19], *Streptococcus thermophilus* is less acidifying than lactobacilli.

Table 3- Quantitative analysis of lactic acid

Sample	Concentration of lactic acid (g/l)
<i>H. pylori</i> + <i>Sc.tr</i>	0,019
<i>H. pylori</i> + <i>Lb.ac</i>	0,058

The work of Midolo and *al.*, [20]; Sutra and *al.*, [8] have reported that inhibition of pathogenic bacteria do not depend the amount of organic acid produced by lactic acid bacteria, but related to the type and the dissociated form or not the organic acid. Indeed, it is the undissociated form of lactic acid which predominates at acidic pH, it is generally more toxic to microbial cells. Lactic acid is a lipophilic weak acid, capable of crossing the membrane and act directly on cytoplasmic pH and finally be bactericidal [21].

Conclusion

Inhibition of pathogenic bacteria by lactic acid bacteria has been demonstrated by our results and published work in the area confirm that lactic acid bacteria have the ability to inhibit the activity of pathogens by producing antimicrobial substances. The results of qualitative analysis by HPLC revealed the presence of lactic acid in both H.p combinations.

The quantitative analysis showed the secretion of a quantity of 0.019 g/l by *Streptococcus thermophilus* and a quantity of 0.058 g/l by *Lactobacillus acidophilus* responsible for inhibiting the growth of *Helicobacter pylori* isolated from gastric biopsies.

These results show that the agent strain and composition are particularly intended to be administered to humans for therapeutic or prophylactic levels of the stomach, especially in the treatment of gastritis or stomach ulcers or pylorus.

Conflicts of Interest

The authors declare that they have no competing interests

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