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Antagonistic activity of Lactic Acid Bacteria Isolated from Raw Goat's Milk *Bifidobacterium* against *Helicobacter pylori*

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ABSTRACT

Different biological control agents are used to fight various pathogens or even harmful organisms in different environments. Among these agents, a wide range of bacteria belonging to the genus Bifidobacterium spp are capable to produce a large number of enzyme and secondary metabolites with the properties of antibacterial activity.

The main aim of the present work was the evaluation of the antagonistic activity of Bifidobacterium spp against pathogenic bacteria Helicobacter pylori, which was responsible for ulcer gastric disease by the using of agar well diffusion method and the study of the bacterial growth in the absence and in the presence of the supernatant of Bifidobacterium spp. The obtained results showed that the tested antagonistic activity of Bifidobacterium spp against Helicobacter pylori has manifested an excellent antibacterial activity with a maximal diameters of inhibition zone over 15 mm respectively. Furthermore, the study of bacterial growth of Helicobacter pylori in the absence and in the presence of the supernatant of Bifidobacterium spp indicated a considerable biomass reduction accompanied with unbalanced growth after adding of the supernatant.

Keywords*: Bifidobacterium spp, antagonistic activity, Helicobacter pylori.*

INTRODUCTION

Bacterial resistance to antibiotics poses a serious challenge to the prospect of chemotherapy, because the traditional antibiotics and its derivatives are becoming nonfunctional**.** The whole world is thus confronted with a looming drug crisis which has motivated the pursuit of new antibiotic compounds with novel mechanisms of action [1]**.** *Helicobacter pylori* is one of the most infectious human pathogens a gram negative, micro-aerophilic spiral shaped, flagellated, *Bacillus* which colonize the mucus layer of the gastric epithelium Sciences [2, 3]. *H. pylori* manifested a positive test for oxidase, catalase, and urease [4]. *H. pylori* infection is common worldwide in the United States, in South America and in Africa with prevalence rates ranging of (30-40%, 80-90%, 70-90 %) respectively [5-9].

Bifidobacterium are anaerobic Gram positive bacilli belonging to the dominant gut microbiota in humans and animals. Particularly, the genus *Bifidobacterium spp* has received much attention because of their association with numerous health-promoting effects, even though some mechanisms of these beneficial effects remain unexplained and their bacterial products have served the development of new pharmaceutical drugs that are widely used to fight bacterial infections [10,11]**.** The antibacterial properties of *Bifidobacterium spp* have therefore led to the characterization of various antimicrobial substances as organics acids (lactic acid and formic acids), diacetyl, and hydrogen peroxide alone or in combination [12]. Therefore, the objective of the present work was the investigation of the antagonistic activity of *Bifidobacterium spp* against *Helicobacter pylori* and the study of bacterial growth of *Helicobacter pylori* in the absence and in the presence of the supernatant of *Bifidobacterium spp*.

MATERIALS AND METHODS

Milk sample collection

Eight raw goat's milk samples were collected directly from dairy farms of Sidi Bel Abbes (Algeria). For the sample collection, the udder of goat was washed twice with sterilized water and disinfected with 70% ethanol. Furthermore, a volume of 100 ml of milk from each goat was collected aseptically in sterile bottles. The samples were transported immediately under refrigeration (4°C) to the laboratory for further analysis.

Isolation and identification of lactic acid bacteria

The bacteriocin producing strains were isolated from milk, by the dilution of a volume of 10 ml samples of milk in 90 ml saline solution, and the suitable serial dilutions were plated on the MRS Agar culture medium (Fluka, France) [13], incubated at 37°C for 48 hours the typical colonies were isolated and purified. The short-time conservation of the pure isolates has been achieved on a solid MRS culture medium; the obtained colonies were maintained at 4°C and renewed every month. The identification of isolates bacteriocin producing strains used in two steps was performed according to the methods of Badis and co-workers [14]. The first step was based on the coloration of Gram, the presence of the catalase and the production of spores of isolates strains, whereas, the second step was focused on the study of the morphological analysis such as macroscopic and microscopic observation and the type of fermentation. The determination of the physiological and biochemical criteria of the selected producing bacteriocin strains was based on the study of the bacterial growth on the MRS culture medium, supplemented with 0.05% (w/v) L-cysteine-hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) incubated anaerobically under an atmosphere generated using the OxoidAnaeroGenTM System (Oxoid Ltd., Basingstoke, Hampshire, England) at a temperature 37°C for 48 hours.

Biopsy specimens and culture

Gastric mucosal antral biopsy specimens were collected from each of 52 consecutive gastritis patients undergoing endoscopy. Gastric biopsy specimens were introduced in sterile tubes containing a volume of 0.5 ml of transport culture medium, composed of sterile physiological normal saline and kept at temperature of 4°C. The interval between the removal of the specimens and the inoculation on the culture media did not exceed 3 hours. The biopsy specimens were removed from the transport tubes and introduced in sterile tube containing a volume of 0.5 ml of a fresh saline. The biopsy specimens were then finely minced in a tissue grinder to dislodge the organisms. A volume of one hundred microliters (100 µL) from each solution was inoculated on the Mueller-Hinton agar culture medium plates, incubated at temperature of 37°C for up 7 days, in the presence of 100% humidity and under microaerophilic gas mixture, which was composed of 10% CO, 5% O_2, and 85% N (Campy-path; unipath S.P.A., Garvagabnate Milanese, Milan, Italy). The agar plates were checked for bacterial growth from the third days until to the seventh days. Furthermore, the obtained grown isolate was identified as *H. pylori* based on the followed positive test such as catalase, oxidase, and urease reactions; typical colony morphology (small round colonies), and the presence of characteristic curved Gram-negative bacilli on Gram-stained smears. The identified colonies were then inoculated in the culture medium containing alkaline peptone.

Microbial strains

The used antagonistic bacterial strain *Bifidobacterium spp* was isolated from milk product, whereas the used pathogenic bacterial strains *Helicobacter pylori* were isolated from Gastric mucosal antral biopsy specimens at the Center Hospital University (CHU) of Djillali Liabes of Sidi Bel Abbes (West Algeria). Furthermore, the microorganisms were maintained by routine culture on agar slants, stored at 4°C between transfers. At least two additional subcultures (24 h, 37°C) were made in fresh medium before the use in the experiment.

Detection of antagonistic activity by the well diffusion assay

The described well diffusion assay by Schillinger and Lucke [15] was used for the in-vitro test of antagonistic activities of *Bifidobacterium spp* against other bacteria. Plates containing solidified Nutrient Agar (20 ml) were overlaid with 10 ml of soft Nutrient agar and inoculated with 0.05 ml of an overnight culture of pathogenic bacteria. Four wells, three of the periphery and one at the centre, each 7 mm in diameter were made in the agar using cork borer and 0.1 ml of culture supernatant of test bacterium were transferred into each well. The plates were incubated for 24h at temperature of 37°C, examined for clear inhibition zone around the well. The assay was carried out in duplicate for all the test organisms.

Inoculum preparation

The used isolates were inoculated in nutrient broth, incubated at 37°C for 48 h and maintained as stock cultures in starch agar slants, grown at room temperature for 2 days and stored at 4 °C for regular sub culturing. The used culture media had the following composition (in g/L): 10 g starch, 10 g of nutrient broth, 15g agar, served as inoculation media for all the experiments. The pure culture was inoculated into sterile broth medium and incubated at 37°C on a rotary shaker at 75 rpm for 72h. The fresh overnight culture was used as inoculum for the growth study [16, 17]**.**

Preparation of culture supernatant

The used antagonistic strain *Bifidobacterium spp* was inoculated in a nutrient broth, incubated at temperature 37°C for 24 hours. The bacterial cells were removed by centrifugation at 6000 g for 10 min and the supernatant was used immediately for further investigation.

RESULTS

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants and microbes [18]**.** In the present study, the antagonistic activity of *Bifidobacterium spp* against *Helicobacter pylori* has been investigated. To explore the effect of some antibiotic such as Amoxicilline, Gentamicin, Tetracycline, Chloramphenicol, Cefotaxime and Ofloxacin on the bacterial growth of *Helicobacter pylori*, a disk method has been used. Furthermore, the study of the bacterial growth of *Helicobacter pylori* in the absence and presence of supernatant of *Bifidobacterium spp* has been investigated by the using the disk and the wells diffusion methods. The illustrated results (Table 1) of the effect some antibiotic (Chloramphenicol, Amoxicilline, Gentamicin, Oxyline, Tetracycline, Ofloxacin, Cefotaxime, Colistin) on the bacterial growth indicated a excellent antibacterial activity, with a maximal diameters of inhibition zone (29, 24, 24, 22, 19, 16, 13, 13) respectively. The obtained results of the antagonistic activity of *Bifidobacterium spp* highlighted against *Helicobacter pylori* showed a excellent antibacterial activity of the tested bacterial strain, with a clear zones inhibition at least 7 mm (Figure 3). Furthermore, the obtained diameters of zones inhibitions by the methods of disks and well diffusion assay were over 15 mm respectively.

Figure-1: Microscopic observation 100 × of the 24 hours old isolated, antagonistic *Bifidobacterium spp* from raw milk, showing rod shape, Gram positive and spore forming. Bars = 10µm.

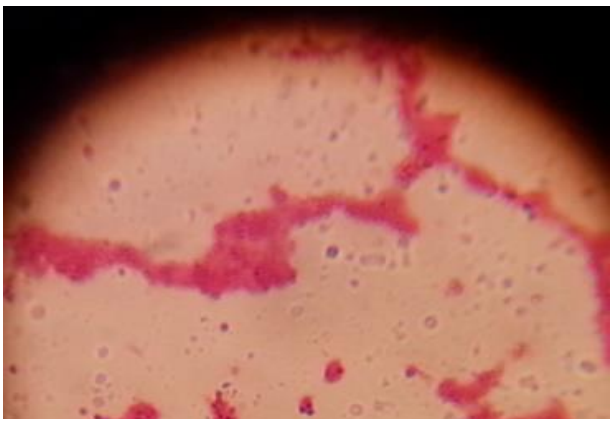

Figure-2: Microscopic observation 100 × of the isolated pathogenic bacterial strain *Helicobacter pylori* from , incubated under micro-aerophilic condition at 37°C for 5 days. Bars = 10µm.

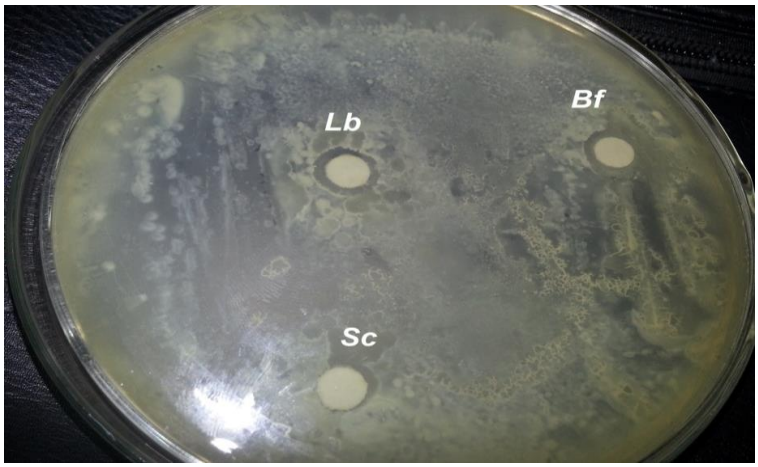

Figure -3: Illustration of antagonistic activity by the produced zone inhibition (mm) of the harvested supernatant of *Bifidobacterium spp*, cultivated on the MRS supplemented with 0.05% (w/v) L-cysteine-hydrochloride against *Helicobacter pylori*.

Table 1: Illustration of the antibacterial activity by the produced zone inhibition *Helicobacter pylori* in the presence of some antibiotic.

Tested Antibiotic	**The produced inhibition zone (mm) *Helicobacter pylori***
Amoxicilline	24
Gentamicin	24
Tetracycline	19
Chloramphenicol	29
Cefotaxime	13
Oxyline	22
Ofloxacin	16
Colistin	13

Study of *Helicobacter pylori* growth in the presence of supernatant of *Bifidobacterium spp*

In order to explore the effect of the used compounds present in the supernatant of *Bifidobacterium spp* against the investigated pathogenic bacteria, the bacterial growth of *Helicobacter pylori* in the presence and in the absence (control) of the supernatant of *Bifidobacterium spp* has been investigated. For this purpose, *Helicobacter pylori* was inoculated in culture medium (broth nutriments) with initial optical density of 0.5 measured at 578 nm according the protocol described by Abbouni and coworkers [19, 20]**.** A volume of 1 ml of the recovered supernatant of *Bifidobacterium spp* was added 7 hours after the onset of the bacterial growth. The obtained results (Figure 4) showed a considerable inhibition of the growth of *Helicobacter pylori*, after the adding a volume 1 ml of the supernatant of *Bifidobacterium spp* in the culture medium of *Helicobacter pylori* during early exponential growth phase. The growth curve of the test strain is plotted as a function of time. In conclusion, the molecules present in the supernatant of *Bifidobacterium spp* has induced unbalanced growth and furthermore the arrest of the cell cycle of *Helicobacter pylori* in comparison with the untreated biomass (balanced growth).

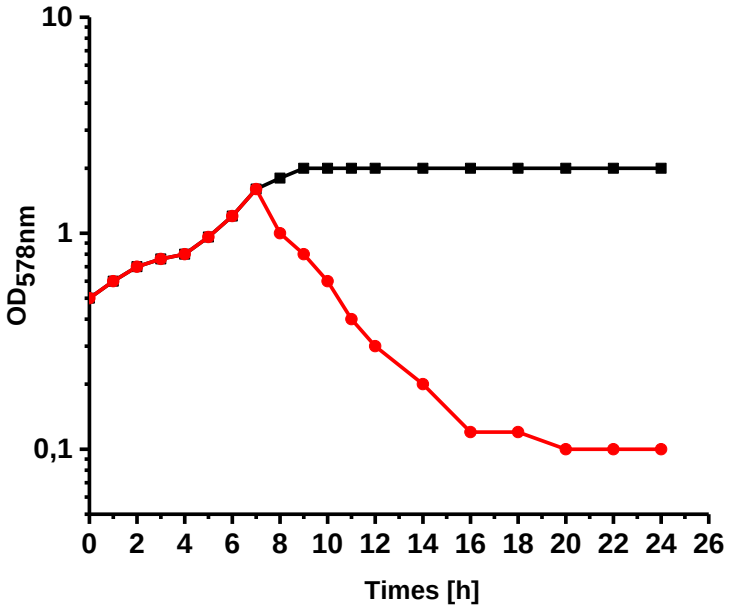

Figure-4: The study of *Helicobacter pylori* growth, inoculated on MRS culture medium, supplemented with 0.05% (w/v) L-cysteine-hydrochloride incubated at 37°C for 24 hours, in the absence (–■ –) and the presence (–● –) of the 7 hours old supernatant of *Bifidobacterium spp.*

DISCUSSION

Many studies suggested the ability of members belonging to the genus of *Bifidobacterium spp* to produce a various secondary metabolite, with antibacterial activity [21]. The investigated antagonistic strain of *Bifidobacterium spp* against of *Helicobacter pylori* has indicated a considerable inhibition of bacterial growth with the following diameters of inhibition zone over 15 mm respectively. Hays and co-workers [22] has reported that *Bifidobacterium spp* manifested a feeble antagonistic activity against gram-negative bacteria. Whereas, Brink and co-workers showed that of *Bifidobacterium spp* reduced the growth of *Helicobacter pylori* [23]. To explore the molecules responsible for antibacterial activity, the selected antagonistic strains of *Bifidobacterium spp* was inoculated in the culture medium and incubated at temperature of 30°C for 24 hours and the supernatant was harvested by using the centrifugation. Furthermore, the supernatant was investigated for antibacterial activity against of *Helicobacter pylori*. The obtained results indicated that the tested supernatant has considerably induced the inhibition of bacterial growth of of *Helicobacter pylori*.

Bendali and co-works [24] has reported that t the 18 hours old culture of *Bifidobacterium spp* culture filtrate has showed high activity against *Pseudomonas aeruginosa*. Whereas, the achieved study by Ashok Kumar and co-workers [25] indicated that *Bifidobacterium spp* strain, isolated from donkey milk, showed a maximum activity against *E. coli* and optimum activity against *S. aureus.* Furthermore, the obtained results indicated that *Bifidobacterium spp* manifested a weak activity against the tested Gram-negative bacteria, which could be explained by to the production of bacteriocins as previously reported by Furtado and co-workers [26].

Bifidobacterium spp species in the human intestinal system act as a barrier to infection and contribute to the control of the enteric microbiota by competing with other microorganisms for adherence to epithelial cells and inhibiting the growth of potential pathogens. Hence, the use of probiotic strains of *Bifidobacterium spp* is potentially interesting both as preventive and curative agents.

CONCLUSION

The delivery of viable bifidobacterial in yoghurt to the consumers remains a problem. Insufficient survival of *Bifidobacterium* spp in commercial and experimental products has been reported and viability of these organisms are in the selection and use of strains that are resistant to acids, bile, and oxygen and possess better *in vivo* colonizing ability such as *B. longum.* From our results, we could demonstrate the potential value of antagonistic activity of *Bifidobacterium spp* against pathogenic bacteria such as *Helicobacter pylori*. It would be of interest to find out which functional group is responsible for the antagonistic activity and also whether any of them is a novel compound with antimicrobial activity which would make it a promising candidate for the production of new antimicrobials. A Further study required involving the purification of the chemical compounds of the secondary metabolite produced by *Bifidobacterium spp* by the using a modern technique such HPLC, IRM, will require for determination of this active molecules.

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