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Assessment of the effect of *Artemisia annua* leave extract infusion pH under dark conditions on *Staphylococcus aureus*, *Salmonella paratyphi* and *Escherichia coli*

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ABSTRACT

Objectives: The influence of abiotic factors of water which may affect *Artemesia annua (A. annua)* efficiency as a disinfectant is not clearly elucidated. This study aimed to assess the potential effects of various water pH values of the infusion of *A. annua* leaves extract on some bacteria of hygiene and sanitary evaluation.

Methodology and Results: *A. annua* leaves were collected in Bangante (West region, Cameroon) and bacteria were isolated from the surface water (lotic hydrosystems) in Yaoundé (Cameroon, Central Africa). The bacteria used were *S. paratyphi*, *S. aureus* and *E. coli*. Experiments were carried out in aquatic microcosms under dark conditions. The pH values considered were 4, 5, 6, 7, 8 and 9. These values were adjusted using diluted HCl and NaOH. The results showed the temporal changes of cell abundance from one bacteria species to another with respect to the pH value in one hand, and also with respect to the number of cell species in solution on the other hand. In monospecific culture condition, the extract of *A. annua* seemed to appear as a source of carbon and energy for bacteria growth. In mixed cultures the influence of pH in the presence of the extract of *A. annua* in the dark seemed to foster trophic relations among bacteria in some cases and stimulate the production of inhibitory substances in other cases.

Conclusions and application of findings: Molecules contained in the extract of *A. annua* were potentially implicated in the physical and chemical changes of the medium, enabling the cell growth observed. *A. annua* extract did not have a significant bacterial inhibitory property in the water in the darkness. Although *A. annua* extract is often used as antibacterial component, it should not be used in the bacteriological treatment of drinking water under dark condition whether the water pH is acidic, neutral or alkaline.

Key words: *A. annua* extract, water pH, *S. aureus*, *S. paratyphi*, *E. coli,* temporal abundance, variation.

INTRODUCTION

Phytotherapy is increasingly used against infections in many parts of the world. Plants of the genus Artemisia, Asteraceae, are among those most exploited and several of its species were thus described as having antibacterial and antifungal effects. These included among others *Artemisia*

nilagirica, *A. siberie*, *A. annua* and *A. salina*. *A. nilagirica* is used against *Escherichia coli, Yersinia enterocolitica, Salmonella typhi, Enterobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa, Bacillus subtilis* and *Shigella flaxneri* (Ahameethunisa and Hopper, 2010*)* and it inhibits the metabolism of *Microsporum* (Vijayalakshmi *et al*., 2010). *A. siberie* has a potential inhibitory effect on *P. aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. *A. annua* negatively affects the culture of *Vibrio fischeri* (Lutgen *et al*., 2008). As for *A. salina,* it is known to act on *Cladosporium sphaerospermum*, *S. aureus*, *E. coli, Bacillus cereus* and *P. aeruginosa* (De Almeida *et al*., 2000). *A. annua* is grown on an industrial scale in many parts of the world for the manufacture of therapeutic combinations based on artemisinin or Artemesia combination therapies (ACTs), used in the treatment of malaria (Ferreira *et al*., 1996). Artemisinin, a bioactive molecules of the plant proved effective against schistosomes, flukes (Xiao *et al*., 2002), and some viruses (Han *et al*., 2008). This molecule inhibits the development of *Cryptosporidium, Giardia intestinalis, Entamoeba histolytica*, and various species of *Leishmania* (Utzinger *et al*., 2007; Kumar *et al*., 2004). The effectiveness of this molecule and its synthetic derivatives against *E. coli, Streptococcus faecalis, Salmonella abony* was demonstrated by Vikas *et al*. (2000). The use of infusions of *A. annua* showed its disinfecting power (capacity) in the bacteriological contamination of water (Lutgen and Michels, 2008; Allahdin *et al*., 2008). However, the influence of intrinsic abiotic factors on water is still not clearly elucidated in this method of disinfection. Abiotic factors such as pH, electrical conductivity, concentrations of dissolved oxygen and carbon dioxide affect the growth of microorganisms in water (Mezrioum and Baleux, 1992; Nola *et al*., 2002; Nougang *et al*., 2011). Besides, the effectiveness of water treatment procedures depends largely on pH. For the techniques of coagulation and flocculation, it is customary to adjust the pH to a value around neutrality to form possible flocs (Maudling and Harris, 1968). The effectiveness of chlorine as a disinfectant in water decreases as the pH increases. It is maximal at pH having a value around neutrality (Morris, 1971; Kott *et al*., 1975). According to WHO (2004), water and food can serve as routes of transmission of many bacteria of public-health concern mostly in developing countries. The diseases often reported include gastroenteritis, cutaneous infections, typhoid**,** colitis among others (Balbus and Embrey, 2002; Paiva de Sousa, 2006; Kuitcha *et al*., 2008). The causing bacteria include *S. typhimurium*, *S. aureus* and *E. coli* and their behaviors differ according to the general conditions of the environment and the nature of the disinfectant. Wegerlin and Sommer (1996) developed SODIS (Solar water Disinfection) and SOPA (Solar Pasteurization) methods which are based on the synergistic effects of UV rays and heat treatment of water by infrared heat. Water disinfection using the solar disinfection (SODIS) process relies on the synergistic effect of sunlight and temperature upon bacteria (Conroy *et al*. 2001; Reed 2004). A great part of the research in understanding the mechanism of this process has been done using transparent plastic bottles exposed to sunlight under different operating conditions. Many micro-organisms including bacteria respond to light. In recent years research focused on determining the bacterial inactivation rate under several physical environmental factors such as light intensity, radiance, temperature and turbidity (Sinton *et al*. 2002; Yukselen *et al*. 2003; Kay *et al*. 2005). However, little data are available on the combined influence of the darkness and various pH in the disinfection process. The present study aims to assess in the absence of light and under various conditions of pH, potential inhibitory effect of the extract of the leaves of *A. annua* on the three bacteria of health importance as cited above in aquatic microcosms.

MATERIALS AND METHODS

Collection of *Artemisia annua* leaves and extracts preparation: *Artemisia annua* leaves were harvested from Banganté, located in the western region of Cameroon (Central Africa). This region is located between latitude 5 and 16 north, and between longitude 10 and 11 west. Its climate is tropical and humid and has two main seasons: a dry season which last from October to March and a rainy season that runs from late March to October. The topography of the area is mountainous with plains and plateaus many of whose height range between 1000 and 1500 meters. Temperatures range from 15 to 27 °C with peaks in some areas of up to 37 °C. Nights are usually cool, especially between July to October. The soils of the western region are mostly lateritic, clay and volcanic in some area (UNDP, 2010). The leaves were harvested in July 2011 and then dried in the laboratory at room temperature (22 ± 2 °C) for 1 month. Thereafter, the leaves were ground into powder. An infusion was prepared from 20 g of dried ground leaves and mixed in 1 liter of boiling distilled water. The infusion was then filtered and left at room temperature (Muller *et al*., 2004; Blanke *et al*., 2008). The preparation of *A. annua* leaves in the form of infusion is customary to the ancient traditional Chinese medicinal method (Onimus *et al*., 2011).

Collection and identification of bacteria used: The bacterial strains used were isolated from surface waters of Yaoundé in Cameroon (Central Africa). They belonged to the species of *S. aureus*, *S. paratyphi* and *E. coli*. The isolation was performed respectively on Baird-Parker agar (Bio-Rad), Wilson-Blair (Biokar Diagnostics) and Endo (Biokar Diagnostics) by membrane filter technique, followed by incubation for 24 hours at 37 °C for *S. aureus* and *S. paratyphi*, and 44 ° C for *E. coli* (Rodier, 1996; APHA, 1998). Biochemical identification of the cells was done according to standard techniques described by Holt *et al*. (2000). Each pure strain was then cultured in trypto-casein broth (Bio-Rad) for 24 hours at 37 °C. The cells were then washed 3 times by centrifugation at 3600 rev/min for 15 minutes at 10 °C in NaCl (8.5 g/l). The pellet was then dissolved and 5 ml of each suspension was introduced into a test tube, and then preserved in cold glycerol.

Experimental protocol: Five (500) ml capacity flasks each containing 250 ml of NaCl solution (8.5 g/l) were used in this study. They were organized into three series: A, B and C of 7 round-bottom flasks (Erlenmeyer) each: f1, f2, f3, f4, f5, f6 and f7. For each series, the values of pH in flasks f1, f2, f3, f4, f5 and f6 were adjusted to 4, 5, 6, 7, 8 and 9 respectively. The pH was adjusted with HCl (0.1M) and NaOH (0.1M). According to Colin (2002), pH values greater than 9 or less than 4 have a bactericidal potential. Flasks f7 contained only NaCl (8.5 g/l), and served as a control. All seven flasks were sterilized in an autoclave. Each flask was then covered with aluminum foil and placed in the dark. Before the experiments, each frozen tube containing *S. aureus* or *E. coli* or *S. paratyphi* was thawed at room temperature. Then, 100 µl of the culture was transferred to a tube of 10 ml of nutrient broth (Oxford), and incubated at 37 °C. After 24 h, 100 µl of the suspension was added to 100 ml of nutrient broth and incubated for 24 h at 37 °C. The cells were then harvested by centrifugation at 3600 rev/min for 15 minutes at 10 °C and washed twice with a solution of sterile NaCl (8.5 g/l). The pellets were then re-suspended in 50 ml of sterile NaCl solution. After a series of dilutions, 1 ml of the suspension was added to 250 ml of sterile NaCl in each of the 7 Erlenmeyer (f1, f2, f3, f4, f5, f6 and f7) as shown above. Based on our preliminary study, the cell concentration was adjusted to 2.83 log (CFU/ml). Then, 10 ml of the extract of *Artemisia annua* prepared as described above was added to Erlenmeyer f1, f2, f3, f4, f5 and f6. The extract was not added to the Erlenmeyer flask f7 which was considered as the blank. The flasks were then incubated at room temperature (23 ± 2 ° C) in the dark. The experiments were carried out in three stages. In the first, a single strain of bacteria was added to each solution and it was called the "mono-species culture." In the second, two different strains were added to each flask at the same time and it was called the "dual-species culture." In the last step, the three strains were added at the same time in the flask, and called the "tri species culture." The analyses were performed in triplicate for each step using the three sets A, B and C.

Incubation of bacterial suspensions and analysis: Erlemeyers flasks containing the blank as well those containing the sample with *A. annua* prepared as described above were incubated for 10 hours at room temperature (23 ± 2 ° C) in the dark. Bacteriological analysis of the content of the Erlemeyers flasks was performed every 2 hours. The contents of each flask were first homogenized and then µl 100 was taken and analyzed by the technique of surface spreading on agar culture medium. The standard agar culture medium

contained in a Petri dish was used when the experiments were conducted in monospecific culture conditions. In bi- or tri- culture conditions, selective culture media were used. In this case, the culture media Baird-Parker agar (Bio-Rad), Wilson-Blair (Biokar Diagnostics) and Endo (Biokar Diagnostics) were respectively used for the isolation of *S. aureus*, *S. paratyphi and E. coli* contained in Petri dishes. These Petri dishes were then incubated for 24 hours at 37 °C for *S. aureus* and *S. paratyphi*, and 44 °C for *E. coli* (Rodier, 1996; APHA, 1998). The numbers of colony forming units were later determined.

Data analysis; The mean abundance of each species observed in each experimental condition were calculated and illustrated with histograms. Degrees of correlations were calculated between cell abundance and values of pH of the medium for each incubation period and experimental condition. Then for each pH value, the regression log (CFU number) depending on the duration of exposure to the extract of *A. annua* was drawn. The slope of each line was considered as the rate of change of apparent abundance of bacterial cells every 2 hours. The slope value was then divided by 2 to estimate the hourly rate of cellular evolution. When this value was positive, it indicated cell growth and when it was negative, it indicated an inhibition of cells. All data processing was performed using the program Statistical Package for Social Science (SPSS) version 12.0.

RESULTS

Temporal evolution of cell abundance: In mono specific culture, there was a gradual increase in the solution at pH 5 than at pH 9, as there were concentrations of culturable cells in the presence of the extract of *A. annua* at that pH. In these media, the cell abundance increased from the initial concentration of 2.83 log (CFU/ml) to maximum values of 3.43 log (CFU/ml) for *E. coli*, 3.83 log (CFU/ml) for *S. paratyphi* and 4.43 log (CFU/ml) for *S. aureus*. Highest concentrations of culturable cells were recorded after 10 hours of incubation (Figure 1). These values were obtained at pH 7, 8 and 9 for *S. paratyphi* and *E. coli*. The highest concentration of *S. aureus* was recorded at pH 8 and 9. For the three bacterial species, the lowest concentrations of culturable cells were recorded at pH 4 (Figure 1). In the control solutions, cell abundance increased to 3.53 log (CFU/ml) in *S. aureus*, 4.13 log (CFU/ml) in *S. paratyphi* but decreased to 1.91 log (CFU/ml) in *E. coli*, after 10 hours of incubation (Figure 1).

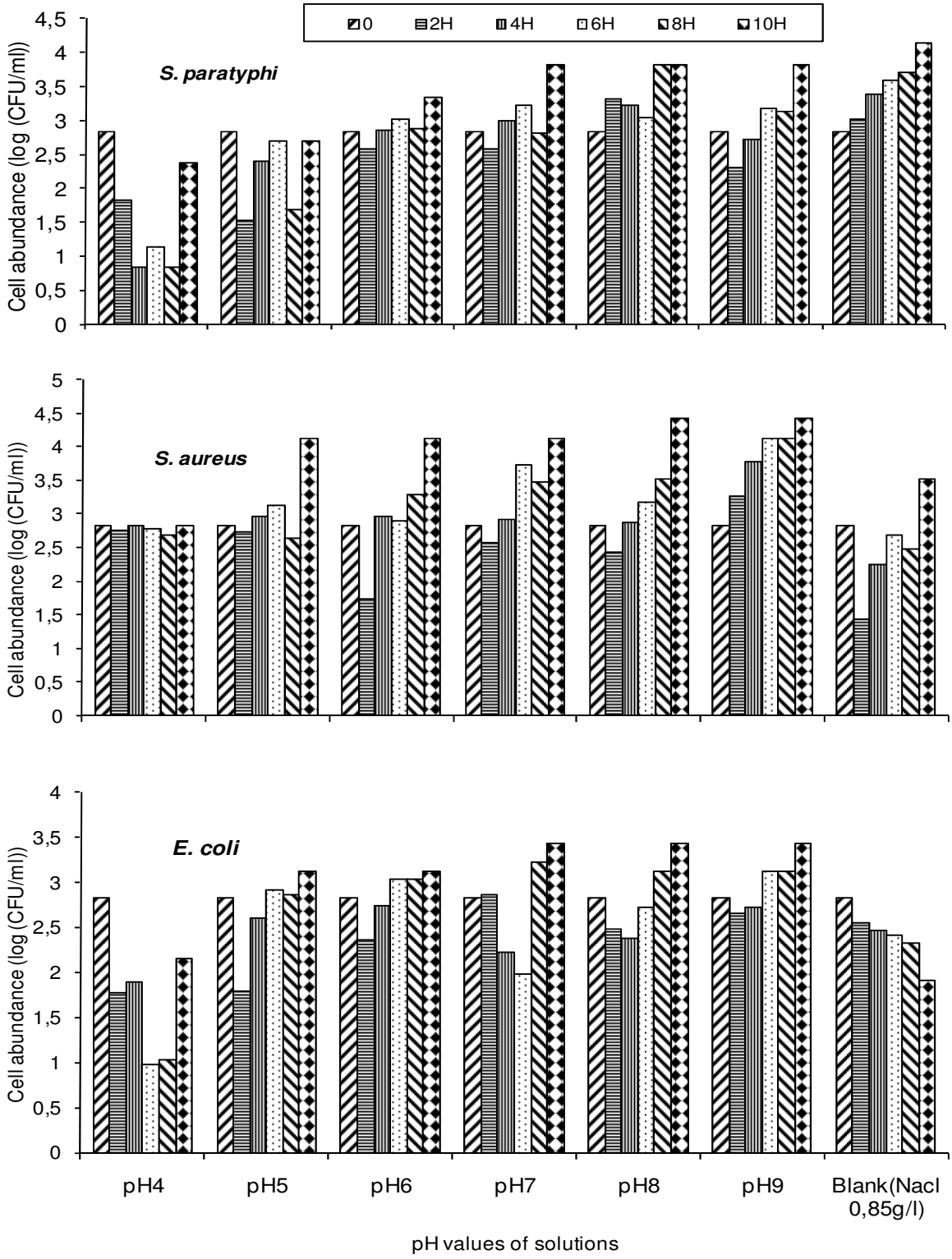

Figure 1: Variation of cell abundance with respect to the pH value when each solution contains only one bacteria species.

In bi-specific culture, there was an increase in abundance of the *E. coli # S. paratyphi* pair in solutions of pH 5 to pH 9 in the presence of the extract of *A. annua*. Cell concentrations reached maximum values of 3.45 log (CFU/ml) for *E. coli* and 4.26 for *S. paratyphi*. These maximum values were recorded at pH 8 and 9 after 10 hours of incubation (Figure 2). The same trend was observed with the pair *E. coli # S. aureus* (Figure 2) with highest concentrations of culturable cells of 3.37 log (CFU/ml) and 4.16 log (CFU/ml), respectively, for *E. coli and S. aureus*. Cell abundances were lower at pH 4 containing *S. aureus*, *S. paratyphi* and *E. coli*. However, when the bacteria *E. coli* and *S. paratyphi* were simultaneously present, the cultivable cells became rarer after 2-4 hours of incubation in the presence of the extract of *A. annua* (Figure 2). In the simultaneous presence of *S. aureus* and *S. paratyphi*, concentrations of culturable cells initially increased and then decreased in all solutions containing the *A. annua* extract after a certain time. This period ranged from 4 to 6 hours for *S. aureus*. These concentrations left from a maximum value of 3.60 log (CFU/ml) to minimum value 0.30 log (CFU/m) (Figure 2). For *S. paratyphi*, the evolution of the abundance of culturable cells was quite different from that of *S. aureus*. The decrease in the concentration of culturable cells was observed after a period ranging from 2 to 4 hours of incubation. In all control solutions of the three bacteria, there was a relative increase in abundance of culturable cells of *S. paratyphi*, but a relative decrease in the concentrations *of E. coli* and *S. aureus* (Figure 2).

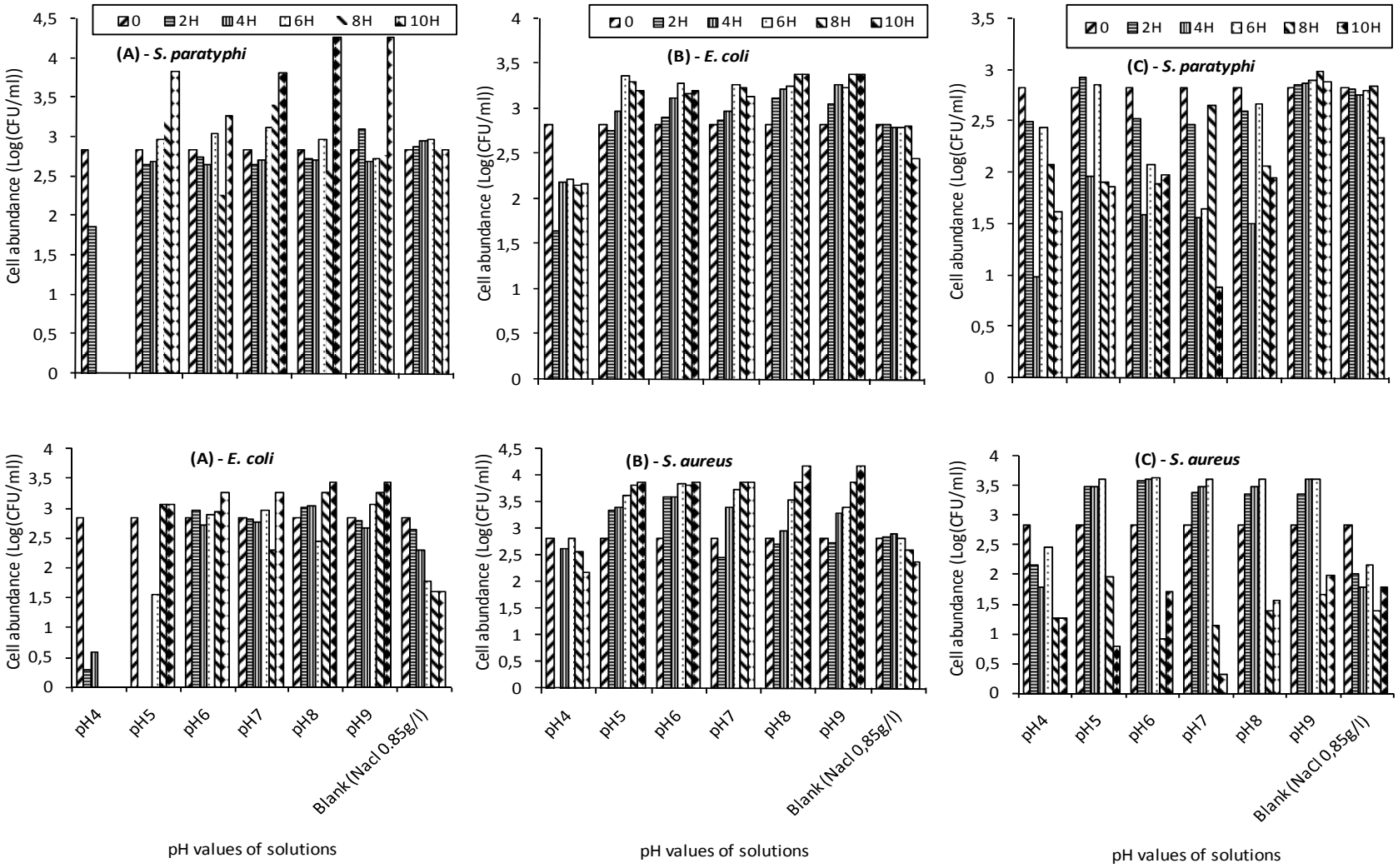

Figure 2. Variation of cell abundance with respect to the pH value when the solution contains dual *E. coli* and *S. paratyphi* (**A**), *E. coli* and *S. aureus* (**B**) or *S. aureus* and *S. paratyphi* (**C**).

In the presence of the three bacterial species, there was a relative decrease in the concentrations of culturable cells of *E. coli* and *S. paratyphi* after 4-6 hours of incubation in solutions containing the extract of *A. annua* at all pH values (Figure 3). Whereas, a gradual increase in abundance of *S. aureus* was observed during the whole incubation period in all solutions containing the extract of *A. annua*, except in the solution at pH 4 and the control (Figure 3).

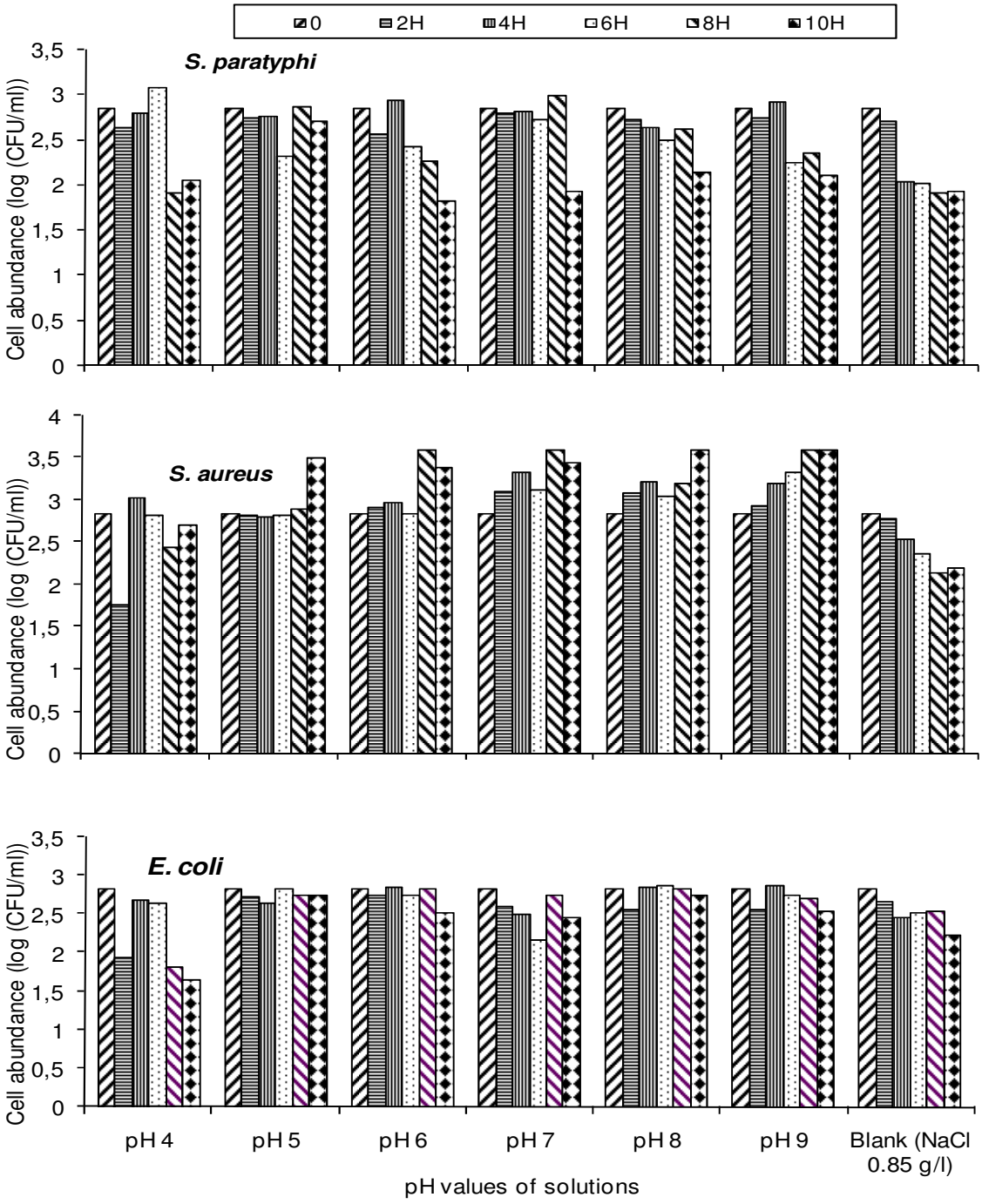

Figure 3: Variation of cell abundance with respect to the pH value when each solution contains the three bacteria species at the same time.

Assessment of the rate of variation of cell type abundance: Variations of cell type abundance were evaluated hourly. This was then referred to growth rate when the variation was positive, and the inhibition rates the variation was negative. It was noted that in monospecific culture, growth rates of bacterial cells gradually increase when the pH of the medium increased, in the solutions containing the extract from *A. annua*. When considering pH 5-9, these rates ranged from 925 to 2501/h for *S. aureus*, from 83 to 196/h for *E. coli* and 9 to 502/h for *S. paratyphi* (Table 1). Lowest growth rates were recorded at pH 5 and pH 6 and highest at pH 8 and pH 9. The growth of *S. aureus* was relatively faster than of *S. paratyphi* and *E. coli*. At pH 4, cell metabolism of the 3 species was relatively inhibited. At this pH, the inhibition rates were 42/h, 34/h and 6/h respectively for *E. coli, S. paratyphi* and *S.aureus* (Table 1). In the control solutions, growth rates of bacterial cells were 1116/h for *S. paratyphi* and 212/h for *S. aureus*. Growth of *S. paratyphi* was relatively faster than *S. aureus*. In the control solutions that is to say in the absence of *A. annua* extract, cell metabolism of *E. coli* was inhibited at the rate of 49/h (Table 1).

Table 1: Hourly changing rate of the cell abundance (and regression coefficient) of each bacteria species at each of the considered pH value, when the solution contains only one bacteria species.

Considered bacteria species	Changing rate of the cell abundance (h^{-1})						
	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	Blank Solution (NaCl 0.85g/l)
S. paratyphi	-34 (0.230)	-9 (0.017)	131 (0.568)	459 (0.486)	629 (0.689)	502 (0.576)	1116 (0.763)
S. aureus	-6 (0.096)	925 (0.436)	1002 (0.515)	1099 (0.655)	2035 (0.513)	2501 (0.899)	212 (0.383)
E. coli	-42 (0.362)	83 (0.512)	94 (0.699)	185 (0.469)	195 (0.599)	196 (0.745)	-49 (0.836)

In bi-specific culture solutions containing *A. annua* extract, the growth rate of bacterial species was highly variable from one pair of bacteria to another. When the bacteria *E. coli* and *S. aureus* were simultaneously present, the growth rates of *E. coli* ranged from 51 to 87/h and those of *S. aureus*, from 317 to 668/h, in the range of pH 5 to pH 9. Highest growth rates were recorded at pH 8 and pH 9, respectively, for *S. aureus* and *E. coli* (Table 2). When pair was *E. coli* and *S. paratyphi*, the growth rates of *E. coli* ranged from 36 to 109/h, and those of *S. paratyphi* 38 to 632/h, still in the range of pH 5 to pH 9. Highest growth rates were recorded at pH 8 and pH 9, respectively, for *S. paratyphi* and *E. coli*. For the pair *S. aureus # S. paratyphi*, there was in the majority of cases, cellular inhibitions. Inhibition rates ranged from 25 to 103/h for *S. aureus* and from 20 to 34/h for *S. paratyphi*. Highest Inhibition rate for *S. aureus* was observed at pH 6, whereas that of *S. paratyphi* was observed at pH 5 (Table 2). In control solutions, cellular inhibitions were observed in the presence of each pair of bacterial species (Table 2). When the three bacterial species were simultaneously present in the solutions containing the extract of *A. annua*, inhibitions of *E. coli* and *S. paratyphi* cells were observed in most cases. The highest inhibition rate for *S. paratyphi* was 31/h, observed at pH 9, while that *of E. coli* was 23/h recorded at pH 4 (Table 3). Cells of *S. aureus* grew, with the exception at pH 4. The highest growth rate was182/h, observed at pH 9. Overall, the rates of evolution of cell abundance of each of the 3 bacterial species, in absolute terms, varied relatively from one bacterial species to another, from one pH value to another and from one type of bacterial combination to another (Tables 1-3).

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Table 2: Hourly changing rate of the cell abundance (and regression coefficient) of each bacteria species at each of the considered pH value, when the solution contains dual species of bacteria at the same time.

Dual-species of bacteria considered		**Changing rate of the cell abundance (h^{-1})**						
Dual-species	Bacteria species	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	Blank Solution (NaCl 0.85g/l)
S. paratyphi #	*S. paratyphi*	-25 (0.575)	-34 (0.463)	-26 (0.614)	-20 (0.314)	-24 (0.480)	8 (0.411)	-15 (0.406)
S. aureus	*S. aureus*	-25 (0.526)	-79 (0.116)	-103 (0.131)	-69 (0.094)	-63 (0.080)	-68 (0.075)	-23 (0.486)
S. aureus #	*S. aureus*	-9 (0.062)	351 (0.958)	317 (0.853)	412 (0.917)	668 (0.794)	655 (0.776)	-24 (0.645)
E. coli	*E. coli*	-17 (0.305)	74 (0.554)	51 (0.650)	52 (0.573)	84 (0.942)	87 (0.911)	-15 (0.507)
E. coli #	*E. coli*	-24 (0.432)	42 (0.306)	45 (0.482)	36 (0.221)	88 (0.520)	109 (0.781)	-32 (0.845)
S. paratyphi	*S. paratyphi*	-26 (0.501)	248 (0.557)	38 (0.239)	258 (0.689)	632 (0.424)	618 (0.410)	0,52 (0.001)

Table 3: Hourly changing rate of the cell abundance (and regression coefficient) of each bacteria species at each of the considered pH value, when the solution contains all of the three bacteria species in solution at the same time.

Considered bacteria species	**Changing rate of the cell abundance (h^{-1})**						
	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	Blank Solution (NaCl 0.85g/l)
S. paratyphi	-23 (0.182)	-5 (0.038)	-30 (0.550)	-14 (0.143)	-22 (0.823)	-31 (0.651)	-30 (0.741)
S. aureus	- 4 (0.008)	90 (0.460)	124 (0.509)	126 (0.648)	117 (0.608)	182 (0.922)	-30 (0.911)
E.coli	-23 (0.429)	-3 (0.041)	-11 (0.375)	-12 (0.219)	9 (0.126)	-11 (0.237)	-21 (0.767)

Correlation between changes in cell abundances and pH values: When each bacterial species was present singly (i.e. in monospecific conditions), only one pH value favored significantly ($P<0.05$), an increased in cell abundance of *E. coli* in the presence of the extract of *A. annua* at pH 9 (Table 4). For *S. paratyphi*, it was pH 8 and pH 9 that favored significantly higher cell abundance ($P<0.05$). For *S. aureus*, there were pH 7, pH 8 and pH 9 which favored significantly an increase in the number of cells in the presence of *A. annua* extract (Table 4). Considering bi-specific culture conditions in the presence of *A. annua* leave extract, the cell abundance of *E. coli* increased significantly ($P<0.05$) at pH 5 to pH 8 when this bacterium was co-cultured with *S. aureus* (Table 4). At pH 9, an increase of cell abundance was also observed both in the presence of *S. aureus* and of *S.paratyphi* (Table 4). A significant increase in the cell abundance of *S. paratyphi* was observed only at pH 4, pH 7 and pH 5 when this bacterium was co-cultured with *E. coli*. In the presence of the extract of *A. annua*, a significant increase in the cell abundance of *S. aureus* was recorded at pH 4 in the presence of *S. paratyphi*, and at pH 5 and pH 9 in the presence of *E. coli* (Table 4). In tri-specific culture conditions in the presence of *A. annua* leave extract, the abundance of *E. coli* did not undergo any significant change at any pH value ($P>0.05$). Significant abundance of *S. paratyphi* were recorded at pH6, pH8 and pH 9 ($P<0.05$). In this tri-specific culture condition, cell abundance of *S. aureus* increased significantly ($P<0.05$) at pH 7 to pH 9 (Table 4).

Table 4: Correlation coefficients between cell abundances and the incubation durations at each pH value and in each of the experimental conditions (mono species, dual species and tri species of bacteria)

pH value and number of cell species present in solution at the moment			Bacteria species and correlation coefficient		
			E. coli	***S. paratyphi***	***S. aureus***
pH 4	Mono-species		-0.497	-0.315	-0.318
	Dual-species	*E. coli # S. paratyphi*	-0.749	-0.844*	/
		E. coli # S. aureus	-0.255	/	0.226
		S. aureus # S. paratyphi	/	-0.465	-0.826*
	Tri-species		-0.656	-0.668	0.145
pH 5	Mono-species		0.569	0.002	0.629
	Dual-species	*E. coli # S. paratyphi*	0.433	0.842*	/
		E. coli # S. aureus	0.812*	/	0.956**
		S. aureus # S. paratyphi	/	-0.709	-0.701
	Tri-species		-0.170	-0.186	0.703
pH 6	Mono-species		0.724	0.768	0.766
	Dual-species	*E. coli # S. paratyphi*	0.681	0.179	/
		E. coli # S. aureus	0.841*	/	0.827*
		S. aureus # S. paratyphi	/	-0.674	-0.628
	Tri-species		-0.632	-0.854*	0.771
pH 7	Mono-species		0.367	0.721	0.895*
	Dual-species	*E. coli # S. paratyphi*	0.152	0.895*	/
		E. coli # S. aureus	0.822*	/	0.879*
		S. aureus # S. paratyphi	/	-0.636	-0.738
	Tri-species		-0.408	-0.570	0.852*
pH 8	Mono-species		0.718	0.822*	0.883*
	Dual-species	*E. coli # S. paratyphi*	0.488	0.598	/
		E. coli # S. aureus	0.927**	/	0.955**
		S. aureus # S. paratyphi	/	-0.502	-0.656
	Tri-species		0.161	-0.866*	0.836*
pH 9	Mono-species		0.872*	0.825*	0.965**
	Dual-species	*E. coli # S. paratyphi*	0.868*	0.548	/
		E. coli # S. aureus	0.927**	/	0.970**
		S. aureus # S. paratyphi	/	0.670	-0.589
	Tri-species		-0.476	-0.864*	0.981**

Number of observations: 6 ; * :P< 0.05 ; ** : P< 0.01 .

DISCUSSION

The results showed that the temporal changes of the cell abundance varied from one bacterial species to another on one hand, and with respect of the pH value and type of bacterial combination on the other hand. In the presence of *A. annua* extract and with only *S. aureus* under neutral and slightly alkaline pH values (8 and 9), cell growth was favored. In the conventional culture conditions, the optimum pH for the growth rate of *S. aureus* was always observed at neutrality (pH 7). According to Holt *et al.* (2000), *Staphylococcus* can grow at pH values varying from 4 to 10 with an optimum growth at pH values between 6.5 and 7.5. This ability to

easily growth under slightly basic pH is observed in nutrient-rich environments. In this study the nutrients were provided by the environment; ions from NaOH and NaCl and molecules from *A. annua*. Growth of *S. aureus* requires an energy source such as organic complex sugars (fructose, glucose, galactose, mannose, ribose, maltose, sucrose, trehalose), alcohols (mannitol), organic acids (acetic acid) and, under certain conditions, amino acids (glutamine, arginine) (Charlier *et al*., 2009). The results can be explained by a combined action of pH and molecules of the *A. annua* extract on one hand, and by a pH influence, altering the physical and chemical properties of the molecules constituting the extract on the other hand. According to Sutherland *et al*. (1994), the effect of pH on the growth of *S. aureus* is linked to several environmental factors and also to the type of acid used to adjust the pH of the medium. In addition*, S. aureus* is a versatile opportunistic pathogen that can survive in different environments. This versatility depends on its ability to acquire and use nutrients from different sources (Yefei, 2010). In aerobic conditions, the final product of the glycolytic pathway of *S. aureus* is acetate. Acetate is accumulated in the culture medium during cell growth. When the medium is depleted in nutrients, cells of *S. aureus* are capable of changing the substrate and use the accumulated acetate as a carbon source (Somerville *et al*., 2002). In the experiments carried out, the growth of *E. coli* and *S. paratyphi* was favored by slightly alkaline pH in the *A. annua* extract solution. Both species also had an optimum growth pH value located around neutrality. Bacteria belonging to the Enterobacteriaceae family are chemo-organotrophic. They have simpler nutritional needs compared to *S. aureus* (Hantke *et al*., 2003; Prescott *et al.*, 2003). They also need in their environment a source of energy and substances for their growth. Leaves of *A. annua* contain organic and inorganic substances and bioactive compounds (Ferreira and Janick, 1996). Molecules from extracts of *A. annua* can be a source of nutrients in the experimental conditions and allow the growth of different species. Generally, bacterial growth is defined as an orderly increase of all the cellular components, leading to an increase in the number of individuals (Mainil, 2005). During the growth, there is a nutrient depletion of the culture medium and an enrichment of nutrient by-products of metabolism which are potentially toxic. Energetic metabolism of *E. coli* and *S. paratyphi* is fermentative. Fermentation of glucose, in both species begins with glycolysis and continues with a mixed acid fermentation. The products formed may be organic acids, alcohols, and gases, particularly with *E. coli* (Le Minor and Veron, 1989). The results showed that the presence of *E. coli* was favorable to the growth of *S. aureus* and vice versa. Concerning the nutrients availability, the doubling of the bacterial population could contribute to a rapid depletion of nutrient molecules and an accumulation of metabolic waste. The evolution of the physical and chemical properties of the environment seems to favor the growth of both species. This could be linked to a trophic and metabolic exchange or due to the syntrophic system in which the catabolites of one species become the essential and indispensable anabolites of the other and vice versa (McInerney *et al*., 2008). Such association has been reported between bacteria of the family Enterobacteriaceae and lactic acid bacteria (Jorgensen *et al*., 2000). *Pseudomonas* has also been noted as having potential in boosting the growth of *S. aureus* in milk (Seminiano and Frazier, 1966). In the presence of *E. coli*, the pH values 4, 5 and 7 boost the growth of *S. paratyphi*. When *S. aureus* and *S. paratyphi* are in co-culture, the pH 4 rather promotes the growth of *S. aureus*. Under these conditions, the pH 4 seems to have an influence on the behavior of *S. paratyphi* by encouraging the uptake of molecules from *A. annua* or the production of molecules that can promote the growth of *S. aureus*. It was observed that *S. typhimurium* produces molecules to enhance the buffering capacity of the cytoplasm under acidic stress conditions (Hall *et al*., 1995). Because this type of stress can cause an increase in the intracellular concentration, the use of lysine allows *S. typhimurium* to maintain a difference of one unit magnitude between extracellular and intracellular pH value (Bearson and Foster, 1997; Metzner *et al*., 2004). When the three bacteria species were simultaneously present, the pH values of 6, 8 and 9 inhibited the growth of *S. paratyphi*, while pH 7, 8 and 9 supported the growth of *S. aureus*. As *S. aureus* is known to not be a good competitor (Haines and Harmon, 1973), these results can be explained by the production of growth inhibitory substance by *S. aureus* targeting *S. paratyphi*. Enterotoxin production in *S. aureus* is maximal at pH values between 6.5 and 7.0 in laboratory conditions (Markus and Silverman, 1970; Su and Wong, 1998).

CONCLUSION

This study showed that the addition of the *A. annua* extract in water contaminated with *E. coli*, *S. aureus* or *S. paratyphi* in dark conditions caused a significant increase in the bacteria abundance in monospecific culture condition, when the pH was between 7 and 9. Acidic environments also favour growth in certain conditions of co-culture. Molecules contained in the extract of *A. annua* are potentially implicated in the physical and chemical changes of the medium, enabling the cell growth observed. These results suggest that the extract of *A. annua* does not have a bacteria inhibitory property in the water treatment in the darkness. The analysis of the chemical composition of the extract of *A. annua*, prepared according to the traditional Chinese method can enable to identify chemical molecules and their evolution in solutions used in these experiments.

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