

The Effect *Allium sativum* (Garlic Extract) as Prebiotic Substance on the Activity of Probiotic Bacteria *Lactobacillus acidophilus* against Some Locally Isolates of Pathogenic Bacteria

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Abstract

The aim of this study was to improve the growth and inhibitory activity of the selected *Lactobacillus* isolate. A total of twenty samples of dairy products (fresh yogurt) were collected. Seven isolates of *Lactobacillus* species. were obtained, and identified as three *Lactobacillus fermentum*, three *Lactobacillus acidophilus* and one of *Lactobacillus brevis*. The growth of *Lactobacillus acidophilus* was counted, also inhibitory activity of *Lactobacillus acidophilus* against some pathogenic bacteria such as (*Salmonella typhimurium* and *Pseudomonas aeruginosa*) was tested on solid medium (nutrient agar) for two different incubation periods before the addition of *Allium sativum* (garlic extract) as prebiotic substances. To improve the growth and inhibitory activity of the selected isolate (*Lactobacillus acidophilus*), three different concentrations (10, 20, 30 %) v/v of it were used by addition of it to De Man, Rogosa and Sharpe agar (MRS) medium as prebiotic substances, and two incubation periods (24, 48) hour. The effect of *Allium sativum* (garlic extract) on the growth of probiotic bacteria was evaluated, measurement of *Lactobacillus acidophilus* growth curve was done after and before adding garlic extract. For analyzing inhibitory activity of *Lactobacillus acidophilus* with the *Allium*, results showed that *Lactobacillus acidophilus* propagated in MRS fortified with 20% and 30% v/v garlic extract with incubation period 24 hr compared to propagating of *Lactobacillus acidophilus* in MRS alone, by giving the highest inhibition zone. It was concluded that *Lactobacillus acidophilus* isolate can grow well and exhibit good effect against Gram-negative bacteria in a medium containing garlic extract (that is added as a substrate supporting probiotic growth and activity). also it was clear that filtrate of *Lactobacillus acidophilus* isolate that propagating in specific broth fortified with 20% garlic extract at 48 hr incubation period produce antibacterial effect better than 10% but as same effect as in 30% against pathogenic bacteria, From above results it was concluded that prebiotic treatment enhance the growth of *Lactobacillus acidophilus* especially at 24 hr. this may due to fructooligosaccharide content in garlic have the best effect on the growth of *Lactobacillus acidophilus* bacteria, but less effect was observed after 48 hr incubation period, and also when propagated in MRS without prebiotic treatment, and which may due to highly decrease in pH (less than 3) and this may cause inhibition the growth of *Lactobacillus acidophilus* cells and hydrolysis of its cells.

Keywords: Prebiotic; Probiotic; Garlic extract; *Lactobacillus*; MRS medium

Introduction

Recently a great attention was paid to use microorganisms or their metabolites in the industry, food safety and treatment of some diseases. Bacteria are the first type of microorganisms used in this approach [1]. Among the bacterial group is the *Lactobacillus* spp. which have a great role as probiotics, due to their presence in mucous membrane of intestine and digestive tract of human as normal microflora. Also, it is safe used in food industry and their ability to produce inhibited materials such as organic acids, H₂O₂, CO₂, amino acid, di-acetyl, acetaldehyde and bacteriocins [2].

Beneficial effects of feeding an exogenous probiotic may be enhanced and extended by simultaneous administration of a prebiotic, that is non-digestible food ingredient improving the human health [3].

Many types of prebiotic were found naturally as prebiotic carbohydrate found naturally in fruits, vegetables and grains as well as other classes like dietary fiber and oligosaccharides. All those materials enhance growth of probiotics in several ways.

These synergistic effect of probiotic and prebiotic contributes to health, one example of prebiotic is disaccharides (lactose) which is primary carbohydrate of mammalian milk, and it is hydrolyzed into two monosaccharides (glucose and galactose) by the intestinal brush border enzyme lactase [4]. Lactose that is not digested by small intestine becomes a substrate for fermentation by bacteria in the colon by β -galactosidase enzyme, producing of short chain fatty acids that restrict the growth and activity of less beneficial species.

Dairy by-products are the main sources of the disaccharide lactose, which is a by-product of cheese industry. It contains diverse biologically components like lactose as a carbohydrate and other components of health benefits [5].

For such reason, this study was designed to Isolation and identification of lactic acid bacteria from local sources as probiotic. Using garlic extract as prebiotic available substances evaluation their effect in enhancing properties of the probiotic bacterial growth and/or activity. Determining the minimum inhibitory concentrations of concentrated filtrate of *Lactobacillus* bacteria before and after addition of garlic extract.

Method

Isolation and identification of *Lactobacillus* spp

Yogurt samples (20) of dairy food product were purchased from Baghdad market for isolation of *Lactobacillus* spp, Lactic acid bacteria were isolated from the samples by capturing a portion of 1 ml of sample and added to sterile test tube with 10 ml MRS broth, incubated at 37 °C for 24 hr, under anaerobic conditions. Serial dilutions prepared and 0.1 ml from last dilution was streaked on the surface of MRS agar containing 1% CaCO₃ in petri dishes, incubated for 24 hr at 37°C. The suspected LAB isolates were identified by microscopic examination (Gram stain) to examine cells shape, Gram reaction, grouping and non-spore forming phenomena. Then biochemical test in which catalase, oxidase, gelatinase tests were, also acid manufacture and clot creation test done then

production of ammonia from arginine tested. Finally to confirm the diagnosis the species of lactobacillus bacteria, carbohydrate fermentation test used to consult lactic acid bacteria species, It was done by using sterilized MRS broth after substituting glucose and meat extract by 1% of each of the autoclaved sugars (lactose, fructose, raffinose, maltose, mannitol, sucrose), also filtered sugars are used for carbohydrate fermentation test such sugars are (arabinose, xylose, galactose), then adding 0.004% of chlorophenol red reagent as an indicator, the pH was adjusted to 6.5 [6].

Also, gelatin medium agar was used to detected gelatin liquification in tubes, by inculcating 1% of LAB isolates, and incubating at 37°C for 48 hr. After that it was put into the refrigerator at 4 °C for 30 min. This test was performed to demonstrate the ability of isolates to hydrolyze gelatine [7]. acid production and clot formation test done in which tubes containing 10 ml of litmus milk medium were inoculated by 1% of the suspected bacterial culture and then incubated at 37°C for 48 hr. to detect color change, crude production and pH decrease as positive result, Growth at 45°C and 15°C tubes containing 10 ml MRS broth were inoculated with 1% of lactic acid bacterial culture then incubate at 15°C and 45°C for 24 hr. After incubation, growth was observed in the tubes and compared with control.

Growth Curve Measurement of *Lactobacillus acidophilus* isolate grow in MRS

To check the effect of garlic extract as prebiotic, on the growth of *Lactobacillus acidophilus*, measurement of *Lactobacillus acidophilus* growth curve were done before then after the addition of garlic extract to MRS medium [8].

200 ml of MRS broth was inoculated with 2 ml culture of *Lactobacillus acidophilus*, incubated on shaker incubator under anaerobic condition of 180 rpm at 37°C, optical density read in spectrophotometer at wave length of 600 nm at the time of inoculation and then each 2 hr for 24 hr. The producers were reiterated via MRS broth with prebiotic substances (garlic extract) in three different concentration (10,20,30% v/v) inoculated with probiotic bacteria *Lactobacillus acidophilus*, to find the best one for enhancing activity on probiotic bacteria *Lactobacillus acidophilus*.

Inhibitory Activity of *Lactobacillus acidophilus* Isolates against pathogenic test bacteria

Determining Inhibitory Effect of *Lactobacillus acidophilus* isolate that chosed randomly from other species against pathogenic bacteria *Salmonella typhimurium* and *Pseudomonas aeruginosa*) by using well diffusion method as following [9].

First, before adding prebiotic, inoculating MRS broth with of *Lactobacillus acidophilus* isolate in a test tube, the tube was incubated at 37°C for two different incubation periods (24 and 48 hr). The culture was centrifuged at 6000 rpm for 10 min this to get the supernatant which contain the filtrate of the grown bacteria in medium. After that, it was filtered through Millipore filter 0.22 μ m unit. Wells diffusion method was donw to detect the inhibitory effect of *Lactobacillus acidophilus* against test pathogenic bacteria by making wells on nutrient agar surface, filling them with the filtrates of lactobacillus acidophilus. After incubation the diameter of

inhibition zones around wells were measured and compared with control.

Secondly, it was determining inhibitory effect of *Lactobacillus acidophilus* propagating in MRS broth that mixed with garlic extract as prebiotic by inoculating 1% of previously activated bacteria (*Lactobacillus acidophilus* isolate) into the MRS broth enriched with garlic extract at different concentrations (10,20, 30 v/v), then adjust pH to 6, and incubate at 37°C for (24 and 48) hr. Then centrifuged at 6000 rpm for 10 min, cell free supernatant was taken and filtered throughout millipore filter (0.22µm). Well diffusion method was applied and inhibition zone diameter was measured as following, each pathogenic bacteria was streaked on surface of nutrient agar plate then wells were made by the aid of a cork borer, the wells was filled with the filtrate of *Lactobacillus acidophilus*

treated with garlic extract, then incubated at 37°C for 24hr, the inhibition zone diameter was measured.

Results and Discussions

Isolation and identification of *Lactobacillus* species

Seven isolates of *Lactobacillus* were isolated from a total of 20 yogurt samples, when grown on MRS agar, suspected *Lactobacillus* isolates produced colonies surrounded by clear zones. Colonies were white to pale in color, round, soft, mucoid, convex and having smooth edges. The gram staining demonstrated that suspected *Lactobacillus* isolates were gram +ve, short or long bacilli, grouped in long and short chain containing (3-8) cells but sometimes were single, non-spore former and non-motile. Biochemical tests were done as exposed in table 1.

Table 1: Biochemical test for identification of *Lactobacillus* sP.

Isolate	arabinose	Xylose	Galactose	Manitol	Maltose	Raffinose	Lactose	Fructose	sucrose
<i>Lb.acidophilus1</i>	(1)+	(-)	(1)+	(-)	(1)+	(1)+	(4)+	(1)+	(1)+
<i>Lb.acidophilus2</i>	(1)+	(-)	(1)+	(-)	(1)+	(1)+	(4)+	(1)+	(1)+
<i>Lb.acidophilus3</i>	(1)+	(-)	(1)+	(-)	(1)+	(1)+	(4)+	(1)+	(1)+
<i>Lb.fermentum1</i>	(2)+	(1)+	(1)+	(-)	(1)+	(1)+	(1)+	(3)+	(1)+
<i>Lb.fermentum2</i>	(1)+	(1)+	(1)+	(-)	(1)+	(1)+	(2)+	(2)+	(1)+
<i>Lb.fermentum3</i>	(1)+	(1)+	(1)+	(-)	(1)+	(1)+	(1)+	(1)+	(2)+
<i>Lb.brevis</i>	(1)+	(1)+	(1)+	(-)	(-)	(1)+	(-)	(2)+	(1)+

Note: +: positive fermentation, -: negative fermentation, (): number of days

Table pointed to that suspected *Lactobacillus* isolates yield clot when grown in litmus milk medium leading to reduction the pH from 6.5-4.5. Furthermore, all suspected isolates provided negative results for the catalase test so no bubbles were detected after addition of hydrogen peroxide to the colonies. The isolates also give negative results for both oxidase and gelatinase tests. In addition, they were not capable to crop ammonia from arginine-supplemented medium when the color of medium waited unchanged (or-

ange) after addition of Nessler reagent, but not all species. Besides that, all isolates were not capable to grow on nutrient agar. Some isolate was able to grow at 45°C, while other able to grow at 15°C.

The seven isolates of *Lactobacillus* species, were different in their capability to ferment the carbohydrate sources used as shown in table 2.

Table 2: Carbohydrate fermentation for differentiation between Lactobacillus ssp

Isolate	Catalase Test	Oxidase Test	Gelatinase Test	Growth in Litmus Milk	NH3 from Arginine	Growth at		Growth on Nutrient Agar
						15°C	45°C	
Lb.acidophilus1	-	-	-	+	-	-	-	-
Lb.acidophilus2	-	-	-	+	-	-	-	-
Lb.acidophilus3	-	-	-	+	-	-	-	-
Lb.fermentum1	-	-	-	+	+	+	-	-
Lb.fermentum2	-	-	-	+	+	+	-	-
Lb.fermentum3	-	-	-	+	+	+	-	-
Lb.brevis	-	-	-	+	+	+	-	-
Isolate	Catalase Test	Oxidase Test	Gelatinase Test	Growth in Litmus Milk	NH3 from Arginine	Growth at		Growth on Nutrient Agar
						15°C	45°C	
Lb.acidophilus1	-	-	-	+	-	-	-	-
Lb.acidophilus2	-	-	-	+	-	-	-	-
Lb.acidophilus3	-	-	-	+	-	-	-	-
Lb.fermentum1	-	-	-	+	+	+	-	-
Lb.fermentum2	-	-	-	+	+	+	-	-
Lb.fermentum3	-	-	-	+	+	+	-	-
Lb.brevis	-	-	-	+	+	+	-	-

It was shown that the Lactobacillus isolates which fermented all sugars but unsuccessful to ferment only mannitol were recognized as Lactobacillus fermentum. Isolates that fermented all sugars with the exception of maltose, mannitol and lactose were classified as Lactobacillus brevis. Isolates that unable to ferment both xylose and mannitol but ferment other sugars were Lactobacillus acidophilus.

According to the above results, three isolates were identified as Lactobacillus acidophilus, three isolates Lb. fermentum and one for Lb. brevis. Lactobacillus acidophilus isolates chosen to detect

its effect on pathogenic bacteria before and after adding prebiotic material randomly.

Growth Curve Measurement of Lactobacillus acidophilus isolate in MRS broth before addition of garlic extract

Growth characterization of the selected lactobacillus acidophilus was done to determine the extent of their growth phase when proliferated in MRS broth alone, comparing with their propagation in MRS broth containing garlic extract as prebiotic substance.

When a bacterium inoculated into a fresh MRS broth culture me-

dium, it exhibits a characteristic of growth curve [8].

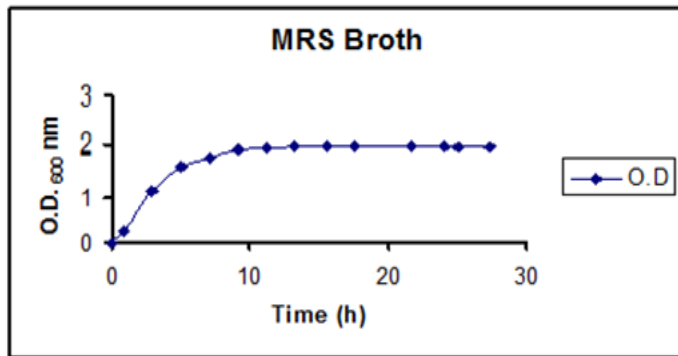


Figure 1: Growth Curve of *Lactobacillus acidophilus* isolate in MRS Medium.

Figure 1 shows that lag phase took about 2 hr. During this phase no upturn in cell number was detected which may be associated to preparation of cells for synthesis of DNA, inducible enzymes looked for cell division and reproduction. This phase existed followed by the log phase when the number of cells was bigger. A logarithmic growth remarked during the first 8 hr, then growth rate become at a slower speed for 24 hr. at which the isolate arrived the stationary stage [10].

Growth Curve Measurement of *Lactobacillus acidophilus* isolate Propagated in MRS Broth Fortified with garlic extract as Prebiotic Substances

Testing the improving effect of garlic extract on probiotic growth was observed. The growth curve of *Lactobacillus acidophilus* isolate propagated in MRS broth containing 10% garlic extract were examined as in figure 2.

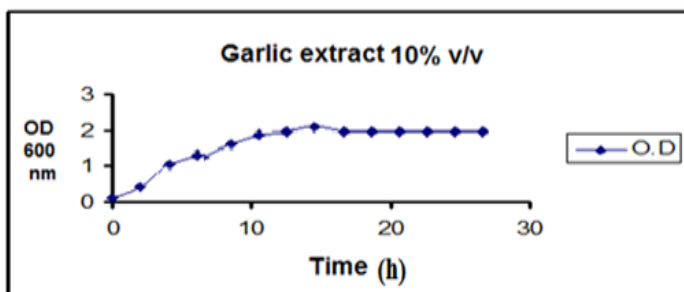


Figure 2: Growth curve of *Lactobacillus acidophilus* with 10 % garlic extract.

As soon as, *Lactobacillus acidophilus* was propagated in MRS broth containing 20% and 30% (v/v) of garlic extract The lag phase also take about 2 hr and result showed that there was increase in the growth of *Lactobacillus acidophilus* with garlic extract as prebiotic substance that mention in figures 3 and 4, noticing that there is no difference in growth curve between 20% and 30 %.

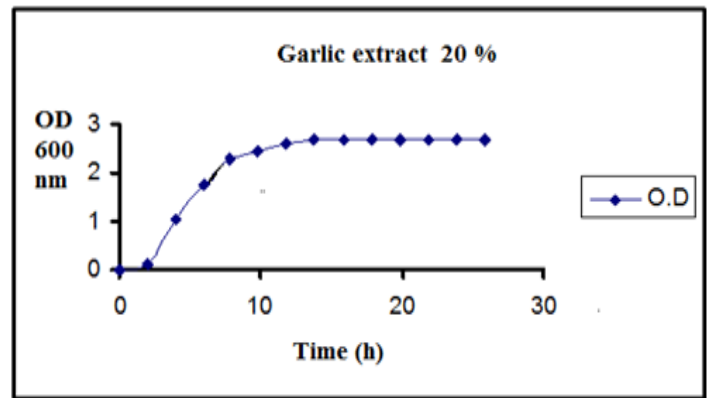


Figure 3: Growth Curve of *Lactobacillus acidophilus* isolate in MRS medium fortified with garlic extract 20% v/v.

In general, in comparing growth of probiotic bacteria *Lactobacillus acidophilus* before and after the addition of garlic extract, it was found that there is increase in growth curve of *Lactobacillus acidophilus* with 20% and 30% of garlic extract. That is mean the enhancement growth of probiotic bacteria by adding garlic extract to the MRS medium, may be due to composition of garlic and its active compound that found in it, such as the fructooligosaccharide (FOS) that found in garlic, it has effect on the growth of *Lactobacillus acidophilus* bacteria when it added to the media it change in growth behaviour [11,12].

Inhibitory Activity of *Lactobacillus acidophilus* isolates against pathogenic test bacteria

Well diffusion method used to evaluate the inhibition activity of selected *Lactobacillus acidophilus*. Filtrates bacteria was applied in this experiment after propagation in MRS broth at two incubation periods (24, 48 hr) against the tested pathogenic bacteria. This done by making wells on nutrient agar plates that have been cultured by the tested bacteria, then the wells were filled with *Lactobacillus acidophilus* filtrate. It was found that *Lactobacillus acidophilus* isolate give highest inhibitory activity when grown in liquid medium for all incubation periods used, when maximum inhibition zone diameters reached 14mm, this is due to the ability of MRS broth to exhibit wide spectrum inhibitory effect against Gram-positive and Gram-negative bacteria as *Lactobacillus* sp. propagated on it [13].

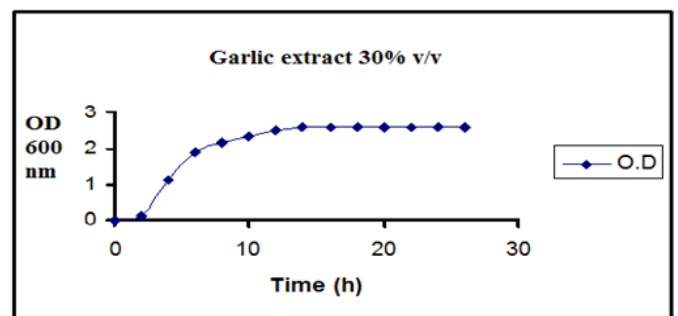


Figure 4: Growth Curve of *Lactobacillus acidophilus* isolate in MRS medium fortified with garlic extract 30%.

Table 3 and figure 5 displays the inhibitory effect of Lactobacillus acidophilus filtrate, as well as, it was established that 24hr period of incubation showed best inhibitory effect against test bacteria when inhibition zone diameter reached to 14 and 15.5 mm for Salmonella typhimurim and Pseudomonas aeruginosa respectively, but after 48 hr the inhibition zone for Salmonella typhimurim still the same while it decrease to 13 mm for Pseudomonas aeruginosa

Table 3: Inhibitory Activity of Unconcentrated Filtrate of Lactobacillus acidophils isolate against Test Bacteria after Propagating in MRS Broth for 24 hr and 48hr.

Inhibition Zone Diameter(mm)		
Pathogenic bacteria isolates	Incubation Periods(hr)	
S. typhimurium	24	48
Pseudomonas	14	14
aeruginosa	15.5	13

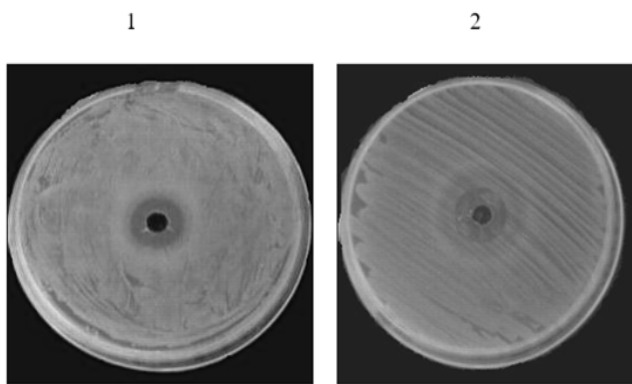


Figure 5: Inhibitory activity of Lactobacillus acidophilus isolate against test bacteria after propagating in MRS broth for 24 hr, 1: for Salmonella typhimurium, 2: for Pseudomonas aeruginosa.

These results in line with Al-Jebory who found that increasing incubation period to 48hr lead to unablty for increase the inhibitory activity instead less activity. While attained results was disagreement with those obtained by Al-Dulemy who found that the inhibitory effect increased after 48 hr [14,15]. The reason for such result may be that the inhibitory materials (plantaracin) are secreted outside the cells after increasing the incubation time causing decrease in the inhibitory activity of Lactobacillus against the pathogenic isolate.

Inhibitory Activity of lactobacillus acidophilus Propagated in MRS Medium after addition of Allium sativum (garlic extract)

Three concentrations of garlic extract (10, 20, 30% v/v) and two incubation periods (24, 48 hr) were used for assess the inhibitory activity of Lactobacillus. acidophilus isolate against the pathogenic bacterium.

Results in table 4 and figure 6 showed that, 10% garlic extract

have not raised Lactobacillus acidophilus activity at all incubation periods when inhibition zone diameter range between 14-15.5 mm, against pathogenic bacteria, but it was clear increasing in inhibition zone diameter when garlic extract concentration raise to 20% and 30%, so cause increase in inhibitory activity as with 20% of garlic extract at 24hr incubation period when inhibition zone diameter increased to a range between 18-19 mm against pathogenic bacteria. This range of inhibition zone diameter was still at the same rang when incubation period is 48hr. The same increases were recorded at 30% of garlic extract at 24hr and 48hr when inhibition zone ranged between 18 -19 mm against pathogenic test bacteria.

Table 4: Inhibition zone produced by Lactobacillus acidophilus isolate propagating in MRS broth fortified with different garlic extract concentration against test bacteria.

Conc. (%v/v)	Incubation period (hr)	Salmonella typhimurium	Pseudomonas aeruginosa
10	24	14	15.5
	48	14	15.5
20	24	19	18
	48	19.5	18
30	24	19	18
	48	18	18

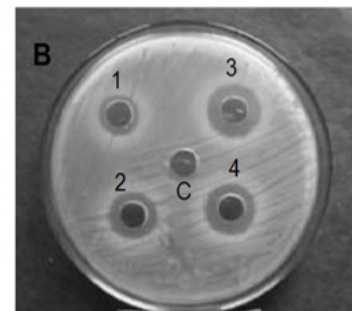
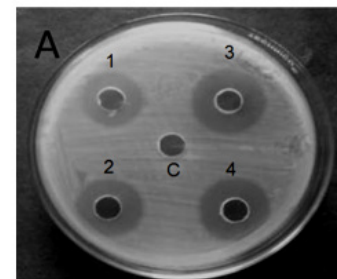


Figure 6: Inhibitory Activity of Lactobacillus acidophilus Filtrate against Test Bacteria after Propagating in MRS Broth Fortified with garlic extract for 24 hr. C: control, 1: Lb. acidophilus in MRS media alone, 2: Lb. acidophilus in MRS media with garlic extract 10% v/v, 3: Lb. acidophilus in MRS media with garlic extract 20% v/v, 4: Lb. acidophilus in MRS media with garlic extract 30% v/v,

A: *Salmonella typhimurium* as pathogenic bacteria, B: *Pseudomonas aeruginosa* as pathogenic bacteria.

Almost similar results were obtained by Kontula et al. who found that LAB may utilize lactose, and the probiotic action of the strains could be enhanced [16].

It was concluded that *Lactobacillus acidophilus* isolate can grow well and exhibit good effect against Gram-negative bacteria in a medium containing garlic extract (that is added as a substrate supporting probiotic growth and activity). From above results it was clear that filtrate of *Lactobacillus acidophilus* isolates that propagating in MRS broth fortified with 20% garlic extract at 48 hr incubation period produce antibacterial effect better than 10% but as same effect as in 30% against pathogenic bacteria.

The compromise of scientists in the European Project ENDO (DGXII AIRII-CT94-1095) was to spot the uniquely well accepted prebiotic action of beta(2-1)fructans (fructooligosaccharides and inulin [17,18]. which may increase the growth of probiotic bacteria *Lactobacillus acidophilus* so act as prebiotic material that increase activity against pathogenic bacteria as mention above.

It is clear that the fructooligosaccharide (FOS) content in garlic have the best effect on the growth of *Lactobacillus acidophilus* bacteria, when the FOS content in garlic was about 3.34% (b/b). This is consistent with the study of Altuntas and Korukluoglu, which reported that FOS is favored and fermented by *Lactobacillus* and *Bifidobacterium*. FOS and galactooligosaccharides are a composite of oligosaccharides (medium-chain carbohydrates) [11,19]. FOS mixtures are found in fruits and vegetables, including onion tubers, such as garlic (1-2%) [12].

This is consistent with Lopes et al. who informed that the addition of foods containing prebiotics such as oligosaccharides, the composition of *Lactobacillus* spp., will be increased by 81%. Supplementation with prebiotics at 0.1-0.2% can enhance the growth and activity of beneficial bacteria [18,20-22].

Conclusion

From above results it was concluded that prebiotic treatment enhances the growth of *Lactobacillus acidophilus* especially at 24 hr. this may due to fructooligosaccharide (FOS) content in garlic have the best effect on the growth of *Lactobacillus acidophilus* bacteria, when the FOS content in garlic was about 3.34% (b/b). but less effect was observed after 48 hr incubation period f, and also when propagated in MRS without prebiotic treatment, and which may due to highly decrease in pH (less than 3) and this may cause inhibition the growth of *Lactobacillus acidophilus* cells and hydrolysis of its cells. Despite that, several healthy important components are found in garlic extract, but it was rarely used due to many causes. Condensed (dried) garlic extract is easier to use due to its potential in various field such as prebiotic. However not much attention was conveyed to use some materials as prebiotics in Iraq.

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