

HPLC Analysis of lactic acid inhibition of *Salmonella typhi* and *Escherichia coli* responsible of diarrheal diseases in Algeria: Alternative treatment *S.typhi.E.coli.BacLac*.

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Abstract

Several works were shown the effect *in vitro* of lactic acid bacteria on pathogenic strains responsible for human gastroenteritis. This work aims to seek strains of lactic acid bacteria could inhibit *in vitro* the growth of pathogenic bacteria involved in gastroenteritis and analyze organic acids responsible of this inhibition. Interactions in agar medium were conducted between lactic acid bacteria in pure or mixed culture and pathogenic bacteria (*E. coli* and *S. typhi*). The best inhibition zones of *E.coli* are found with *Lp* (diameter = 10 mm) and *St**+ *Lb**+ *Bl* (diameter = 10 mm). For *S.typhi*, the combinations *St*3 + *Lb*1+ *Bl* and *St*2 + *Lp* + *Bl* have the major inhibitory effect (diameters of 8 mm and 7 mm, respectively). HPLC method confirmed the presence of lactic acid in bacterial cultures used in this study.

Keywords: HPLC; lactic acid bacteria; *Salmonella typhi*; *Escherichia coli*; organic acids.

1. Introduction

Diarrheal diseases are still today a major public health problem in developing countries. Diarrhea is a common reason for consultation, especially in industrially developing countries where fecal and food hygiene is precarious [1]. According to the World Health Organization (WHO) and UNICEF, about two billion cases of diarrhea are recorded worldwide every year and 1.9 million children under 5 die from diarrhea every year, mostly in developing countries. Among all child deaths from diarrhea, 78% occur in Africa and South-East Asia [2]. These explosive epidemics are related to many factors: rapid population growth in some countries, economic recession, disasters, natural or man-made (war, famine, population massacres) [1]. In industrialized countries, the number of deaths from diarrhea patients is relatively small, but nevertheless diarrhea remains a major cause of morbidity and induces substantial costs of care. In developing countries, enteric bacteria and parasites are more prevalent than viruses typically with a peak during the summer months enteropathogenic *E. coli* (EPEC) causes most often diarrhea in children less than 2 years [2]. Typhoid fever caused by *Salmonella typhi* is still a common cause of mortality. In severe cases, this infection is manifested by high fever of around 40°C [3]. Algeria crossed, for some years now, an epidemiological transition marked by the persistence of communicable diseases (infectious childhood diseases, waterborne disease, zoonoses), characteristics of developing countries, which are becoming more an important place in the burden of diseases [4]. It has been confirmed that different probiotic strains including *L. reuteri* ATCC 55730, *Lactobacillus rhamnosus* GG, *L. casei* DN-114 001 and *Saccharomyces cerevisiae* (boulardii) are useful for reducing the severity and duration of the acute infectious diarrhea in children. The oral administration of probiotics reduced the duration of acute diarrhea in children by about one day [2]. Thus, the objective of this work was to study *in vitro* the interaction of lactic acid bacteria with pathogenic bacteria responsible for gastroenteritis in Algeria (*E. coli*, *S. typhi*), in pure culture, mixed or added bifidobacteria to highlight the best probiotic combinations, and proceeding with the analysis of organics acids in bacterial cultures by High Performance Liquid Chromatography (HPLC).

2. Experimental

Lactic acid bacteria:

- *Streptococcus thermophilus* (St1, St2, St3) and *Lactobacillus bulgaricus* (Lb1, Lb2, Lb3) isolated from raw cow's milk and identified in the laboratory lactologie, Department of Food Science, University of Chlef.
- *Streptococcus thermophilus* (St *), *Lactobacillus acidophilus* (Lb *), *Bifidobacterium longum* (Bl) and *Bifidobacterium adolescentis* (Ba); isolated from stools of children and identified in Molecular Biology laboratory, University of Bejaia.
- *Lactobacillus paracasei* (LP) isolated from cow's milk and identified in laboratory lactologie, University Pamplona - Navara Spain.

Both strains are kept frozen at -18°C in reconstituted milk. A few microliters of bacteria is transplanting in 10 ml of milk. The incubation is done at:

- 45°C for strains *Streptococcus thermophilus*; *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* [5];
- 37°C for *Bifidobacterium adolescentis* and *Bifidobacterium longum* [6];
- 30°C for *Lactobacillus paracasei* 1 [6].

After 72h, 1 ml of each tube was raised aseptically in 9 ml of MRS broth for *Lb.ac*, 9 ml of M17 (Terzaghi and Sandine) broth for *Sc.tr*, then incubated for 3h. The streptococci was counted in M17 agar [7], the lactobacilli in MRS (De Man, Rogosa and Sharpe) agar [7] and the bifidobacteria in MRS (De Man, Rogosa and Sharpe) agar cysteine [8]. The cultures was mixed by using combinations with *Streptococcus thermophilus*, *Lactobacillus bulgaricus*,

Lactobacillus acidophilus and *Lactobacillus paracasei* 1 and incubated during 48 h at 45°C for *Stx + Lb* y, at 30°C for *Stx+Lp* [9].

After enumeration, the best combinations were supplemented with *Bifidobacterium adolescentis* or *Bifidobacterium longum* and incubated at 37°C for 48 h [9].

Pathogens bacteria:

Two very common bacterial pathogens in Ain Defla were isolated of stools samples in Hektoen agar: *Escherichia coli* responsible of diarrhea children and *Salmonella typhi* of typhoid fever [10]. After culture at 37°C for 24 h, *E. coli* colonies are flat, dry, and yellow with salmon center; *Salmonella* colonies are green with a black center.

Preparation of bacterial culture:

In this study, we inoculate:

- *Lactobacillus* strains in 5 tubes containing 5 ml of MRS broth;
- *Streptococcus thermophilus* strains in 4 tubes containing 5 ml M17;
- *Bifidobacterium* strains in 2 tubes containing 5ml MRS cysteine broth ;
- the best microbial mixed in tubes containing 5ml of MRS broth;
- *E. coli* and *S. typhi* into two tubes containing 5 ml of nutrient broth;

We incubated these preparations at 37°C for 24 h.

Agar diffusion method:

Petri dishes containing M17, MRS and MRS cysteine agar were inoculated by a few milliliters of pathogenic bacteria culture; and they let dry at 37°C for 30 min. After, we deposit on the surface of the agar blotting paper discs impregnated beforehand by lactic acid bacteria strains and let dried at 37°C for 30 min. Finally, we incubate the Petri dishes at 37 ° C for 24 to 48 h [11].

Analyses of organics acids by HPLC:

Preparation of Lactic Acid Bacteria-Pathogenic Bacteria(PB) culture: For this step, we inoculated : A tube of 5ml of MRS broth with 250 µl of *Lb.* and 250 µl of *PB* .A tube of 5ml of M17 broth with 250 µl of *Sc.tr* and 250 µl of *PB* . A tube of 5ml of MRS broth with 250 µl of (*Stx + Lby*) and 250 µl of *PB* . A tube of 5ml of MRS broth with 250 µl of (*Stx + Lby+ Bifz*) and 250 µl of *PB* . The cultures were centrifuged at 12400 rpm/min for 10 min to remove bacterial cells from the middle. The supernatant obtained was treated at 100°C/60min, 100°C/30min and 121 °C/15min to eliminate the bacteriocins[12].

High Performance Liquid Chromatography (HPLC)

Analysis of samples was performed at 25°C using Waters 1525 binary HPLC pump, Waters 2487 detectors 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 µm). A syringe previously rinsed with the sample is used to collect 8 ml. 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[12]. 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:

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

3. Results and discussion

Interaction pathogenic-lactic acid bacteria:

Among all pure cultures of lactic acid bacteria strains, *Lactobacillus bulgaricus* 2, *Lactobacillus bulgaricus* 1 and *Bifidobacterium longum* gave the best growth rates estimated at $1,59 \times 10^8$, $1,42 \times 10^8$ and $1,25 \times 10^8$ bacteria / mL, respectively, while the strains of *Streptococcus thermophilus* 2, *Streptococcus thermophilus* 1, *Lactobacillus paracasei* 1, *Streptococcus thermophilus* 3, *Streptococcus thermophilus*, *Bifidobacterium adolescentis*, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* 3 gave moderate growth rates estimated at 7.6×10^7 , 6×10^7 , 4.51×10^7 , 2.69×10^7 , 2.11×10^7 , 2×10^7 , 1.48×10^7 and 1.28×10^7 bacteria / mL, respectively.

A significant growth rates are found with *St3* + *Lb1*, *St1* + *Lb3*, *St** + *Lb3*, *St1* + *Lb1* and *St** + *Lb** (6.4×10^7 , 5.9×10^7 , 5.6×10^7 , 4.8×10^7 and 3.1×10^7 bacteria / mL, respectively). The growth rates of lactic acid bacteria is higher with the addition of *Bifidobacterium longum*.

Interaction lactic acid bacteria- E.coli:

In pure cultures, 70% of lactic acid bacteria inhibit *E. coli*, another inhibition zones are found with mixed cultures (Tab1, Fig.1, Fig.2). The strain *Lb. paracasei* 1 and the combination *St** + *Lb** + *Bl* gives a great inhibitory effect (diameter = 10mm) among all cultures.

Interaction lactic acid bacteria -S. typhi:

We recorded the largest inhibition zone with *St1* + *Lb3* (7mm) (Tab1). For mixed cultures supplemented with *B. longum*, the combination *St3* + *Lb1* + *Bl* gave better inhibition zone (diameter = 8 mm), then *St2* + *Lp* + *Bl* (diameter = 7mm) (Fig. 3). The results show that the inhibition observed in the mixed cultures supplemented with *Bifidobacterium longum* is greater than those found in mixed cultures supplemented with *Bifidobacterium adolescentis*.

High performance liquid chromatography (HPLC):

Qualitative Analysis

The two standard injected by spectrophotometer gave two peaks, one for lactic acid (RT = 4.887 min) and one for acetic acid (RT = 5.344 min) [12]. Qualitative analysis of the lactic acid, gave small differences (D) relative to the retention time (RT) of the standard lactic acid for all selected bacterial cultures (Tab.2). While qualitative analysis of acetic acid, gave large differences (D) relative to the retention time of the standard acetic acid to all the selected bacterial cultures (Tab.3). These results demonstrate that the inhibition of pathogenic strains (*E. coli* and *S. typhi*) is due to the secretion of lactic acid (Fig.4, 5,6,7).

Quantitative analysis:

In bacterial cultures we detect only the lactic acid. According to the results (Tab.4), we can record a quantities of lactic acid secreted by *Lp* (0.069 g / L), *St** + *Lb** + *Bl* (0.075 g / L), *St3* + *Lb1* + *BL* (0.070 g / L) and *St2* + *Lp* + *BL* (0.077 g / L).

3. Discussion:

Lactic acid bacteria have the ability to inhibit, *in vitro*, the action of Gram positive and negative pathogens [13]. Several works were shown the effect of lactic acid bacteria on pathogenic strains responsible for peptic disease. Guetarni and al., [14] isolated thirty strains of lactic acid bacteria. The bacterial genera found in samples of raw milk are represented par strains of *Lactococcus* and *Enterococcus*. *E. faecium* (B13) strain showed a highly significant inhibition. *In vivo*, *H. pylori* was successfully detected in the gastric mucosa of BalB/C mouse. *E. faecium* (B13) reduced the colonization of *H. pylori* in the stomach with a rate of 43% in a week. In another study [15] homofermentative lactic acid bacteria strains were isolated from raw milk and tested for their antagonistic power against strains of *Helicobacter pylori*. The search for the antagonist substance was performed following agar and broth diffusion method. The substances produced by *Enterococcus faecium* strain (B13) lose their inhibitory activity after treatment with trypsin and pepsin, but retain the antibacterial activity after heat treatment for 30 min at 60°C, 80°C for 10 min and 100°C for 5 min. This protein is stable in acid and alkaline pH. *Lactobacillus acidophilus* and *Lactobacillus paracasei* in mixed culture have an inhibitory effect on *E. coli* [16]. Several strains of probiotic bacteria have been isolated and identified; these strains have an inhibitory effect on *E. coli* and *Salmonella*. Two strains of *Lactobacillus paracasei* subsp. *paracasei*, obtained from nine samples of artisanal *Caprino Aspromonte* cheese, made from raw goats' milk, have Anti-*E. coli* effect [17]. Another probiotic bacterium, *Lactobacillus acidophilus* strain La-5 has been shown to be effective against enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 [18]. *B. longum* can inhibit the binding of *E. coli* on Caco-2 cultures and destroy *Salmonella* [19, 20, 21]. *B. longum* correctly resists the acidity and H₂O₂ secreted by the yogurt ferments (*Sc. thermophilus* and *Lb. bulgaricus*) hence the importance of employment in fermented milks [22]. *In vitro* *Lactobacillus acidophilus* binds to the Caco-2 cells, and if it is sufficient, it can occupy the field 'and prevent pathogenic bacteria to join [23]. The difference in affinity between *Sc. thermophilus* and those of *Lb. bulgaricus*; *Lb. acidophilus* and *Lb. paracasei* 1 recorded in our work, can be explained by the difference in growth characteristics of *Sc. thermophilus* and *Lactobacillus spp.*, individual or associatively [24]. The highest growth rate is of *Stx* + *Lby*, this may be due to the positive association of lactic bacteria strains in mixed culture [25]. This positive association can be explained by the synergy between *Sc. thermophilus* and *Lactobacillus spp.*, each species produces one or more substances, absent originally in the culture medium, which stimulates growth of the other species, with an increase of proteolysis by the genus *Lactobacillus* and a higher production of lactic acid by the two species [26]. Strains of *Sc. thermophilus* are often little proteolytic, due to a low level or absence of a protease of the wall, hence their growth is limited, amino acids and peptides initially presents in the medium being insufficient to cover their needs [27]. Inhibition of *E. coli* by mixed culture may be due also to the large production of acid by *Lactobacillus* relative to *Streptococcus thermophilus* [28]. Lactic acid inhibits the intestinal survival of *Salmonella*. This acid is solely responsible for the strengthening of the gastric barrier to *Salmonella* [29]. The inhibitory activity *in vivo*, has been proven where treatment with lactobacilli, given during acute diarrhea in children under three years, showed a reducing of diarrhea of approximately 0.7 days, and stool frequency of 1.6 stools / day from the second day [30]. It was also shown that the incorporation of *Lactobacillus acidophilus* in the diet of infants fed by bottle reduces the incidence of diarrhea and other digestive disorders [31]. The effect of *L. acidophilus* L1 was revealed *in vivo* by injecting a group of mice with *L. acidophilus* 1 suspension or its infiltrate 30 min after injecting the *E. coli* A99. Intraurethrally and the histopathological sections revealed the disappearance of inflammation signs caused by *E. coli* A99 when It was injected alone [32]. The antibacterial effects of supernatant culture of *Lactobacillus* species, *L. casei*, *L. acidophilus*, *L. helveticus* and *L. delbrueckii* ssp. *bulgaricus* isolated from yougourt samples were stable at different temperatures conditions (56–100°C) for 30 and 60 min; also, it was stable at pH 3–10 [33].

Table.1: diameters of inhibition zones (mm) of lactic acid bacteria against pathogenic bacteria

Lactic acid bacteria	<i>Escherichia coli</i>	<i>Salmonella typhi</i>
Pathogenic bacteria		
<i>St1</i>	0	0
<i>St2</i>	0	0
<i>St3</i>	5	0
<i>St*</i>	0	4
<i>Lb1</i>	7	0
<i>Lb2</i>	4	0
<i>Lb3</i>	3,5	4
<i>Lb*</i>	8	5
<i>Lp1</i>	10	0
<i>Ba</i>	5	0
<i>Bl</i>	6	4
<i>St2+Lp</i>	5	4
<i>St3+Lb1</i>	4	4
<i>St1+Lb3</i>	4	7
<i>St*+Lb3</i>	4	5
<i>St1+Lb1</i>	4	0
<i>St*+Lb*</i>	5	4
<i>St2+Lp+Ba</i>	0	0
<i>St3+Lb1+Ba</i>	0	0
<i>St1+Lb3+Ba</i>	0	5
<i>St*+Lb3+Ba</i>	0	4
<i>St1+Lb1+Ba</i>	0	0
<i>St*+Lb*+Ba</i>	5	4
<i>St2+Lp+Bl</i>	5	7
<i>St3+Lb1+Bl</i>	0	8
<i>St1+Lb3+Bl</i>	5	6
<i>St*+Lb3+Bl</i>	4	6
<i>St1+Lb1+Bl</i>	8	4
<i>St*+Lb*+Bl</i>	10	6

Tab2. Qualitative analysis of the lactic acid

Samples	RT (min)	D (difference)
<i>E.coli+Lp</i>	4,961	0,074
<i>E.coli+ (St*+Lb*+Bl)</i>	4,959	0,072
<i>S.typhi+ (St3+Lb1+Bl)</i>	4,969	0,082
<i>S.typhi+ (St2+Lp+Bl)</i>	4,949	0,062

These strains may help in the treatment of *E. coli* O157:H7-associated diseases and can be applied as a biological preservative in the food industry [34]. Bacterial associations were carried out between *H. pylori* strain isolated to gastric biopsy from a patient having ulcer and two strains of lactic acid bacteria: *Streptococcus thermophilus* and *Lactobacillus acidophilus*. Supernatants obtained by centrifugation (12400 rpm / min during 10 min) of the bacterial cultures treated with various temperatures: 100°C / 60 min, 100°C / 30 min and 121 °C / 15 min, are analyzed by HPLC. Qualitative and quantitative analysis showed the presence of lactic acid in supernatants with a quantity of 0.019 g / L secreted by *Streptococcus thermophilus* and 0.058 g / L secreted by *Lactobacillus acidophilus* [13]. In our study, lactic acid is the major inhibitor substance *in vitro* of *E. coli* and *S. typhi*. Lactic acid is a weak mono, with a pKa of 3.86. Only the undissociated form (CH₃CHOHCOOH) can cross the plasma membrane and dissociate within the cell, releasing ions that acidify the cytoplasm. In addition to the effect of pH, undissociated acid brought down the electrochemical gradient of protons, killing sensitive bacteria [35,36, 37] that the introduction of yogurt in the child's diet suffering from gastroenteritis allows weight gain, decreased hospitalization time and the frequency of watery stools. According to these studies, the introduction of the yogurt was done after the rehydration period. The use of probiotics in various health problems is increasingly documented. Yogurt is obtained by the fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. Several mechanisms action of probiotics are proposed such as synthesis of antimicrobial substances, competition for nutrients required for growth of the pathogen, competitive inhibition of the accession of the pathogen and a change of toxins or toxin receptor [38].

Tab3. Qualitative analysis of the acetic acid

Samples	RT (min)	D (difference)
<i>E.coli</i> + <i>Lp</i>	4,961	0,074
<i>E.coli</i> + (<i>St</i> *+ <i>Lb</i> *+ <i>Bl</i>)	4,959	0,072
<i>S.typhi</i> + (<i>St</i> 3+ <i>Lb</i> 1+ <i>Bl</i>)	4,969	0,082
<i>S.typhi</i> + (<i>St</i> 2+ <i>Lp</i> + <i>Bl</i>)	4,949	0,062

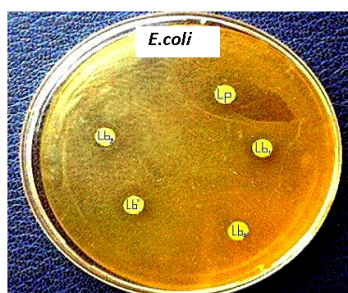


Fig.1: Interaction of acid lactic bacteria in pure culture with *E. coli*



Fig.2: Interaction of acid lactic bacteria in mixed culture with *E. coli*.



Fig.3: Interaction of acid lactic bacteria in mixed culture with *S.typhi*.

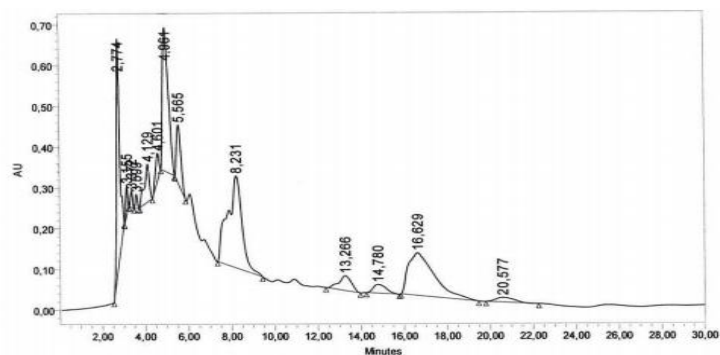


Fig.4: analyse of organic acids in supernatant of culture *E.coli*+*Lp*

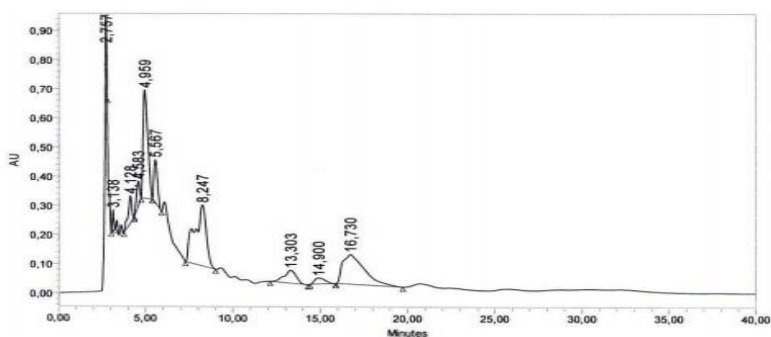


Fig.5: analyse of organic acids in supernatant of culture *E.coli*+ *St**+*Lb**+*Bl*

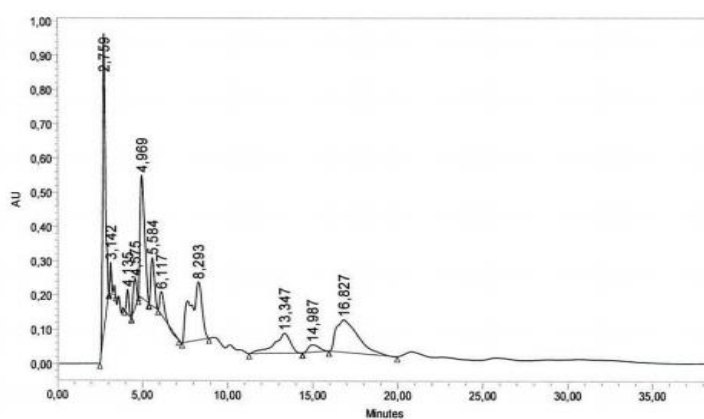


Fig.6: analyse of organic acids in supernatant of culture *S.typhi*+ *St3*+*Lb1*+*Bl*

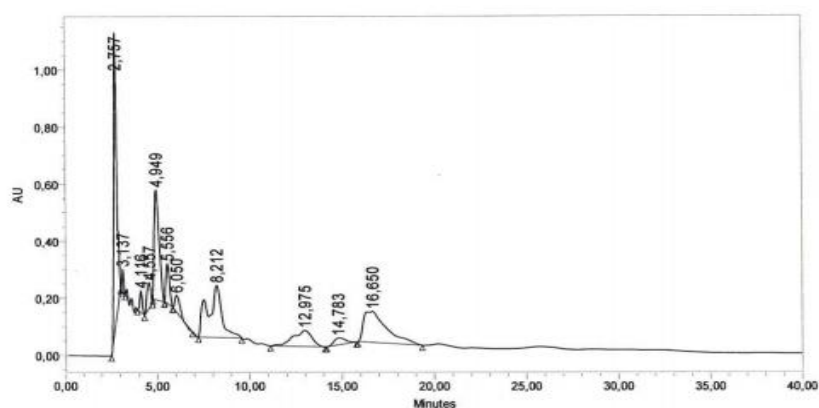


Fig.7:analyse of organic acids in supernatant of culture *S.typhi*+ *St2*+*Lp*+*Bl*

4. Conclusion

The interaction of lactic acid bacteria that have given the best microbial loads after counting and isolated pathogenic bacteria showed inhibitions in pure and mixed cultures. *Lactobacillus bulgaricus* 2, *Bifidobacterium longum* and *Lactobacillus bulgaricus* 1, *St2* + *Lp*, *St3* + *Lb1*, *St1* + *Lb3*, *St** + *Lb3*, *St1* + *Lb1* and *St** + *Lb** gave high levels of bacteria among all pure and mixed cultures of lactic acid bacteria enumerated. The addition of bifidobacteria to six best combinations, counting from mixed cultures, gave higher bacterial loads with *Bifidobacterium longum*. The cultures *L. paracasei* 1 (*Lp*) and *St**+ *Lb**+ *Bl* have a significant inhibitory effect of *Escherichia coli*. For *Salmonella typhi*, the results of interactions showed the best zones of inhibition with *St3* + *Lb1*+ *Bl* and *St2* + *Lp* + *Bl*. The qualitative and quantitative analysis of organic acids by high performance liquid chromatography (HPLC) was confirmed the presence of lactic acid in supernatants cultures and it may be alone or in combination with other substances responsible in the inhibition of *E. coli* and *S. typhi*.

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