5.1 Conclusions

1. Among nine *Lactobacillus* isolates, *Lactobacillus plantarum* was the most dominant in dairy samples as well as having best inhibitory effect against all test bacteria.

2. Selected *Lactobacillus plantarum* isolate, have the ability to produce β-galactosidase metabolizing lactose.

3. Inhibitory effect of probiotics bacteria was increased when various prebiotic substances were added.

4. Fresh whey (40%) v/v and dried whey (3%)w/v enhanced the inhibitory effect of probiotic bacteria.

5. Third fold concentrated filtrate of *Lactobacillus plantarum* that propagated in media containing prebiotic substances give the best inhibitoriest effect compared to that propagated in MRS alone.

5.2 Recommendations

1. Using other different synthetic prebiotic substances like dietary fiber to detect its healthy enhancing properties.

2. Extended studies are needed to show the effect of prebiotic on another species of lactic acid bacteria.

3. *In vivo* studies needed to investigate the synergistic effect of probiotic and prebiotic on gut microbial populations.

4.1 Isolation of *Lactobacillus* Species:

From a total of 17 dairy product samples collected from Baghdad local markets, nine isolates of *Lactobacillus* were obtained. They were firstly identified depending on their ability to form clear zones around the colonies when cultured on MRS agar containing 1%$CaCO_3$, due to the acid produced by the isolates which dissolved the $CaCO_3$. Then the isolates were further identified depending on their cultural, morphological and biochemical characteristics.

4.2 Identification of *Lactobacillus* Species:

4.2.1 Cultural Characteristics:

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. Such cultural characteristic are concerned with those of *Lactobacillus* species (Kandler and Wess, 1986).

4.2.2 Morphological Characteristic:

Microscopical examination after 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 are single, non-spore former and non-motile. So they are related to the *Lactobacillus* spp (Atlas *et al.,* 1995).

4.2.3 Biochemical Tests:

Biochemical tests shown in table (4-1) indicated that suspected isolates were able to produce clot when grown in litmus milk medium

leading to decrease the pH from 6.5- 4.5. Furthermore, all suspected isolates gave negative results for the catalase test when no bubbles were observed after addition of hydrogen peroxide to the colonies. The isolates also gave negative results for both oxidase and gelatinase tests. In addition, they were unable to produce ammonia from arginine-supplemented medium when the color of medium stayed unchanged (orange) after addition of Nessler reagent, unless some species. Moreover all isolates were unable to grow on nutrient agar. Some isolate were able to grow at 45ºC, while other able to grow at 15º C.

In order to differentiate the nine isolates of *Lactobacillus* species, carbohydrates fermentation test was performed. The isolates were different in their ability to ferment the carbohydrate sources used..The isolates which fermented all sugars but not xylose were identified as *Lactobacillus plantarum,* while those fermented all sugars and failed to ferment only manitol were identified as *Lactobacillus fermentum*. Isolates that fermented all sugars except maltose, manitol and lactose were classified as *Lactobacillus brevis.* Finally isolates which were unable to ferment both xylose and manitol but ferment other sugars were considered to be belonging to *Lactobacillus acidophilus* (Hammes and Vogel, 1995).

According to the above result, four isolates were identified as *Lb. plantarum,* three isolates were identified as *Lb. fermentum* and one isolate was identified as *Lb. acidophilus* while the another one was identified as *Lb. brevis.*

4.3 Inhibitory Effect of *Lactobacillus* Isolates against Test Bacteria

4.3.1 on Solid Medium:

Testing the inhibitory effect of *Lactobacillus* isolates was done against some pathogenic test bacteria by propagating on MRS agar medium at different incubation periods (24, 48 and 72) hr.

Table (4-2), showes the inhibitory effect of Lactobacillus isolates grown on MRS agar against all test bacteria. It was found that most isolates possess inhibitory effect at various levels. However, isolates of *Lb.plantarum* were more effective by exhibiting highest inhibitory effect against all test bacteria. Like *Lb.plantarum* 1 isolate exhibited good inhibitory effect against test bacteria when the inhibitory zone reached 10 mm and 12 mm after (24hr) with *Staphylococcus aureus* and *Bacillus cereus,* and 8 mm for both *Pseudomonas aeruginosa* and *Escherichia .coli*, respectively. While increasing incubation period to 48 hr and 72 hr, was showed slight decreases in the inhibition zone observed when it was ranged between (7-10.5mm) after 48 hr incubation period and ranged between 7.5-10.5mm after 72 hr incubation period against test bacteria. *Lb.plantarum* 2 isolate was recorded the highest inhibitory effect after 24hr incubation period when inhibitory zone reached 10, 14.5, 10 and 13mm against *S.aureus*, *B.cereus, P. aeruginosa* and *E.coli,* respectively. Figure (4-1) shows that both Gram-positive isolates (*S.aureus* and *B.cereus)* and Gram-negative isolates *(P.aeruginosa* and *E.coli)* were highly affected by isolate, *Lb.plantarum* 2 after 24hr of incubation. Almost similar results were obtained by Jimenez-Diaz *et al.*(1993) and Nitagu and Gash (1994) who found that *Lactobacillus plantarum* excreted good inhibitory effect against Gram-positive and Gram-negative bacteria. Increasing incubation

period to 48 hr showed no improvement in the inhibitory effect when inhibition zone diameter remain as it is.

Table (4-2). Inhibitory Effect of *Lactobacillus* Isolates Against Test Bacteria on Solid Medium (MRS agar) after Different Incubation Periods.

Isolates	Incubation Periods (hr)	Inhibition zone diameter			
		Tested Bacteria			
		S.aureus	***E. coli***	***p.aeruginosa***	***B.cereus***
Lb.plantarum1	**24**	10	8	8	12
	48	9.5	8.5	7	10.5
	72	9	8.5	7.5	10.5
Lb.plantarum2	**24**	10	13	10	14.5
	48	10	13	10.5	14
	72	8	9.5	10	11
Lb.plantarum 3	**24**	11	9	8	10
	48	12	12	12.5	10.5
	72	11.5	12.5	11	9
Lb.plantarum.4	**24**	11	11	11.5	10.5
	48	13	12	12	13
	72	11	13	11	11
Lb.Fermentum.1	**24**	12	11.5	9.5	8
	48	10	11	9	6
	72	10	11	9	6
Lb.Fermentum.2	**24**	5	7	5	8
	48	8	7.5	7	10
	72	5	5.5	6	7
Lb.Fermentum.3	**24**	5	11	10	8
	48	5	11.5	10.5	8
	72	-	10	9	-
Lb.acidophilus.	**24**	10	9.5	9	11
	48	12	11	11.5	12
	72	12	11	11.5	12
Lb. brevis.	**24**	5	6.5	5	5.5
	48	10	9	11	14
	72	9	9	10	12

(-)= No Effect

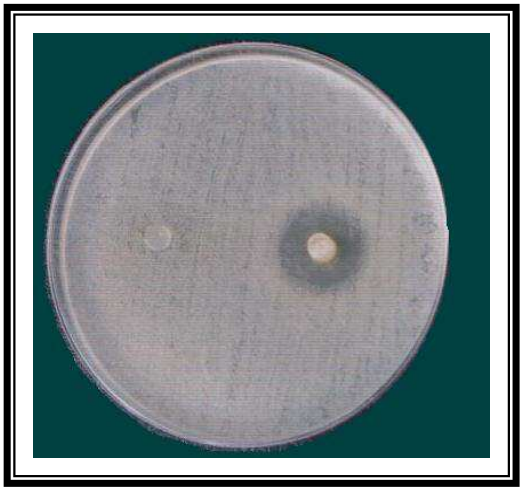
In contrast, after 72hr less inhibitory effect was observed, when inhibition zone diameter decreased to 8, 11, 10 and 9.5 mm against *S.aureus, B.cereus, P.aeruginosa* and *E. coli,* respectively. *Lb.plantarum 3* and *Lb.plantarum* 4 isolates also have inhibitory effect but less than *Lb.plantarum1* and *Lb.plantarum 2* isolates, when inhibition zone diameter ranged between (8-11mm) after 24hr with *Lb.plantarum3* isolate and ranged 10.5-11mm with *Lb.plantarum* 4 isolate, with same incubation period. However extending incubation period to 48hr show better inhibitory effect for both isolates when inhibition zone reached to 12, 10.5, 12.5 and 12mm, against *S.aureus, B.cereus, P.auroginosa* and *E. coli*, respectively with *Lb.plantarum3*, while reached to 13, 11 and 12mm against *S.aureus, B.cereus, P.auroginosa and E.coli* with *Lb. plantarum*4. This result almost agreed with those obtained by Al-Dulemy (2000) who found that the inhibitory effect of LAB increased after 48hr of incubation. But Al-Jeboury (2005) found that LAB gave good inhibitory effect after 24hr. On the other hand, after 72hr incubation no increases in inhibitory effect for both isolates (*Lb.plantarum* 3 and *Lb.plantarum* 4) isolates was obtained, when range of inhibition zone diameter remain without changes (9-12.5mm) with *Lb.plantarum* 3 and (11-13mm) with *Lb.plantarum* 4. *Lb.fermentum* 1 isolate showed varied inhibitory effect with different incubation periods. It has good effect at 24hr against test bacteria when inhibition zone diameter were 12, 8, 9.5 and 11.5mm against *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively. With increasing incubation period to 48hr and 72hr, inhibitory effect decreased and inhibition zone diameters were 10, 6, 9 and 11mm for *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively. *Lb.fermentum2i*solate have less inhibitory effect against all test bacteria at all incubation period, when maximum inhibitory effect recorded for this isolate after 24hr was 8mm for *B.cereus*, maximum

inhibitory effect of the same isolate after 48hr was 10mm against *B.cereus* also. Increasing incubation period to 72hr, result in decreasing inhibitory effect to very low level when it reached 5, 7, 6 and 5.5mm for *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively.

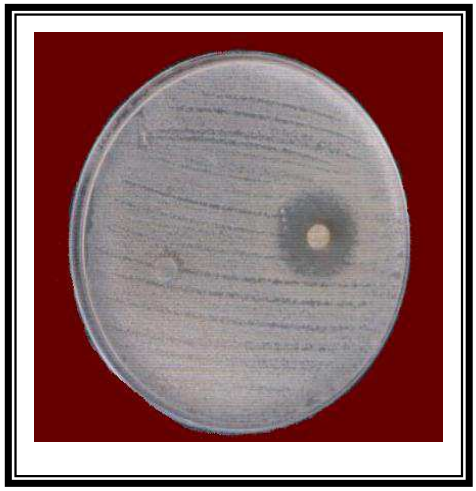
Lb.fermentum3 isolate, have no inhibitory effect against S.aureus after 24hr of incubation but it has good inhibitory effect on G- bacteria also on B. cereus, this agreed with result obtained by Al-Obidy (1997) how found that LAB gave inhibitory effect after 24hr. While increasing incubation period to 48hr have the same effect when inhibition zone remain as it is. While after 72hr on incubation period *Lb. fermentum 3* isolate have no effect on Gram-positive *S.aureus* and *B.cereus,* but showed slight effect on Gram-negative bacteria *P. aeruginosa* and *E. coli* when inhibition zone reached to 9 and 10 mm, respectively. *Lb.acidophilus*, exhibited moderate inhibitory effect after 24hr of incubation against the test bacteria when slight increases were recorded in the inhibition zone, which diameters were estimated as 10 and 11mm for Gram-positive(*S.aureus* and *B.cereus*) and 9-9.5mm for Gram-negative(*P.aeruginosa* and *E.coli).* The same increases was also recorded when incubation period increased to 48hr and 72hr for such isolate, when inhibition zone diameters reached to 12, 12, 11.5 and 11mm for *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively The last isolate *Lb. brevis* differ from other above isolates, it has slight inhibitory effect against all test bacteria in all incubation period especially 24hr, inhibitory zones were 5, 5.5, 6 and 5mm against *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively. But it increased when incubation period become 48hr with diameter 10, 14,11 and 9mm with *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively.

A) *S.aureus*

B) *B.cereus*



C) *P.aeruginos*



D) *E.coli*

Figure (4-1). Inhibitory Effect of *Lb.plantarum* 2 Isolate against Test Bacteria after Propagating on Solid Medium for 24 hr.

. Present results were nearly close to the results obtained by Al-Yas (2006) who found that inhibitory effect of LAB increased after 48hr of incubation. Generally, incubation period 24hr resulted in production of more inhibitory effect by almost all Lb. isolates especially *Lb.plantarum2*. Aktypis *et al.*(1998) referred such differences in the inhibitory effect at different incubation periods may be needed to the nature of LAB isolates used against test bacteria. While Vingholo *et al.*, (1995) referred that to the test bacteria itself. For its highest inhibitory effect, *Lb.plantarum2* isolate was selected for further experimenting in this study.

4.3.2 in Liquid Medium

Well diffusion method was used to determine the inhibition effect of selected LAB isolate (*Lb.plantarum* 2). Filtrates of this bacteria was applied in this experiment after propagation in MRS broth at different incubation periods (24, 48 and 72 hr) against test bacteria. By filling the wells of nutrient agar plates that have been cultured by test bacteria with the filtrate. Table (4-3) exhibits the inhibitory effect of *Lb.plantarum2* filtrate. It was found that 24hr period of incubation showed best inhibitory effect against Gram-positive bacteria when inhibition zone diameter reached to 16 and 13.5 mm for both *S.aureus* and *B.cereus*, respectively and 15.5 and 13mm for *P.aeruginosa* and *E.coli* respectively.It was found that *Lb.plantarum2* isolate give highest inhibitory effect when grown in liquid medium than on the solid medim for all incubation periods used, when maximum inhibition zone diameters reached 16mm, which is higher than that recorded by the solid media. This due to the ability of MRS broth to exhibit wide spectrum inhibitory effect against Gram-positive and Gram-negative bacteria (Gupta *et al.*, 1998). Figure (4-2), shows inhibitory effect of filtrate of *Lb.plantarum* 2 isolate when propagated in MRS broth after 24hr period of incubation.

Increasing incubation period to 48hr showed less inhibitory effect against Gram-negative bacteria when inhibition zone diameter reached to 10 mm for *E.coli*, and no change was observed against *P.aeruginosa*. Also, in Gram-positive bacteria such as *B.cereus* after such period, less inhibitory Effect was obtained when inhibition zone diameter reached to 10 mm. While increasing period to 72hr resulted in less inhibitory effect effect for *Lb.plantarum* filtrate against all tested bacteria, when all inhibition zone diameters decreased to 8.5, 9, 8 and 7.5mm against *S.aureus*, *P.aeruginosa, B.cereus* and *E.coli,* respectively. These results agreed with those obtained by Al-Jebory (2005) who found that increasing incubation period to 48hr and 72hr were unable to increase the inhibitory effect instead less Effect was recorded. While obtained results was disagreement with those obtained by Al-Dulemy (2005) who found that the inhibitory effect increased after 48 hr. 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 effect.

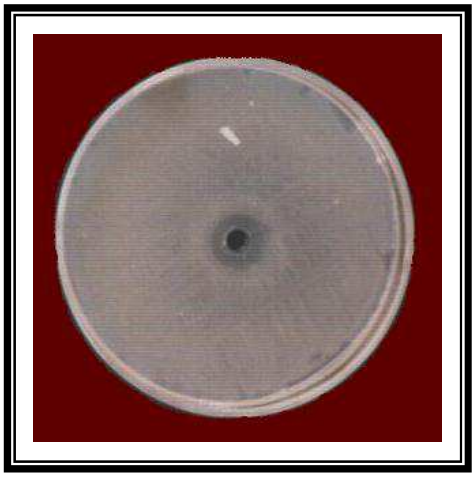
Table (4-3): Inhibitory Effect of Unconcentrated Filtrate of *Lb. plantarum*2 Isolate Against Test Bacteria after Propagating in MRS Broth for 24 hr.

Test bacteria	Diameter of inhibition zone (mm)		
	Incubation Periods(hr)		
	24	48	72
S. aureus	16	16	8.5
P. aeruginosa	15.5	15.5	9
B. cereus	13.5	10	8
E. coli	13	10	7.5

A) *S.aureus*

B) *B.cereus*

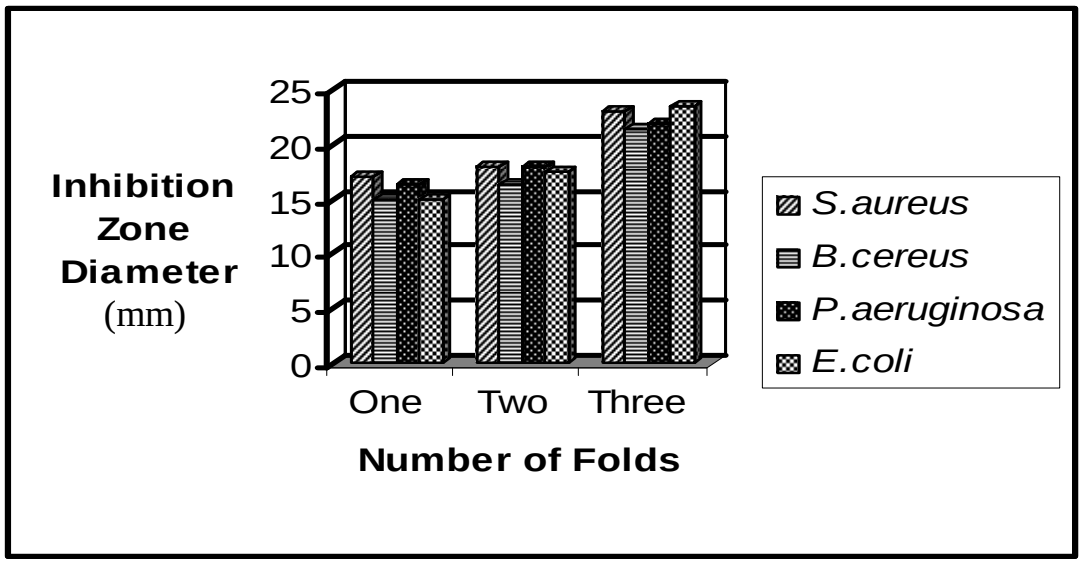
C) *P.aeruginosa*



D) *E.coli*

Figure (4-2). Inhibitory Effect of Unconcentrated Filtrate of *Lb.plantarum*2 Isolate against Test Bacteria after Propagating in MRS Broth for 24 hr.

Pfeiffer and Radler (1982) found a relationship between the diameter of inhibition zone and concentration of the inhibitory substances, so filtrate of *Lb.plantarum2* isolate that propagated in MRS broth after 24hr incubation period was concentrated to three folds, by freezer-dryer, and as shown in figure (4-3) the inhibitory effect increased against all tested bacteria when the filtrate of it was concentrated. One fold concentrated filtrate show slight increases in effect against test bacteria when inhibitory zones ranged between 15-17mm. While two fold filtrate showed noticeable inhibitory effects with zones diameter of 18, 18, 16.5 and 17mm against *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively. Three fold filtrates exhibited the highest inhibitory effects, when diameter of inhibition zone was increased and reached to 23 and 23.5mm for both *S.aureus* and *E.coli*, respectively and recorded 21.5 and 22 mm for *P.aeruginosa* and *E.coli* (figure 4-4). So increasing inhibitory effect was associated with increases concentration.



Figure(4-3). Effect of Concentrated Filtrate of *Lb. plantarum2* Isolate when Propagated in MRS Broth.

A) *S.aureus*

B) *B.cereus*

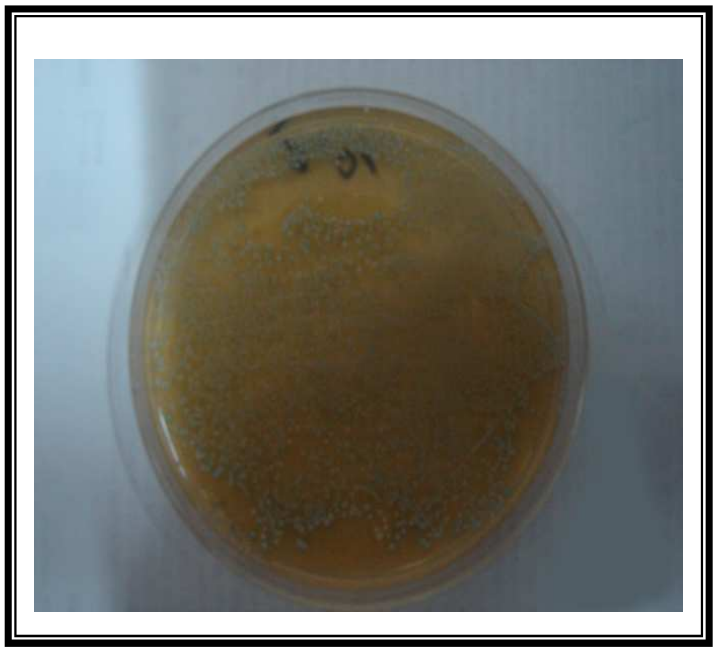
C) *P.aeruginosa*

D) *E.coli*

Figure (4-4). Inhibitory Effect of Three Folded Concentrated Filtrate(12.5%) of *Lb.plantarum*2 Isolate against Test Bacteria after Propagating in MRS Broth for 24 hr.

4.4 Production of β-galactosidase by *Lb.plantarum:*

Culture of *Lactobacillus plantarum* was grown in X-gal-MRS medium to investigate its ability of β-galactosidase production *in vitro*. Results in figure (4-5) showed that the isolate was able to produce β-galactosidase after hydrolyzing the chromogenic substrate X-gal and forming blue color. Turning to blue color took more than two days due to the need for oxygen when the bacterium was incubated under anaerobic conditions.



Figure(4-5) : Ability of *Lactobacillus plantarum*2 Isolate for Production of β-galactosidase (*in vitro*) on Xgal-MRS Agar Medium.

4.5 Inhibitory Effect of *Lb.plantarum* Propagated in Fortified MRS Medium.

a) in MRS Medium fortified with Lactose:

Five lactose concentrations (1, 2, 3, 4 and 5%w/v) and three incubation periods (24, 48 and 72hr) were used for improving the inhibitory effect of *Lb.plantarum*2 isolate on the test organism.

Results in table (4-4) shows that, 1% lactose have not increase *Lb.plantarum* Effect at all incubation periods when inhibition zone diameter range between 8-11mm, against test organism, but it was clear that increasing lactose concentration to 2% and 3% cause increase in inhibitory Effect as with 2% of lactose at 24hr incubation period when inhibition zone diameter increased to a range between 13-16 mm against test bacteria. This range of inhibition zone diameter was increased at high level and become 16-18.5 mm when incubation period was 48hr and 72hr.

Table (4-4). Inhibition Zone Produced By *Lb.plantarum* 2 Isolate Propagating in MRS Broth Fortified with Different Lactose Concentration against Test Organism

Lactos Conc. (%w/v)	Incubation period(hr)	Diameter of inhibition zone(mm) on Test Bacteria			
		S.aureus	***B.cereus***	***P.aeruginosa***	***E.coli***
1	**24**	9	9	10	9.5
	48	9.5	8	11	9.5
	72	9.5	8	10.5	9
2	**24**	13	15	16	14
	48	18.5	17	18.5	16
	72	18	17.5	18	16
3	**24**	18	17	16.5	17
	48	17	16	15.5	16
	72	17	16	15.5	16
4	**24**	12	14	9.5	10
	48	10	12	8	11
	72	10	11	12	10.5
5	**24**	8	6.5	8	10
	48	6	7	9.5	10
	72	5	7	9.5	8

The same increases were recorded at 3% of lactose at 24hr and 48hr when inhibition zone ranged between 15.5 -18 mm against test bacteria, but at 72hr incubation period there was slight decreases in the inhibition zone diameter when reached to 17, 16, 15.5 and 16mm against *S.aureus, B.cereus*, *P.aeruginosa* and *E.coli*, respectively. This result was higher than that recorded when *Lb.plantarum* propagated in MRS alone (without prebiotic) while give inhibition zone diameter between 13-16mm, almost similar results were obtained by Kontula *et al.*(1999) who found that LAB may utilize lactose, and the probiotic action of the strains could be enhanced. The effect of *Lb.plantarum* 2 isolate decreased when increasing lactose concentration to 4% at all incubation periods against all tested bacteria, especially Gram-negative bacteria (*P.aeruginosa* and *E.coli*) at 24hr incubation period, when inhibition zone diameter decreased to 9.5 and 10mm, respectively. The same decreases was recorded for 5% of lactose, like at 24hr incubation period, inhibition zone diameters decreased to a range between 6.5-10mm, and become 6-10 mm and 5-9.5 mm, with increasing incubation period to 48hr and 72 hr. This may due to the effect of high concentration of lactose on β-galactosidase enzyme which metabolizes lactose in the medium. It was concluded that *Lb.plantarum 2* isolate can grow well and exhibit good effect against Gram-positive and Gram-negative bacteria in a medium containing disaccharide (that is added as a substrate supporting probiotic growth and Effect) and this enhanced by LAB enzymes that help in breakdown lactose to glucose and galactose (Lonnerdal, 2003). From above results it was clear that filtrate of *Lb.plantarum* 2 isolate that propagating in MRS broth fortified with 2% lactose at 48 hr incubation period gives highest inhibitory effect against test bacteria. (Figure 4-7). So this filtrate was concentrated into three fold by freezer-dryer. Results in Figure (4-6), shows that the inhibitory effect

were increased with increasing folds. One fold concentrated filtrate exhibit inhibitory effect more slightly than filtrate without concentration, when inhibition zone diameter ranged between 16.5-19mm (figure 4-6). Two fold concentrated filtrate have noticeable increases in inhibitory effect when inhibition zone diameter increased to 20, 19.5, 21 and 18mm with *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively. This higher than that recorded by two-fold concentrated filtrate of *Lb.plantarum* 2 isolate when propagated in MRS alone, when inhibition zone diameters reached to 18, 16.5, 18 and 16.5mm, respectively. Third fold exhibiting the highest inhibitory effects when diameters of inhibition zone reached to 29, 28.5, 29 and 27mm with *S.aureus, B.cereus, P.aeruginosa* and *E.coli*, respectively. So prebiotic (lactose) can improve the effect of probiotic bacteria(Macfarlane and Cummings, 1999).

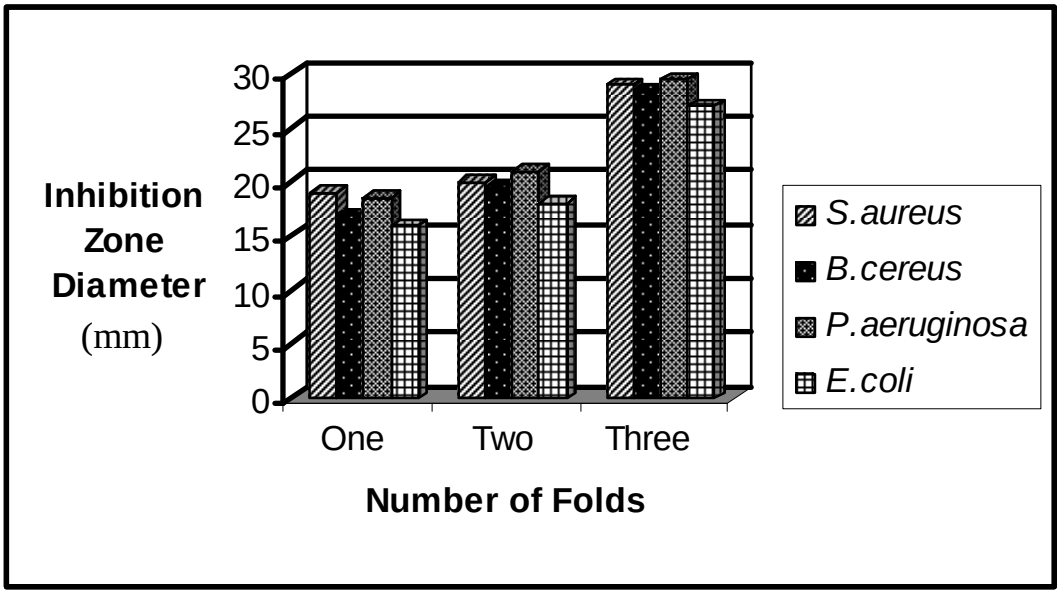


Figure (4-6). Effect of Concentrated Filtrate of *Lb. plantarum2* Isolate when Propagated in MRS Broth Containing, Lactose 2%

A) *S.aureus*

B) *B.cereus*

C) *P.aeruginosa*

D) *E.coli*

Figure (4-7). Inhibitory Effect of Unconcentrated and Concentrated Filtrate of *Lb.plantarum*2 Isolate Propagated in MRS Fortified with Lactose after 48 hr Incubation.

100 = Filtrate Only

50 = One Fold

25 = Two Fold

12.5 = Three Fold

b) MRS Fortified with Fresh Whey:

From result in table (4-5) it was shown that different concentration of whey effect on inhibitory effect of *Lb.plantarum* at different incubation periods when inhibition zone was increased in some concentration of whey. Concentration of whey at 10% and 20% , with incubation period 24 hr, have good inhibitory effect with inhibition zone diameter of 7-10 mm.

Table(4-5). Inhibition Zone Produced by *Lb.plantarum2* Isolate Propagating in MRS Broth Fortified with Different Fresh Whey Concentration against Test Bacteria

Whey Conc. (%v/v)	Incubation period(hr)	Diameter of inhibition zone(mm) on Test Bacteria			
		S.aureus	***B.cereus***	***P.aeruginosa***	***E.coli***
10	**24**	8.5	9	7.5	7
	48	9	10.5	7.5	7
	72	10	8.5	9	5
20	**24**	10	9	8	7.5
	48	12.5	13	8	7
	72	13	13.5	13.5	13.5
30	**24**	13.5	14	13	14
	48	13.5	12.5	12	15
	72	13	13.5	12.5	14
40	**24**	15.5	15	16.5	17.5
	48	19	18.5	19	17
	72	18.5	18	19	17.5
50	**24**	10.5	8	10	9.5
	48	11.5	7.5	9.5	9
	72	11	7.5	9.5	10

Increasing incubation period to 48 hr with the same fresh whey concentrations (10% and 20%), show increases in inhibitory effect against Gram-positive bacteria (*S.aureus* and *B.cereus*) when range

of inhibition zone diameter reached to 9-13mm. But Gram-negative bacteria not affected when inhibition zone diameter remain as it is.

This result agreed with that obtained by Sharba (2006), how found that lactose utilization was increased with increasing incubation period to 48 hr, increasing incubation period to 72 hr the effect increased slightly against all tested bacteria when inhibition zone diameter ranged between 8.5-13.5mm. At 30% concentration of fresh whey, filtrate of *Lb.plantarum* have better inhibitory effect at 24hr and 48hr, against all tested bacteria, in 24hr incubation period inhibition zone diameter ranged 13-14mm, and increased slightly when incubation period 48hr and become 13.5-15mm. Increasing period to 72hr, have no increases in Effect when inhibition zone diameter remain as it is. At 40% concentration of fresh whey, *Lb.plantarum* have maximum inhibitory effect compare with other concentrations, especially at incubation period of 48 hr. when inhibition zone reached to 19, 18.5, 19 and 17 mm against *S.aureus*, *B.cereus*, *P.aeruginosa* and *E.coli,* respectively. Such inhibitory Effect was remaining as it, after increasing period of incubation to 72hr, when diameter of inhibition zone not changed. Increasing fresh whey concentration to 50%, inhibitory effect was decreased, to low level at all incubation period compared to the above concentrations of fresh whey, and this may due to inoculums size insufficient to consume increases of lactose concentration, found in fresh whey. Like at 24hr incubation period, inhibitory effect was decreasing for all test bacteria, when inhibition zone diameter decreased to a range 7-9.5mm, such decreases in inhibition zone diameter was also recorded for 48hr and 72 hr incubation period, when it was ranged between 7.5- 11.5mm for both. From above result it was clear that, the filtrate of *Lb.plantarum2* isolate propagated in MRS broth fortified with

40% fresh whey, at 48hr incubation period, have maximum inhibitory effect against all tested bacteria. So this filtrate was concentrated to three fold by freeze-dryer. From figure (4-8), it was clear that increasing the inhibitory effect was associated with increases number of folds. It was shown that one fold of concentrated filtrate have effect against all tested bacteria in a range 17.5-19.5 mm, but this effect increases with two fold concentrated filtrate and inhibition zone diameter was at range 21.5- 23 mm. Third fold have maximum inhibitory effect compare with one and two fold when inhibition zone diameter reached to 28.5, 30, 31 and 29mm against *S.aureus, B.cereus, P.aeruginosa* and *E.coli* respectively and considered as higher than that recorded for concentrated filtrate when *Lb.plantarum* propagated on MRS alone. Result in figure (4-9).

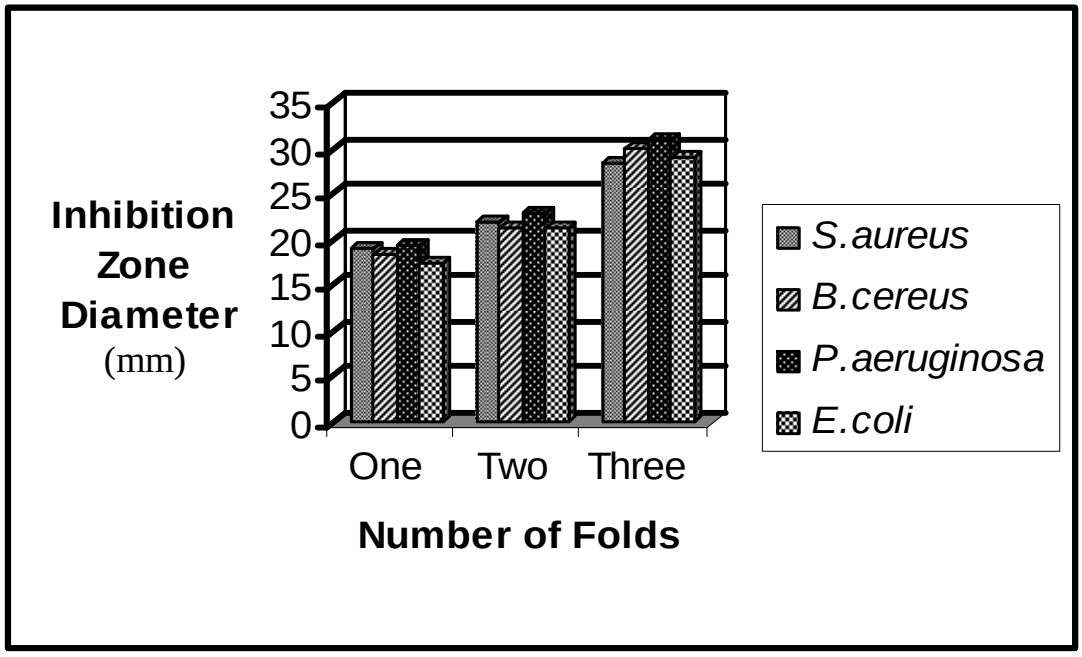
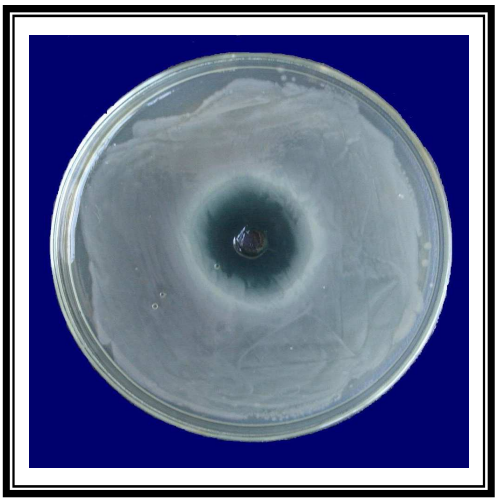
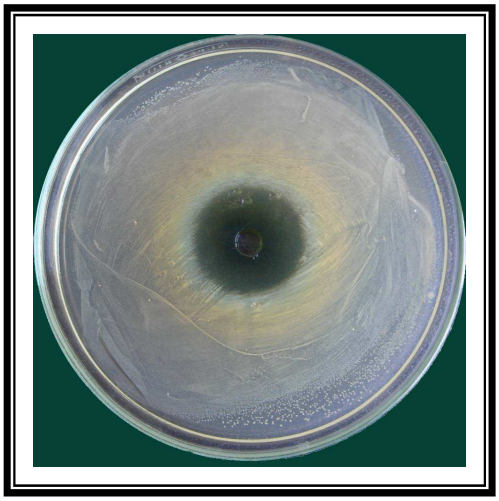


Figure (4-8). Effect of Concentrated Filtrate of *Lb. plantarum2* Isolate when Propagated in MRS Broth Containing Fresh Whey 40%v/v

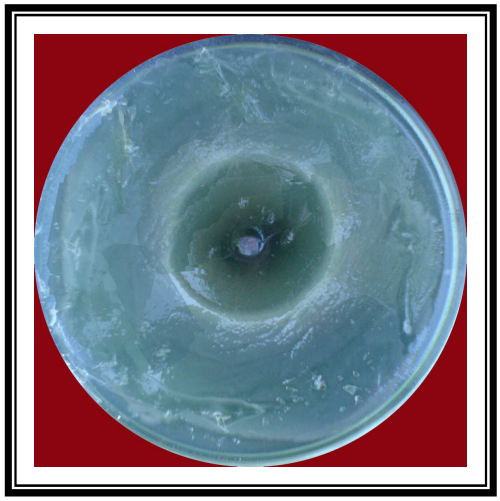
A) *S.aureus*



B) *B.cereus*



C) *P.aeruginosa*



D) *E.coli*

Figure (4-9). Inhibitory Effect of the Three Folded Concentrated Filtrate (12.5%) of *Lb.plantarum2* Isolate Propagated in MRS Fortified with Fresh Whey after Incubation for 48 hr.

c) MRS Fortified with Dried Whey:

Inhibitory effect of filtrate of *Lb.plantarum*2 grown in MRS fortified with different concentration of dried whey at three incubation period was tested, against test bacteria. Table (4-6) shows that the inhibitory effect of *Lb.plantarum* varied with different concentrations of dried whey. 1% concentration of dried whey, have no improving effect on *Lb.plantarum* Effect against all test bacteria at 24hr, when inhibition zone diameter remain in the same range when it propagated in MRS alone (13- 16 mm). Increasing incubation period to 48hr, resulted in slight decreases in inhibitory effect against Gram-positive bacteria (*S.aureus* and *B.cereus*), with inhibition zone reached 11.5 and 9 mm, while no effect was observed against Gram-negative bacteria. While at 72hr incubation period, no increase in the Effect was obtained when inhibition zone diameter ranged 9 -13 mm. Increasing dried whey concentration to 2% at 24hr, have good inhibitory effect when inhibition zone diameter increased against all test bacteria, to 13 and 12.5 mm, for *S.aureus* and *B.cereus* and to 13.5 and 12 mm for both *P.aeruginosa* and *E.coli*. After 48hr incubation period there was continuous increases in inhibitory effect against all test bacteria when inhibition zone diameter reached to 14 mm for *P.aeruginosa*. The best inhibitory effect was obtained at 3% concentration of dried whey at 24hr incubation periods when inhibition zone diameter ranged to 10-13 mm, but inhibitory effect was increased and inhibition zone diameter reached to 19, 18.5, 20 and 18mm, against *S.aureus, B.cereus, P.aeruginosa* and *E.coli* this at 48hr incubation period. Diameters of inhibition zone were remain at the same range when incubation period become 72hr.

Table(4-6). Inhibition Zone Produced by *Lb.plantarum 2* grown in MRS Broth Fortified with Different Dried Whey Concentration against Test Bacteria

Dried Whey Conc. (%w/v)	Incubation period(hr)	Diameter of inhibition zone(mm)on Test Bacteria			
		S.aureus	***B.cereus***	***P.aeruginosa***	***E.coli***
1	**24**	13	10.5	15.5	16
	48	11.5	9	12.5	15.5
	72	12.5	9	12	13
2	**24**	13	12.5	13.5	12
	48	13.5	13	14	13.5
	72	13.5	12.5	14	13
3	**24**	12.5	13	10	11.5
	48	19	18.5	20	18
	72	18	18.5	17	19.5
4	**24**	11	13	14	11.5
	48	11	12.5	12	12
	72	9	9.5	6	10
5	**24**	9	8	7.5	10.5
	48	9	8	7.5	10.5
	72	7.5	8	7.5	8

At concentrations 4% and 5%, inhibitory effect of *Lb.plantarum* filtrate was decreased, at all incubation periods, as with 4% dried whey, inhibition zone diameter was at range 11.0 – 14 mm at 24 hr, and to 11-12.5 mm, at 48hr. This range decreases largely to 6-10 mm, at 72hr incubation period. The same decreases was observed with 5% dried whey, when inhibition zone diameter ranged 7.5-10.5 mm, at 24hr and 48hr incubation period, and to 7.5-8 mm when increase incubation period to 72hr. From all above result, it was concluded that filtrate of *Lb.plantarum2* isolate that propagated in MRS broth fortified with 3% dried whey at 48hr incubation period give highest inhibitory effect against all tested bacteria, so it was concentrated by freezer-dryer to three-fold. Increasing number of fold leading in increases inhibitory effect, results in figure (4-10), show that the inhibitory Effect was

increased at each fold of concentration against all tested bacteria. One-fold of the concentrated filtrate exhibit good inhibitory effect when it ranged to 17.5- 20.5 mm, with all tested bacteria. While, fold increased to two, inhibition zone diameter reached to highiest value with *S.aureus* when it reached to 23 mm and 21, 22.5 and 22mm with *B.cereus, P.aeruginosa* and *E.coli,* respectively. Three fold concentrated filtrate have the highest inhibitory effect when inhibition zone diameter reached to 30mm with both *B.cereus* and *P.aeruginosa* and 29 mm, 29.5 mm for both *S.aureus* and *E.coli.* Figure (4-11)

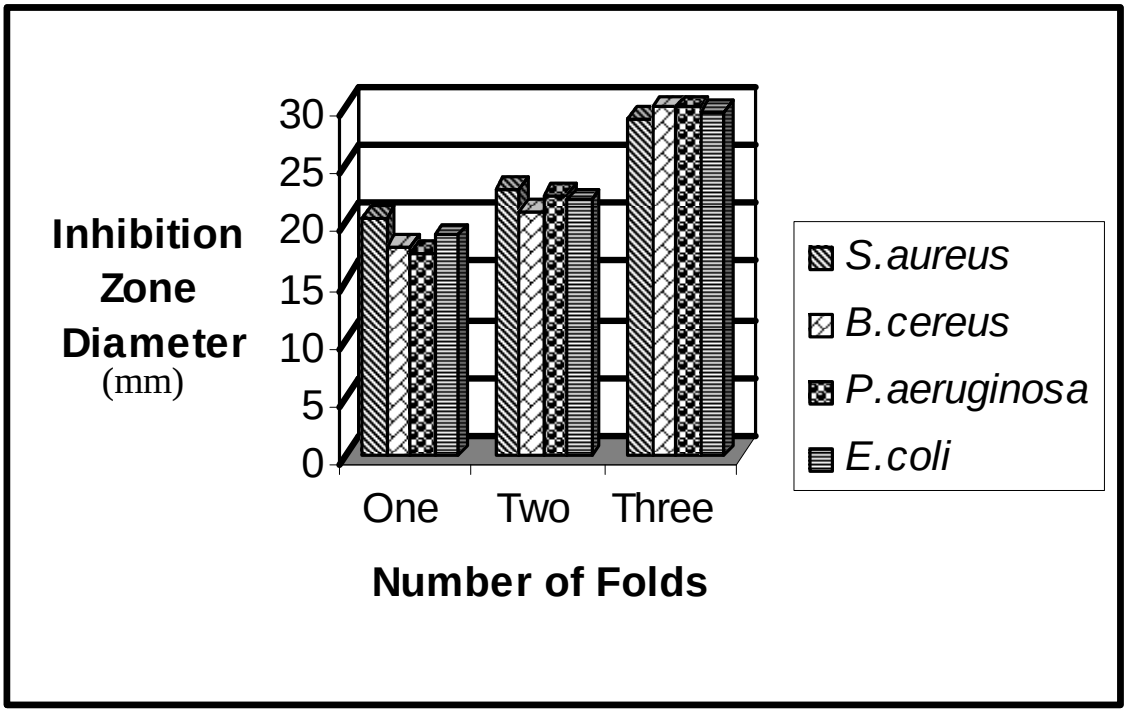
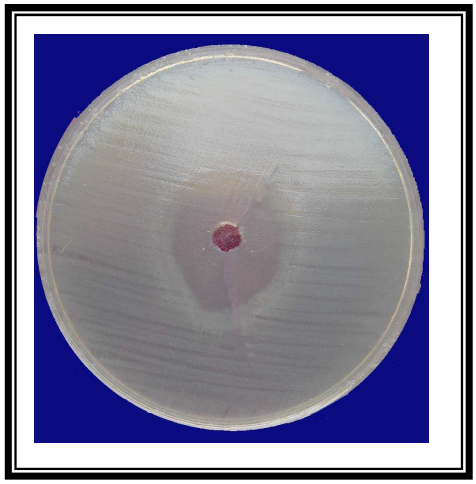
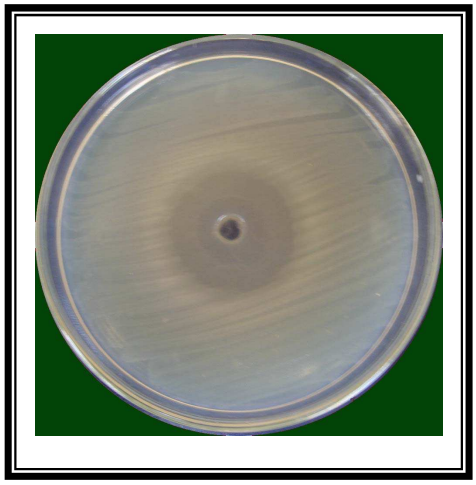


Figure (4-10). Effect of Concentrated Filtrate of *Lb. plantarum2* Isolate when Propagated in MRS Broth Containing Dried Whey 3%.

A) *S.aureus*



B) *B.cereus*



C) *P.aeruginosa*

D) *E.coli*

Figure (4-11). Inhibitory Effect of Three Folded Concentrated Filtrate (12.5%) of *Lb.plantarum*2 Isolate against Test Bacteria after Propagating in MRS Broth Fortified with Dried Whey for 24 hr.

4.6 Minimum Inhibitory Concentration (MIC,s) of Concentrated Filtrates of LAB When Propagated in:

A- MRS Medium:

To determine MICs of the *Lb.plantarum* filtrates required to inhibit microbial growth, serial dilutions were prepared from the three-fold filtrates of *Lb.plantarum2* isolate, as previously mentioned (3.2.10). Table (4-7) contains MIC of the concentrated filtrate of *Lb.plantarum* propagated in MRS broth. Results of the table declared that the first two concentrations (1:9 and 2:8) had no observed effect against all bacteria when heavy growth of these bacteria was noticed after incubation. While growth of *S.aureus* and *B.cereus* decreased to the moderate level at the following two concentrations (3:7 and 4:6) and at 4:6 for the other two test bacteria *P.aeruginosa* and *E.coli.*

Table (4-7). Minimum Inhibitory Concentrations (MIC,s) of Concentrated Filtrates of *Lb.plantarum*2 Isolate Propagating in MRS Broth against Test Bacteria.

Concentrated Filtrates: Nutrient Broth									
Isolate	**1:9**	**2:8**	**3:7**	**4:6**	**5:5**	**6:4**	**7:3**	**8:2**	**9:1**
S. aureus	+++	++	++	++	+	–	–	–	–
P.aeruginosa	+++	+++	+++	++	++	+	–	–	–
B. cereus	+++	+++	++	++	+	–	–	–	–
E. coli	+++	+++	+++	++	++	+	–	–	–

Heavy Growth = +++ Medium Growth = ++ Light Growth = + No Growth = -

Sharp decreases in growth to the light level were recorded by *S. aureus* and *B. cereus* after treatment with a concentration of (5:5) of the filtrate and with (6:4) for *E.coli* and *P.aeruginosa.*

The last three concentrations of *Lb.plantarum* isolate (7:3, 8:2, 9:1) were quite enough to retard any growth of all the test bacteria.

From the above results it may be concluded that filtrate concentrations of (6:4) is the MIC,s for *P.aeruginosa* and *E.coli*, and 5:5 for *B.cereus* and *S.aureus*. Such results agreed with those obtained by Al-Jeboury (2005) who found that the MIC of *Lb.plantarum* concentrated filtrates were (50%) and (60%) that completely inhibited the growth of *Lb.plantarum*.

B) MRS Fortified with Different Substances:

To determine the differences in MIC values of concentrated filtrates of *Lb.plantarum2* isolate after propagating in MRS broth containing prebiotic substances, steps in (4.6.A) were repeated.

Results in table (4-8 a), shows the MIC of the concentrated filtrate of *Lb.plantarum* propagating in MRS fortified with 2% lactose. Values of MIC were decreased to 5:5 (concentrated filtrate: nutrient broth) for *P. aeruginosa* and *E.coli*, and 3:7 for *S.aureus* and *B.cereus*, this differ from that when propagating in MRS alone. (Table 4-7). Similar results were obtained when *Lb.plantarum* propagated in MRS fortified with dried whey. (Table 4-8 c). While sharp decreases in MICs values were noticed for the filtrate of *Lb.plantarum 2* isolate propagated in MRS fortified with fresh whey when MICs value were 3:7 for *S.aureus*, *B.cereus* and *E.coli* and be 4:6 for *P.aeruginosa*. (Table 4-8 b).

Table (4-8). Minimum Inhibitory Concentrations(MIC,s) of Concentrated Filtrates of *Lb.plantarum*2 Isolate Propagating in MRS Broth Fortified with a) Lactose b) Fresh Whey c) Dried Whey against Test Bacteria.

a)

Concentrated Filtrates+Lactose: Nutrient Broth									
Isolate	**1:9**	**2:8**	**3:7**	**4:6**	**5:5**	**6:4**	**7:3**	**8:2**	**9:1**
S.aureus	+++	++	+	–	–	–	–	–	–
P.aeruginosa	+++	+++	++	++	+	–	–	–	–
B.cereus	++	++	+	–	–	–	–	–	–
E.coli	++	++	++	+	+	–	–	–	–

b)

Concentrated Filtrates+Fresh whey: Nutrient Broth									
Isolate	**1:9**	**2:8**	**3:7**	**4:6**	**5:5**	**6:4**	**7:3**	**8:2**	**9:1**
S.aureus	++	+	+	–	–	–	–	–	–
P.aeruginosa	+++	++	++	+	–	–	–	–	–
B.cereus	++	+	+	–	–	–	–	–	–
E.coli	+++	++	+	–	–	–	–	–	–

c)

Concentrated Filtrates+ Dried whey: Nutrient Broth									
Isolate	**1:9**	**2:8**	**3:7**	**4:6**	**5:5**	**6:4**	**7:3**	**8:2**	**9:1**
S.aureus	+++	++	+	–	–	–	–	–	–
P.aeruginosa	+++	+++	++	++	+	–	–	–	–
B.cereus	+++	++	+	–	–	–	–	–	–
E.coli	+++	+++	++	++	+	–	–	–	–

Heavy Growth = +++ Medium Growth = ++ Light Growth = +

No Growth = -

4.7 Growth Curve Measurement of *Lp.plantarum2* Grown in:

4.7.1 MRS Broth:

Growth characterization of the selected *Lactobacillus* isolates was done to determine the extent of their growth phase when propagated in MRS broth alone, and comparing it with that propagated in MRS broth containing prebiotic substances.

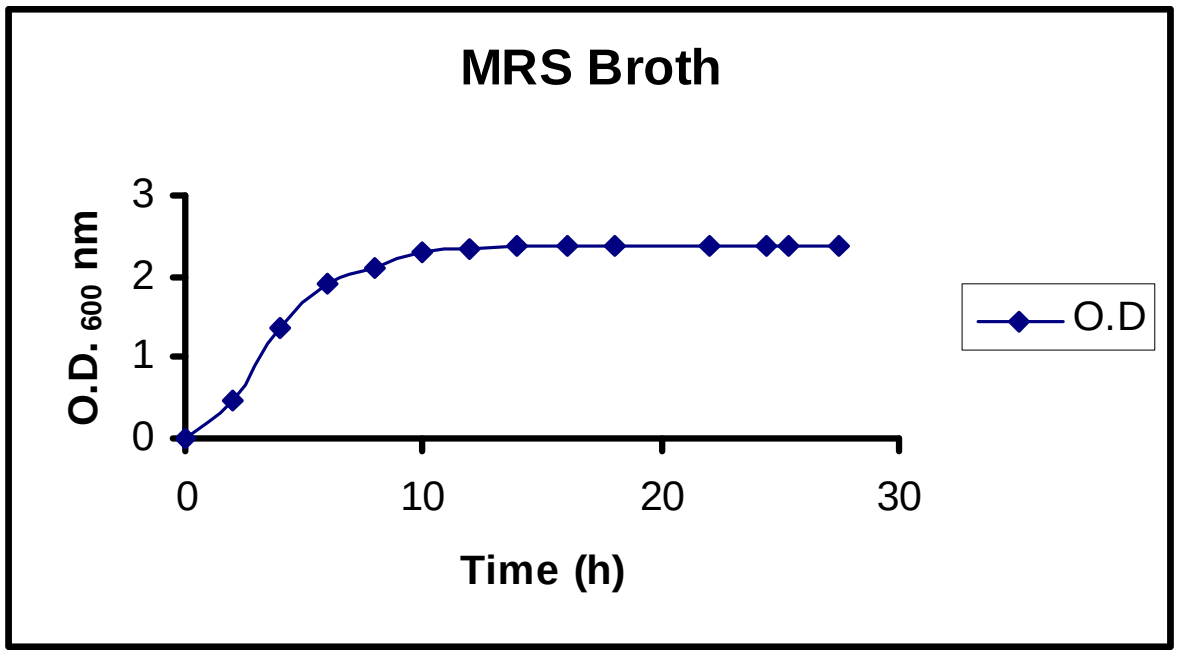
When a bacterium inoculated into a new MRS broth culture medium, it exhibits a characteristic of growth curve which is consisted, in the normal growth, from four phases: lag, log (exponential), stationary and death phases(Atlas *et al.*, 1995)

Figure (4-12) shows that lag phase took about 2 hr. During this phase no increase in cell number was detected which may be related to preparation of cells for synthesis of DNA, inducible enzymes needed for cell division and reproduction. This phase was followed by the log phase when the number of cells was increased. A logarithmic growth was noticed during the first 8 hr, then growth rate become at a slower speed for 24 hr. Mostly, the isolate entered the stationary stage (Emanule *et al.*, 2005).

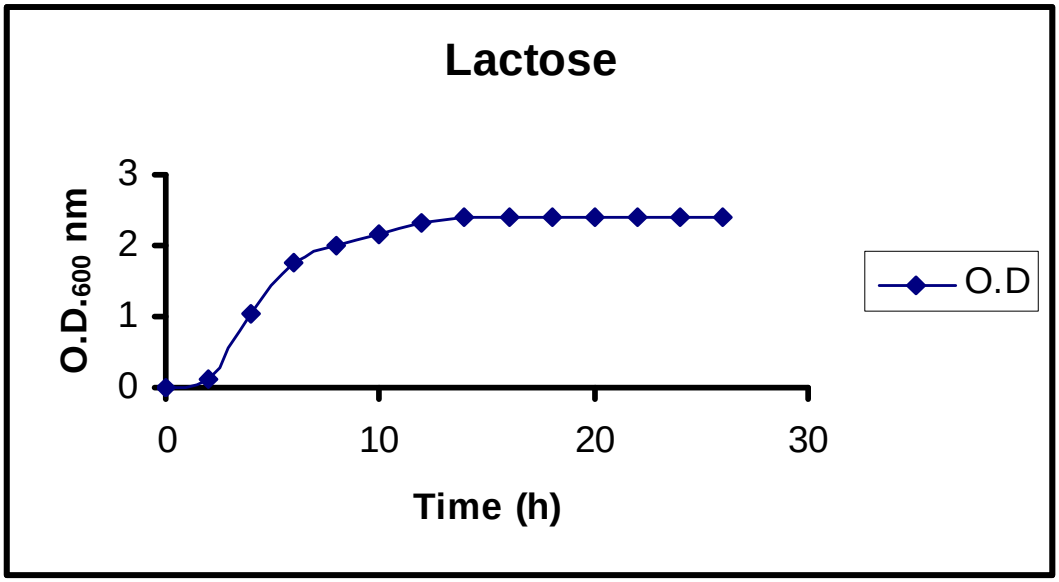
4.7.2 MRS Broth Fortified with Prebiotic Substances:

Testing the improving effect of prebiotic substances on probiotic growth was done. The growth curve of *Lb.plantarum*2 isolate grown in MRS broth containing 2% lactose were examined as in figure(4-13). The lag phase also take about 2 hr and same result was obtained figure (4-14) and (4-15) when *Lb.plantarum* was propagated in MRS broth containing 40% fresh whey and 3%dried whey. This may due to the similar conditions in the media (temperature and nutrient compound), from

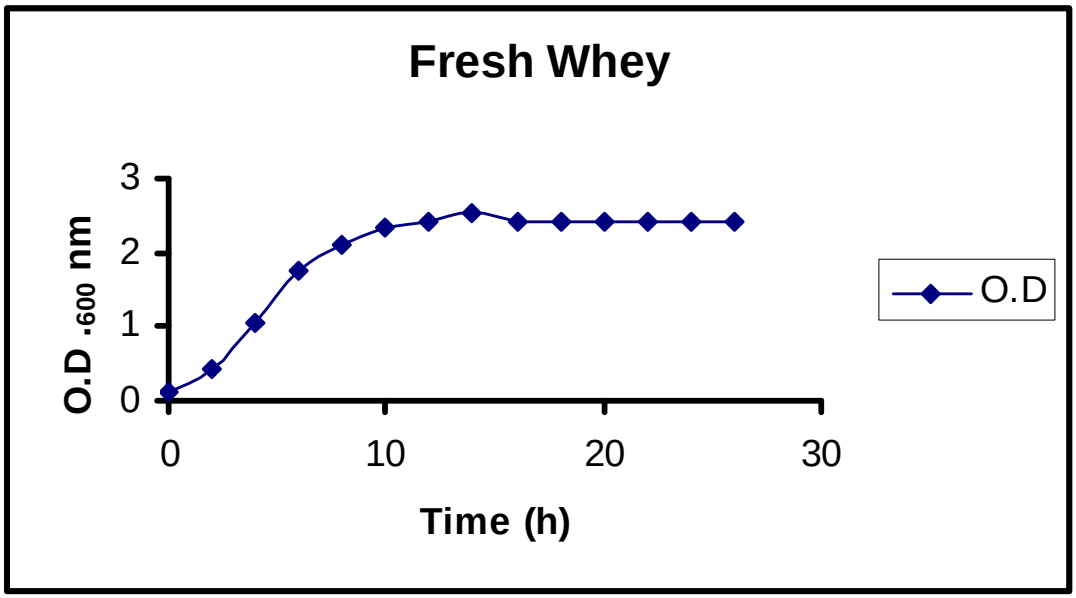
which the organism taken and the one to which they are transferred. The logarithmic growth stage of *Lb.plantarum* took about 10 hr when it propagated in MRS with 2% lactose and it took about 12 hr when it propagated in a medium with 40% fresh whey and 3% dried whey. These periods of log phase of *Lb.plantarum* growth when propagated in MRS broth fortified with these substances considered to be longer as compared with that propagated in MRS alone. Such differences in the period of each phase may be due to the addition of new carbon sources (lactose), while makes the bacteria take more time to ferment it. Meulen *et al.*(2004) found that the addition of prebiotic substances in media changing in growth behavior and this result was likely in agreement with our result.



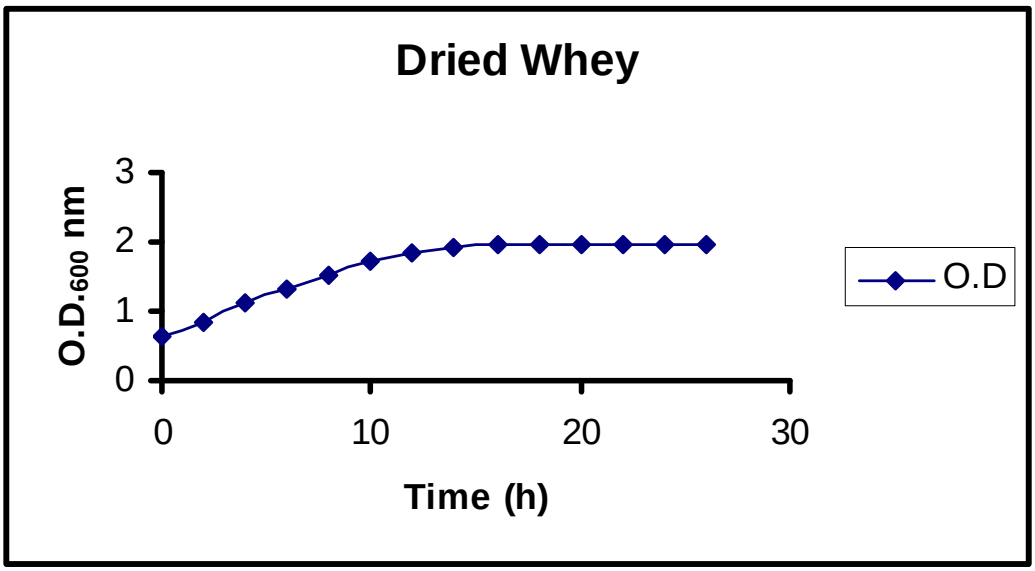
Figure(4-12). Growth Curve of *Lb.plantarum2* Isolate in MRS Medium



Figure(4-13). Growth Curve of *Lb.plantarum2* Isolate in MRS Medium Fortified with 2% Lactose



Figure(4-14). Growth Curve of *Lb.plantarum2* Isolate in MRS Medium Fortified with 40% Fresh Whey



Figure(4-15). Growth Curve of *Lb.plantarum2* Isolate in MRS Medium Fortified with 3% Dried Whey

4.8 *Lb.plantarum 2* Cells Number before and after Prebiotic Treatment:

The effect of three prebiotic substances, with defined concentrations on bacterial numbers of selected probiotic strain *Lb.plantarum*2 were studied, by using plate count method (Bryant and Burkey, 1953). Table (4-9), shows the number of *Lb.plantarum* before and after prebiotic treatment at different incubation periods and from result it was shown that lactose, fresh whey and dried whey enhance *Lb.plantarum* growth and resulted in increasing bacterial numbers at different incubation periods (24, 48 and 72 hr), but in general the highest increasing in *Lb.plantarum* numbers were recorded after 48 hr incubation period. Like after 24 hr incubation period with lactose 2% w/v there is slight increases in number of cells when it reached to 4.1×10^8 while it was 2.2×10^8 with those without lactose enrichment. Increasing incubation period to 48 hr shows better enhancing in growth when cell number reached to 3.3×10^9 . While with those without lactose enrichment the number of

cells reached to 2.9×10^{8}. Increasing incubation period to 72 hr shows lower effect on growth. Largest increasing in *Lb.plantarum* number was recorded after treatment with fresh whey 40% at 48 hr incubation period when the number of cells reached to 5.1×10^{9}. At this period good effect was obtained on *Lb.plantarum* growth as compared with number of cells after 24 hr and 72 hr when it becomes 2.4×10^{8} and 2.1×10^{7}, respectively. The same increasing in number of *Lb.plantarum* was recorded when it was propagated in MRS broth fortified with dried whey 3% w/v, but less than that recorded for whey .

It was found that after 24 hr incubation period with 3% w/v dried whey, the number of cells reached to 1.2×10^{8} and reached to 1.1×10^{9} when incubation period become 48 hr. While increasing period of incubation to 72 hr result in less cell numbers. From above results it was concluded that prebiotic treatment enhance the growth of *Lb.plantarum* especially at 48 hr. incubation period but opposite effect was observed after 72 hr incubation period for all three treatment, 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 *Lb.plantarum* cells and hydrolysis of its cells.

Table (4-9). Number of *Lb.plantarum* before and after Prebiotic Treatment

Prebiotics	*Lb. plantarum* Cell Numbers		
	24 hr	48 hr	72 hr
MRS alone	2.2×10^{8}	2.9×10^{8}	1.8×10^{8}
Lactose 2%w/v	4.1×10^{8}	3.3×10^{9}	2.1×10^{8}
Fresh Whey 40% v/v	2.4×10^{8}	5.1×10^{9}	2.1×10^{7}
Dried Whey 30%w/v	1.2×10^{8}	1.1×10^{9}	6.2×10^{7}

1- Introduction:

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. Among bacterial group is the *Lactobacillus* spp. which have a great role in probiotics, due to it's presence in mucous membrane of intestine and digestive tract of human as normal microflora, safe used in food industry and it's ability to produce inhibitor materials such as organic acids, H_2O_2 , CO_2 , amino acid, di-acetyl, acetaldehyde and bacterocins (Runar, 1998).

Beneficial effects of feeding an exogenous probiotic may be enhanced and extended by simultaneous administration of a prebiotic , that is non-digestible food ingredient beneficially affects the host by improving human health (Meulen *et al.*, 2004).

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

These synergistic effect of probiotic and prebiotic contributes to health, ones example of prebiotic is disaccharides (lactose) that is primary carbohydrate of mammalian milk, and it is hydrolyzed into two monosaccharides (glucose and galactose) by the intestinal brush border enzyme lactase (Lin *et al.*, 1991). Lactose that is not digested by small intestine becomes a substrate for fermentation by bacteria in the colon by β- galactosidase enzyme. Resulting in production of short chain fatty acids that restrict the growth and activity of less beneficial species (Vaux *et al.*, 2000).

Dairy by-products are exclusive sources of the lactose disaccharide, which is a by-product of dairy industry mainly from cheese processing. It

contains divers biologically components like lactose as a carbohydrate and other components of health benefits (Lois and McBean, 2003).

Despite that, several healthy important components are found in whey, but it was rarely used due to many causes. Condensed (dried) whey 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. For such reason, this study was aimed to:-

1. Isolation and identification of lactic acid bacteria from local sources as probiotic.

2. Evaluation the ability of the selected isolate (*Lactobacillus plantarum*) for β-galactosidase production.

3. Using prebiotic available substances to test their effect in enhancing properties of the probiotic bacterial growth and/or activity.

4. Determining the minimum inhibitory concentrations of concentrated filtrate of *Lactobacillus* bacteria before and after addition of prebiotic substances.

3.1 Materials

3.1.1 Apparatus and Equipments:

Apparatus or Equipment	Company (Origin)
Autoclave	Gallenkamp (England)
Anaerobic jar	Rodwell (England)
Centrifuge (Portable)	Hermlex labortech Nik (Germany)
Cooling centrifuge	Harrier (England)
Compound light microscope	Olympus (Japan)
Distillater	GFL (Germany)
Electrical incubator	Gallenkamp(England)
Electrical oven	Gallenkamp(England)
Freeze-Dryer	Christ (Germany)
Glass Pasteur pipettes	John poutten Ltd (England)
Millipore filter unit(0.4 µm)	Millipore and Whatman(England)
Millipore filter unit(0.22µm)	Millipore and Whatman(England)
Micropipette	Oxford (U.S.A)
pH meter	Metter GmbH-Teledo(England)
Shaking incubator	Gallenkamp
Spectophotometer	Aurora Instruments Ltd (England)
Sensitive balance	Delta Range (Switzerland)
Vortex	Stuart Scientific Co.Ltd (England)
Waterbath	Gallenkamp(England)

3.1.2 Chemicals

Chemicals used in this study were classified according to the manufacture companies, as follow:

- **BDH (England)**

Calcium carbonate, Glycerol, Sodium hydroxide, Hydrochloric acid, Peptone, Glucose, Iodine, Argenine, Agar-agar, litmus milk, Mannitol, Lactose, Maltose, Sucrose, Xylose, Sodium chloride.

- **Biolife (Italy)**

Meat extract, Yeast extract and Skim milk.

- **Fluka (Switzerland)**

Manganese sulphate (hydrate), Magnesium sulphate (hydrate), Crystal violet, Hydrogen peroxide.

- **Merck (Germany)**

Sodium acetate trihydrate and Triammonium citrate.

- **Oxoid (England)**

Gelatine.

- **Difco (USA)**

Fructose, Raffinose, Arabinose, Galactose and N, N, N, N-tetramethyl-p-phenylenediamine dihydrochloride, X-Gal.

- **Sigma (USA)**

Tween - 80.

3.1.3 Culture Media:

3.1.3.1 Ready to Use (Powdered) Media:

Media	Company(Country)
Nutrient Agar	Biolife (Italy)
Nutrient Broth	Oxoid (England)
MRS-Agar	Hi-media laboratory limited.
Brain Heart Infusion Broth(BHI)	Oxoid

3.1.3.2 Laboratory- Prepared Media:

The following media were prepared in the laboratory from their contents:

- Man-Rogoza-Sharp (MRS) Broth.
- Gelatin Medium
- Arginine -MRS Medium
- Litmus Milk Medium
- Fermentation Medium
- MRS Broth Media Fortified with Lactose, Fresh Whey and Dried Whey.
- X-Gal MRS Agar

3.1.4 Solutions and Reagents:

3.1.4.1 Normal Saline Solution:

It was prepared by dissolving 0.85g of NaCl in 100 ml of distilled water the pH to 7.0 (Atlas *et al.*, 1995).

3.1.4.2 Nessler's Reagent: It was supplied ready by (BDH) company.

3.1.4.3 Phenol Red Reagent:

It was supplied by (Fluka) company.

3.1.4.4 Catalase Reagent (H_2O_2) (Atlas *et al*., 1995):-

A concentration of (3%) H_2O_2 was prepared for catalase production enzyme.

3.1.4.5 Oxidase Reagent (Baron *et al*., 1994):-

A solution of (1%) N, N, N, N-tetramethyl-p-phenylene dihydrochloride was prepared in sterile distilled water was needed for oxidase production.

3.1.5 Bacterial Isolates:

The following bacterial isolates were obtained from the Department of Biotechnology / College of Science, Al-Nahrain University, Baghdad.

Isolates
Escherichia coli MS1 isolated from urinary tract infection
Staphylococcus aureus B1 isolated from burns
Pseudomonas aeruginosa H3 isolated from burns
Bacillus cereus HI2 isolated from canned food

3.1.6 Prebiotic Raw Materials:

The following two prebiotic materials (in addition to the lactose) were used:

Prebiotic	Supplied by
Fresh Whey	Agricultural College/Baghdad University.
Dried whey	Zhashkov Dairy Plant Company/Ukraine

3.2 Methods:

3.2.1 Sterilization (Baily *et al.*, 1990):

Three methods of sterilization were used:-

3.2.1.1 Autoclaving

All media and solutions were sterilized by autoclaving at 121 ºC (15Ib/in^2) for 15 min.

3.2.1.2 Heat Sterilization

Electric oven was used to sterilize glasswares by heating at 160-180 ºC for 2-3 hr.

3.2.1.3 Membrane Sterilization

Millipore filters (o.22mm), was used for sterilization of bacterial filtrates and some sugar solutions that were needed for the fermentation media.

3.2.2 Media Preparation:

3.2.2.1 Ready-to Use Medium:

The media listed in (3.1.3.1) were prepared according to the instructions of the manufacturer. They were brought to boil in water to dissolve all constituents completely, and then the pH was adjusted before sterilized by autoclaving. They were incubated at 37 ºC for 24 hr to ensure sterilization.

3.2.2.2 Laboratory Prepared Media:-

3.2.2.2.1 Rogoza-Broth Medium (MRS):-

This medium was prepared as described by Deman *et al.*(1960) by dissolving the following ingredients:

Peptone	10 g
Beef extract	10g
Yeast extract	5g
Glucose	20g
Tween-80	1ml
Sodium acetate hydrate	5g
Triammonium citrate	2g
$MgSO_4.7H_2O$	200mg
$MnSO_4.4H_2O$	50 mg

In 950 milliliter of distilled water. Then pH was adjusted to 6.0, before autoclaving. This medium was used for growing lactic acid bacteria.

3.2.2.2.2 Fermentation Media:

It was prepared according to Forbes *et al.* (1998) by using sterilized MRS broth after substituting glucose and meat extract by 1% of each of the autoclaved sugars (lactose, fructose, raffinose, maltose, manitol, sucrose) or filterated sugars (arabinose, xylose, calactose). After adding 0.004% of chlorophenol red reagent, the pH was adjusted to (6.2-6.5). This medium was used for the identification of *Lactobacillus* spp.

3.2.2.2.3 Litmus Milk Medium: (Baily *et al.*, 1990; Baron *et al.*, 1994).

A quantity of (5)g of litmus milk medium and (100)g of skim milk powdered was dissolved in 100 ml D. W. then sterilized by autoclaving at 121ºC for 10 min. This medium was used for identification of *Lactobacillus* spp.

3.2.2.2.4 Gelatin Medium (Stolp and Gadkari, 1984):

Gelatin 12% (w/v) was dissolved in MRS broth medium, then sterilized by autoclaving. It was used for identification of *Lactobacillus* spp.

3.2.2.2.5 MRS Arginine Broth Medium (Harrigan and MacCane, 1976):

This medium was prepared by dissolving 0.3% (w/v) of L-Arganine monohydrochloride in 100ml MRS broth, sterilized by the autoclave. It was used for identification of *Lactobacillus* ssp.

3.2.2.2.6 Fortified MRS Broth Media:

a) MRS Fortified With Lactose:

This medium was prepared by adding lactose at different concentrations (1 %, 2 %, 3 %, 4 %, 5 %) w/v to MRS broth (Item 3.2.2.2.1), then the medium was sterilized by autoclaving for 7 min at 121º C.

b) MRS Fortified With Fresh Whey:

This medium was prepared by dissolving the content of laboratory prepared MRS (3.2.2.2.1) into different concentrations (10, 20, 30, 40, 50 %) v/v of fresh whey. The medium was sterilized by autoclaving for 7 min at 121 ºC.

c) MRS Fortified With Dried Whey:

The medium was prepared by adding different concentration of dried whey (1, 2, 3, 4, and 5 %) w/v into MRS broth (Item 3.2.2.2.1), and then the medium was sterilized by autoclaving for 7 min. at 121º C.

3.2.2.2.7 X-Gal MRS Agar:

A quantity of 0.4g/ml of X –gal solution was sterilized by Millipore filter (0.2µm), after cooling down it was a septically added to previously autoclaved MRS agar before let for solidification (Ibrahim and Sullivan, 2000). It was used for detection the bacterial ability for production of β-galactosidase *in vitro*.

3.2.3 Samples Collection:

Seventeen samples of dairy products (yoghurts and crude milk) were obtained from Baghdad local markets. Samples were then transferred as

quickly as possible to laboratory in sterile containers under aseptic conditions.

3.2.4 Isolation of *Lactobacillus* spp:-

Lactic acid bacteria were isolated from the sample according to Harrigen and MacCane (1976) as following:

A portion of 0.1 ml of sample was added to a previously sterilized test tube containing 9.9ml MRS broth, then incubated at 37 ºC for 24 hr. under anaerobic conditions (in anaerobic jar). After incubation, serial dilutions were made, and 0.1 ml from last dilution was streaked on the surface of MRS agar containing 1% $CaCO_3$ in Petridishes, and then incubated for 24 hr. at 37 ºC. After incubation, one colony that surrounded by a clear zone was transferred and streaked on surface of a plate containing MRS agar for purification , plate was then incubated at 37 ºC for 24 hr. After that, part of the growth was transferred to MRS broth in a test tube and incubated under anaerobic condition.

3.2.5 Identification of *Lactobacillus* Species.

The suspected LAB isolates were identified by the following tests:

3.2.5.1 Microscopic Examination (Harely and Prescott, 1996)

A loopfull of lactic acid bacteria culture was fixed on a microscopic slide, and then stained by Gram stain to examine cells shape, Gram reaction, grouping and non-spore forming phenomena.

3.2.5.2 Biochemical Test

3.2.5.2.1 Catalase Test (Atlas *et al*., 1995):

A loopfull from each of the suspected isolates was transferred to a sterile glass slide and a drop of H_2O_2 (3%) was added to it. Positive result was observed through formation of gas bubbles indicating the ability of bacteria to produce catalase enzyme.

3.2.5.2.2 Oxidase Test (Atlas *et al*., 1995).

A clump of suspected colonies from bacterial growth was picked up with a sterile wooden stick and smeared on filter paper that moistened with a few drops of a freshly prepared oxidase reagent.

3.2.5.2.3 Gelatinase Test (Baron *et al*., 1994).

Gelatin medium agar was used to detected gelatin liquification in tubes, by inoculcating 1% of LAB isolates, and incubating at 37 ºC for 48 hr. After that it was put into the refrigerator at 4 ºC for 30 min. This test was performed to demonstrate the ability of isolates to hydrolyze gelatine.

3.2.5.2.4 Acid Production and Clot Formation Test:

Tubes containing 10 ml of litmus milk medium were inoculated by 1% of the suspected bacterial culture and then incubated at 37 ºC for 48 hr. to detect color change, crude production and pH decrease as positive result.

3.2.5.2.5 Production of Ammonia from Arginine (Briggs, 1953):

Tubes containing 10 ml MRS- arginine medium were inoculated by 1% of each lactic acid bacteria then incubated at 37 ºC for 24 hr. After

that, 1ml of bacterial culture was added to a same volume of Nessler reagent. Inability of the isolate to change the color of medium indicates that the bacteria are not producing ammonia.

3.2.5.2.6 Carbohydrate Fermentation Test:

Tubes containing fermentation media were inoculated with 1% of lactic acid bacterial isolates and incubated with the positive control tube (only fermentation medium) and the negative control tube (contained MRS broth) at 37 ºC for 5 days. Changing the color to red indicates (pH alkaline) while to yellow indicates (pH acids) also CO_2 production considered as positive result.

3.2.5.2.7 Growth on Nutrient Agar (Atlas *et al*., 1995):

Lactic acid bacteria was cultured on nutrient agar then incubated at 37 ºC for 24 hr, after that growth was observed.

3.2.5.2.8 Growth at 45 ºC and 15 ºC:

Tubes containing 10 ml MRS broth were inoculated with 1% of lactic acid bacterial culture then incubated at 15 ºC and 45 ºC for 24 hr. After incubation, growth was observed in the tubes and compared with control tube (growth at 37 ºC).

3.2.6 Maintaining of *Lactobacillus* Isolates

Maintenance of bacterial isolates was performed according to Conteras *et al.* (1997) as follow:

3.2.6.1 Daily Working Culture

MRS broth was inoculated with the *Lactobacillus* isolates and incubated at 37 ºC for 24 hr. After incubation, 1% $CaCO_3$ was added to the tubes and stored at 4 ºC.

3.2.6.2 Stock Culture

Lactobacillus isolate was cultured in MRS broth medium for 24hrs at 37C. Then, 1ml of freshly preparation of bacterial growth was added to Bejo bottles containing 20% glycerol, and stored at -20C.

3.2.7 Determining Inhibitory Effect of *Lactobacillus* Isolate against Test Bacteria:

3.2.7.1 on Solid Medium

A culture of *Lactobacillus* was inoculated in MRS broth then incubated at 37 ºC for 24 hr. After that, the culture was streaked on surface of MRS agar then incubated at 37 ºC for (24, 48 and 72) hr. (Silva *et al.*, 1987).

After incubation, discs was made by the aid of cork borer (5mm). The disc was fixed on the surface of nutrient agar plate that is previously spreaded with test bacteria, and then incubated at 37 ºC for 48 hr. After incubation, inhibition zone around the discs was measured.

After measuring inhibition zone diameter, selection of isolate giving best inhibitory effect was done.

3.2.7.2 in Liquid Media:

After inoculating MRS broth alone with 1% of *Lactobacillus* isolate that selected in (3.2.7.1) culture in a test tube, the tube was

incubated at 37 ºC for different incubation periods (24, 48 and 72) hr. (Lewus *et al.*, 1991). After incubation, the culture was centrifuged at 6000 rpm for 10 min to get the supernatant which contained the filtrate of grown cell. Then it was filtered through Millipore filter 0.22µm unit. Wells diffusion method that mentioned by Ryan *et al. (*1996) was used to detect the inhibitory effect of *Lactobacillus* against test bacteria by making wells on nutrient agar surface, and filling them with the filtrates of *Lb.plantarum*. After incubation the diameter of inhibition zones around wells were measured and compared with that containing MRS broth as control (Choi and Beuchat, 1995).

3.2.8 Production of β- galactosidase (*In vitro*):

Loopfull of bacterial culture was inoculated in X- gal MRS medium and incubated at 37° C for 2-3 days. Changing to blue color considered to be β-galactosidase producers.

3.2.9 Determining Inhibitory Effect of *Lb.plantarum* Propagating in MRS Fortified with Different Substances:

Bacterial culture were prepared by inoculating 1% of previously activated bacteria *(Lb.plantarum* isolate), into the MRS broth enriched with lactose, fresh whey, and dried whey at different concentrations (Kontula *et al.*, 1999). After adjusting pH to 6, they are incubated at 37°C for (24, 48 and 72) hr. then centrifuged at 6000 rpm for 10 min. Free cells supernatant was taken and filtered throughout Millipore filter (0.22µm).

Well diffusion method was applied to detect the inhibitory effect of *Lb.plantarum* (Ryan *et al.*, 1996), by streaking each pathogenic test bacterial isolates on surface of nutrient agar plate with a sterile spreader,

then wells (5 mm) in diameter were made by the aid of a cork borer on surface of nutrient agar. Each well was filled with the filtrate of *Lb.plantarum* and incubated at 37^{o}C for 24 hr. then inhibition zone diameter was measured.

The filtrate of *Lb.plantarum* that propagated in MRS broth was concentrated to-three fold and also the filtrate of *Lb.plantarum* that propagated in MRS broth medium containing the best concentration of prebiotic substances that have best effect on activity of *Lb.plantarum* was concentrated into three fold and the well diffusion method was repeated also.

3.2.10 Minimum Inhibitory Concentrations (MIC,s) of *Lb.plantarum* Filtrate against Test Bacteria:

Serial ratios (10 ml each) of the three- fold concentrated filtrate of *Lb.plantarum* previously propagated in MRS broth medium were prepared by using nutrient broth for dilution. After the following ratios (0:10; 1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1) as concentrated filtrate: nutrient broth were prepared in test tubes. They were inoculated with 0.1 ml of each pathogenic test bacteria culture then incubated for overnight at 37°C. Growth intensity of each tube was observed and recorded as; light (+), medium (++), heavy (+++), and no growth (-). Growth was estimated by measuring optical density ($OD._{600}$) nm was read for each dilution. Results were matched with the growth intensities mentioned in Midolo *et al.*, 1995.

Same procedure was repeated for three-fold concentrated filtrate of *Lb.plantarum* previously propagated on MRS broth fortified with lactose, fresh whey and dried whey, separately.

3.2.11 Measurment of *Lb.plantarum* Growth Curve.

Two hundred ml of MRS broth without prebiotic substances was inoculated with 1 % exponentionaly growing culture of *Lb.plantarum,* then incubated on shaker incubator in anaerobic condition of 180 rpm at 37 ºC, and optical density (600 nm) at the time of inoculation and then each 2 hrs. for 24 hr.

These producers were repeated by using MRS broth containing prebiotic substances (in three concentrations giving best activity on *Lb.plantarum*).

3.2.12 Enumerating *Lb.plantarum* Cells Number Before and After Addition of Prebiotic Substances:

Counting *Lb.plantarum* numbers before and after addition of prebiotic substances were estimated by plate count method. After preparing serial dilutions from the isolate of *Lb.plantarum*, 0.1 ml from each dilutions were transferred into MRS agar plates, after incubation for 24, 48 and 72 hr at 37° C, numbers of colonies were calculated. The same procedure repeated for *Lb.plantarum* that have been propagated in MRS broth fortified with prebiotic substances (Bryant and Burkey, 1953).

2-1 Microbial Ecology of the Human Gut:-

The human intestinal tract is inhabitated with more than 400 bacterial species, among these, only 30 to 40 species constitute 99% of the mass culturable intestinal flora (Tannock *et al.*, 2004). The effect of these microorganisms can profoundly influence nutrient synthesis and absorption , as well as the host's local gastrointestinal tract (GT) and peripheral immune system (Macfarland and Elmer, 1995).

Mital and Garg (1995) stated that environmental factors, physiological interactions and others govern the distribution of the microflora in different regions of the gut. Of other factors, diet is a major factor that regulates frequency and concentration of individual species and groups of microorganisms colonize the human gut.

2-2 Human Gastrointestinal Microflora:-

Bacterial cells that inhabitant human gastrointestinal tract constituted an enormously complex ecosystem that includes both facultative anaerobic and anaerobic microorganisms. For example, microflora of the stomach are predominantly Gram-positive anaerobes with a concentration of$<10^3$ colony forming units (CFU)/ml. The highest concentrations of these microorganisms are in the large intestines (Balmer *et al.,* 1994).

Number of bacterial cells in the human colon is approximately, 10^{14}which is 10 times more than the total number of cells in the human body(10^{13}) (Saxelin *et al.*, 1991).

At birth the gastrointestinal tract is practically sterile, however, it is very soon colonized by microbes, at first from the mother and then by all ingested food materials (Bezirtzoglou, 1997).

Yaeshima (1996) found that *Bifidobacteria* are dominant flora in the intestine, of infant, and breast feeding may impact positively on the development of bifidobacterial flora because of bifidogenic substances in milk, and by the age of two, the gastrointestinal microbiota is similar to that of an adult. Subsequently, only major changes are detected in microbiota in adults.

The amount of bacteria in the mouth is abundant, decreases in stomach, then increases again in the intestine and finally rapidly increases in the colon, where saccharolytic, facultative anaerobic strains are dominant. The most dominating species belong to the genera *Enterobacter, Bactericides, enterococci* and *Bifidiobacteria,* while *Lactobacilli* comprise <10 % of the microbiota (Tannock *et al*., 2004).

Lactobacilli are perhaps the most well known of these favorable microorganisms. A number of species of *Lactobacilli* resides in the human intestine in a symbiotic relationship with each other and with other microorganisms (Fuller, 1991). Gibson and Roberfroid (1995) declared that only the most acid-tolerant species of *Lactobacilli* can survive in the stomach, while in the small intestine *Enterococci* are also encountered.

The intestinal microbiota can further be divided into beneficial, neutral or harmful microbes having several functions in the colon. Harmful or pathogenic effect which include diarrhea or constipation, infection, liver damage, cancer, encephalopathy and intestinal putrefaction, can be attributed to *Pseudomonas aeruginosa, Proteus*, *Staphylococci , Clostridia* and

Veillonae. Neutral species can induce a disease when they dominate in high numbers .These species include *Enterococci* spp*, E.coli, Streptococci* and *Bacteroides* spp. In addition to lactic acid bacteria and *Bifidiobacteria, Eubacteria* may have health beneficial effects (Moore and Moore, 1995).

2-3 Probiotics:-

The word probiotic derived from Greek and means "for life" (Lilley and Stillwell, 1965), and this name is now mostly used to refer to concentrated supplements of beneficial or good bacteria take by humans and animals.

Probiotics can be defined as organisms and substances which contribute to intestinal microbial balance (Fuller and Gibson, 1997; Guarner and Schaafsma, 1998).

Lilley and Stillwell (1965) were first used the term (probiotic) to describe substance secreted by one microorganism to stimulate the growth of another, and thus was contrasted with the term antibiotic. On the other hand, Fuller (1989) attempted to improve the definition of probiotic with the following distinction (a live microbial feed supplement which beneficially affects the host health by improving it's intestinal balance).

Havenaar *et al*., (1992), defined probiotics as a viable mono- or mixed culture of microorganisms which applied to animal or man, beneficially affects the host by improving the properties of the endogenous microflora. Further more, Salminen (1996) defined it as a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host.

Probiotic also termed as biotheraputic agent because it's therapeutically use to modulate immunity, lower cholesterol, treat rheumatoid arthritis, prevent cancer, improve lactose intolerance and prevent or reduce the effect of atopic dermatitis, crhon's disease, diarrhea, and constipation as well as candidiasis and urinary tract infection (Mercenier *et al.*, 2003).

2-3.1 Probiotic Microorganisms:

Many strains are used as probiotic microorganism. Ideally all of them should have a beneficial effect and do not cause harm to the host. Also all they should be studied comprehensively prior to use in humans and animals to be Generally Regarding As Safe (GRAS). As table (2-1) shows the probiotic microorganisms and their infection potential.

The effects of these strains are based on specific capabilities and enzymatic activities even within one species (Ouwehand *et al*., 1999; Bernet *et al.,* 1993).

The most probiotic microorganisms used are lactic acid bacteria (LAB) including the genus *Lactobacillus,* especially *Lb.acidophuils, Lb.plantarum, Lb.rhamnosus, Lb.fermentum* (Havenaar *et al*., 1992; Greene and Klaenhammer, 1994).

In general, a group of requirements have been identified as important properties for *Lactobacilli* to be effective probiotic microorganisms (Ried, 1999; Salminen *et al.*, 1994). These includes; adherence to cells (Ouwehand *et al.*, 1999), exclude or reduce pathogenic adherence to cells, persist and multiply, produce acids, hydrogen peroxide, bacterocins antagonistic to pathogen growth (Reid and Burton, 2002) and resist vaginal microbicides including spermicides and be safe, and therefore non

invasive, non carcinogenic and non- pathogenic, coaggregated from a normal balanced flora (Spanhaak *et al.*, 1998).

Table (2-1), Probiotic Microorganisms and Their Safety Status. (Donohue and Salminen, 1996).

Organism (genus)	Infection Potential
Lactobacillus	Mainly non pathogenic
Lactococcus	Mainly non pathogenic
Streptococcus	Opportunistic; only *S. thermophilus* is used in dairy product.
Enterococcus	Opportunistic, some strains exhibit antibiotic resistance.
Bacillus	Only *B. subtilis* GRAS status is report in probiotic use.
Bifidobacterium	Mainly no pathogenic, some strains are isolated from human infections.
Propionibacterium	Dairy *Propionibacterium* group is a potential candidate for probiotic.
Saccharomyces	Mainly nonpathogenic, some strains are isolated from human infection.

2.3.2 the History of Lactic Acid Bacteria:-

There is a long history of health claims concerning living microorganisms in food, particularly lactic acid bacteria. In 76 before century, the Roman Historian Plinius recommended the administration of fermented milk products for treating gastroenteritis (Schrezenmeir and Vrese, 2001). Since the advance of some investigators of the microbiology

era have been attributed such health effects to shifts in the intestinal microbial balance (Tissier, 1984).

Well before, it was believed that intake of yoghurt containing *Lactobacilli* results in reduction of toxin producing bacteria in the gut and that result in increasing the longevity of the host. It was found that the long and healthy life style of Bulgarian could be attributed to the consumption of fermented milks (Abee *et al*., 1995).

Over the last 30 years, intensified efforts to identify and characterize lactic acid bacteria have revealed their many critical roles in dairy foods. (Salmine, 1994). Tissier (1984) recommended that administration of *Bifidobacteria* to infants suffering from diarrhea could supressed the putrefactive bacteria causing the disease, he added that *Bifidobacteria* were predominant in the gut flora of breast- fed infants.

In 1926 it was found that *Lactobacillus acidophilus* may survive in the human gut (Apella *et al*., 1992; Ried, 1999), while the significant role of the intestinal microflora to resist to disease was showed in 1954 (Agerholm-Larsen *et al*., 2000; D'Souza *et al*., 2002).

2.3.2.1 Characteristics and Requirements of Lactic Acid Bacteria:

Lactic acid bacteria have common properties of Gram positive have cocci or bacillary shape, single, pair or arranged in chain shape, non spore former, non-motile, anaerobic or microaerophilic and catalase negative. Beside producing lactic acid as the main product of fermentation processes (Holzapfel *et al*., 1998).

Lactic acid bacteria need complex nutritional requirements; they are those groups of microorganisms that have lost their ability to synthesize their own growth factors (Narayanan *et al.*, 2004).

LAB can be propagated on complex culture media containing adequate growth factors usually with yeast extract (as source of vitamins) as well as peptone , manages , acetate and stimulatory tween 80 , as well as a low pH ranging between 4.5-6.2. (De man *et al.*, 1960).

2.3.2.2 Inhibitory Materials Produced by LAB:-

Various species of lactic acid bacteria exert antagonistic action against intestinal and food born pathogens (Oyetayo *et al.*, 2003). They are capable of preventing the adherence, establishment ,replication and pathogenic action of specific enteropathogens (Donnet-Hughes *et al.*, 1999). These antagonistic properties are manifested by :-

a) Decreasing the luminal pH through the production of volatile short chain fatty acids such as acetic acids, lactic acid or propionic acids.
b) Rendering specific nutrients unavailable to pathogens.
c) Decreasing the redux potential of the luminal environment.
d) Producing hydrogen peroxide under anaerobic conditions.
e) Producing specific inhibitory materials.

Sanders (1993) reported that the following inhibitory materials are usually produced by LAB by several investigators:

A) Organic Acids :-

Through fermentation, LAB produces organic acids, mainly lactic and acetic acids that have inhibitory action (Wee *et al.*, 2006).

There actions are due to three reason that include low pH , dissociation constant pK value , and mole concentration (Podolak *et al.*, 1996).

Lactic and acetic acid are lipophilic acids in associated forms that can prevent the microbial cell membrane synthesis, and at higher intracellular pH dissociate to produce H^+ ions that interfere with essential metabolic functions (Cintas *et al.*, 1998).

While both organic acids have good inhibitory action against pathogenic bacteria, acetic acid has more antimicrobial activity against undesirable microorganisms (Richards and Xing, 1995).

B) Hydrogen Peroxide (H_2O_2)

Lactic acid bacteria produce hydrogen peroxide in the presence of oxygen that accumulates in the medium resulted from the inability to produce H_2O_2 degrading enzyme such as catalase. It has been reported that production of H_2O_2 by *Lactobacillus* and *Lactococcus* strains inhibited *Staphylococcus aureus*, *Pseudomonas* spp and Vrious psychrotrophic microorganisms in foods (Conne, 1993; Bae *et al.*, 2005).

C) Bacteriocins:-

Lactic acid bacteria produce a variety of antagonistic factors that include metabolic end-products, antibiotics like substances and bacterial proteins termed bacteriocines (Bae *et al.*, 2005). These antimicrobial agents are species specific and exert lethal activity through adsorption to specific receptors located on the external surface

of sensitive bacteria, followed by metabolic, biological and morphological changes resulting in the killing of such bacteria.

LAB produces a wide range of bacteriocins like nisin, diplococcin, lactocin and lactostrepsin (Eijsink *et al.*, 1998). The most important species that produce bacteriocin are *Lactococcus, Streptococcus, Lactobacillus, Leuconostoc* and *Pediococcus*. The *in vitro* studies have demonstrated that some bacterocins have a broad inhibitory spectrum against many Gram- negative bacteria (Carpa and Ackermann, 2006).

D) **Carbon Dioxide (CO_2)**:-

It's mainly produced by heterofermentative LAB. The precise mechanism of its antimicrobial action is still unknown. However, CO_2 may play a role in creating anaerobic conditions which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer may cause failure in permeability (Gibson and Wang, 1994). CO_2 can effectively inhibit the growth of many food spoilage microorganisms, especially Gram- negative psychrotrophic bacteria (Farber, 1991; Hotchkiss *et al.*, 1999).

E) **Diacetyl and Acetyldehyde**

Diacetyl is produced by strains within all genera of LAB by citrate fermentation. The antimicrobial effect of diacetyl has been known since

the 1903 (Hugenholtz, 1993). It inhibits the growth of Gram-negative bacteria by reacting with arginine binding protein (Marteau *et al.*, 2001).

Jay (1982) showed that Gram- negative bacteria were more sensitive to diacetyl than Gram-positive bacteria; the former was inhibited by diacetyl at 200 mg/ml and the latter at 300mg/ml.

Diacetylactis and the acceptable sensory levels of diacetyl are at 2-7 mg/ml (Motlagh *et al.*, 1992). It's practical use as a food preservatives is limited, diacetyl may act synergistically with other antimicrobial factors and contributes to combined preservation systems in the fermented foods, Acetaldehyde formed during carbohydrate metabolism of heterofermentative LAB also could be reduced to ethanol by reoxidation of pyridine nuclotides, catalyzed by an NAD-dependent alcohol dehydrogenase, acetaldehyde imparts the typical aroma of yoghurt .

Piard and Desmazeaud, (1991), found that the antimicrobial activity of acetaldehyde was (10-100) ppm against food born pathogens (*E.coli, Salmonella typhimurum* and *S.aureus*) .

2.3.3 Importance of Lactic Acid Bacteria in Food Industry:-

Lactic acid bacteria have been used to ferment cultural foods for at least 4000 years (Hull *et al.*, 1992). It's found in a number of fermented food products and used for their proposed health promoting properties (Fernands *et al.*, 1992). These lactic acid bacteria contribute to their preservation, nutrition availability and flavor; they are used in particular in fermented milk (Chenoll *et al.*, 2006).

A number of these dairy products are produced using *Lactobacillus* either alone or in combination with other lactic acid

bacteria. Dairy foods fermented by lactic acid bacteria has long been held in special favor as safe and nutritious foods that may also elicit positive effects on health and well being (Hove *et al*., 1999). Soured milk bore the first pure bacterial culture, bacterium lactis and later the probiotic concept. Acidophuilus milk is an example of fermented dairy product and *Lactobacillus acidophilus* is the organism used to produce it.

Vegetables are fermented with *Lactobacilli* to produce products including pickles and olives. Members of *Lactobacillus* genus are natural contaminants of vegetables and take their place in the fermentation process along with a number of other microorganisms.

Lactobacillus species also play an essential role in bread making, and a number of unique strains have been identified in products, most notably sourdough bread , typical species of *Lactobacilli* identified in sourdough bread include *L.acidophilus, L.faciminis, L.delbruecki, L.plantarum, L.rhamnosus, L.brevis* and *L. fermentum*(Walker and Duffy, 1998).

2.3.4 Importance of Lactic Acid Bacteria to Human Health:-

Many have advanced the theory that certain LAB (those normally reside in the human intestine) may exert a positive influence on human health (Oyetayo and Osho, 2004). *Lactobacillus* is considered as a especially beneficial bacteria due to it's ability to aid in the breaking down into proteins, carbohydrates, and fat in food then helping absorption of necessary elements and nutrients such as minerals, amino acids and vitamins required for human and animal to survive (Bartram *et al*., 1994). They are used to treat disturbed intestinal microflora and increased gut permeability which are

characteristic of many intestinal disorders such as acute rotaviral diarrhea, food allergy, colon disorders and changes associated with colon cancer development (Dunne and Shanahan, 2002).

LAB have many other physiological effects to benefit the host, such as absorption of nutrients that is primary function in intestine by salvaging energy from carbohydrate not digested in the upper gut. Also LAB have a role in the synthesis of B vitamines and metabolism of the bile acids, other sterols and exenobiotics (Cummings and Macfarlane, 1997).

Also LAB have a role in allvation of lactose intolerance symptoms (Priebe *et al*., 2006), this symptom is present in a wide range of the world wide population (Montes *et al*., 1995).

Further more, some species of LAB have anticarcinogenic and hypocholesterolemic properties as for example in *L. acidophilus* due to inhibition of 3- hydroxyl 3- methylglutary reductase which is a rate limiting enzyme in endogenous cholesterol biosynthesis in the body . So the use of probiotics to reduce this risk seems very attractive, especially if consumed as a part of a normal dairy nutrition(Pereira *et al*., 2003;Taranto *et al.,* 1998).

LAB also have many immunomodulatory effects, so the potential health benefits of it include protection against enteric infections , use as an oral adjuvant, the immunepotentation in malnutrition and the prevention of chemically induced tumor(Perdigon *et al*., 2001).

2.4 Prebiotics:-

Prebiotic is defined as a non-digestible food ingredient that beneficially affects the host by improving human health due to it's nature that is undigested or partially digested in the small intestine. (Grizard and Barthomeuf, 1999).

The term prebiotic was first introduced by Gibson and Rberfroid (1995) to the substance that promotes the growth and /or activity of a limited number of bacterial species in the gut. Also prebiotic have selective stimulation effects on certain groups of colonic bacteria in the large intestine (Quigley *et al.,* 1999).

Meulen *et al.,* (2004) stated that in addition to their selective effect on *Bifidiobacteria* and *Lactobacilli,* they influence many aspects of bowel function through fermentation.

Beneficial effects of feeding an exogenous probiotic may be enhanced and extended by simultaneous administration of a prebiotic that the probiotic can utilize it in the intestinal tract (Macfarlane and Cummings, 1999).

In order for prebiotics to be effective, they must escape digestion in the upper gastrointestinal tract and be released in the lower tract to be used by beneficial microorganisms found in the colon; (Rolfe, 2002). So the mechanisms by which prebiotics works are via (Gibson *et al.,* 1995):-

a) Stimulation of microbial growth.

b) Increase in bacterial cell mass.

c) Stimulation of peristalsis by the increased bowel content.

2.4.1 Prebiotic Action:

Prebiotics are vital to probiotic organisms to survive and thrive in the human gut, so prebiotics have been shown to enhance health in a number of ways through support growth of the probiotic bacteria in the gut. For example, short chain fatty acids (SCFAs) are products of prebiotic fermentation by these gut microflora and are crucial for human health, so the probiotic bacteria and SCFAs restrict the growth and activity of less beneficial species (Vaux *et al.*, 2000). This considered as the gastrointestinal effect of prebiotic. Potentially, the most important effects of prebiotic carbohydrates are to strengthen the body's resistance to invading pathogens, thereby prevent episodes of diarrhea (Cummings and Macfarlane, 2002). Beside, prebiotics may have anticarcinogenic activities by maintaining healthy bowel function and hence may play an important role in preventing colon cancer (Duggan *et al.*, 2002). Also prebiotic may have antimicrobial, hypolipidemic and glucose-modulatory activities; they may have activity in improving mineral absorption balance.

Prebiotics have mild laxative effects and many other actions which include lower triglycerides levels in some, the mechanism of this possible effect is unclear, decreased hepatocyte synthesis of triglycerides is one hypothetical possibility (Flickinger *et al.*, 2000).

Oligosaccharide, a type of prebiotic may lower total cholesterol and Low Density Lipoprotein (LDL) cholesterol levels in some, the mechanisms of this possible effect is unclear but it may due to propionate (a product of

oligosaccharide fermentation) may inhibit HMG-Coa reductase, the rate limiting step in cholesterol synthesis (Chinda *et al.,* 2005).

2.4.2 Synergistic Effect of Probiotic and Prebiotics:-

Prebiotic are the food of fuel for the healthy bacteria. Studies show that the consumption of a wide range of prebiotics can improve gut health. However, when a food formulation can combined both into one food the result is called symbiotic (Schrezenmeir *et al.,* 2004). Symbiotic is a combination of probiotic and a prebiotic in which the prebiotic is used to increase the intestinal survival of the health promoting bacteria with the ultimate goal of modifying the gut flora and it's metabolism.

The symbiotic relationship of probiotic and prebiotics significally contributes to health together their by (Fooks *et al.,* 1999):-

a) anticarcinogenic activity.
b) Antimicrobial activity.
c) Lowering triglycerides level.
d) Stabilizing blood glucose levels.
e) Boost the immune system.
f) Ridding the gut of harmful microorganisms.
g) Helping to prevent constipation and diarrhea.

2.4.3 Prebiotic Digestion and Fermentation:-

Prebiotics are all carbohydrates of relatively short -chain length that to be effective must reach the ceacum in some form. A fraction of substances due to their chemical structure must escape digestion by pancreatic and small bowel enzymes in the human gut, and therefore arrives in the large

bowel (Ellegard *et al*., 1997; Bach-Knudsen *et al*., 1995). Any carbohydrate reaches the cecum is a potential substrate for fermentation by the microbiota. *In vitro,* many different materials support bacterial growth and produce various fermentation derived products (Molis *et al.*, 1996; Rossi *et al*., 2005). The major products of prebiotic metabolism are short chain fatty acids (SCFAs), the gasses (hydrogen, carbon dioxide) and bacterial cell mass (Cummings *et al.,* 1995).

2.4.4 Prebiotic Foods:-

Prebiotic carbohydrates are found naturally in many fruits, vegetables and grains such as (bananas, asparagus, garlic, wheat, oat meal, barely and other whole grains, flax seed, tomato, onions and chicory, greens and legumes) like lentils, kidney beans, white beans, black beans.(Rastall and Maitin, 2002).

From the definition of prebiotic"non digestible food ingredient ", prebiotics could also include several classes like:

a) **Dietary fiber**: that is a mixture of many complex substances. It's considered as a fraction of the edible part of plants or their extracts or synthetic analogues (Mure *et al*., 2004). The characteristic property of dietary fiber has been defined as the ability to reach the large intestine in an essentially unchanged state so it resist to digestion and absorption in the small intestine (Joy *et al.,* 1999; Chinda *et al*., 2004). Examples of these are; cellulose, hemicelluloses, lignin, various gums, algal or synthetic polysaccharides and peptic substances (Stark and Madae, 1994).

b) **Oligosaccharides**: they found in many natural products such as plant cells and milk (O'Sullivan, 1996). Disaccharides have also been introduced

to be like prebiotic oligosaccharides in case when they enter the colon (Playne and Crittenden, 1996). Oligosaccharide include; fructooligosacchariedes that are widely found in the nature (onion, oats, wheat); they composed of sucrose and from one to five molecules of fructose. Also there are glucooligosacchriedes, maltooligosaccharides and Xylo-oligosaccharides (Voragen, 1998). Various oligosaccharides are classified as prebiotics and added to the processed foods as additives (Vander Meulen *et al.*, 2006).

2.4.4.1 Lactose:-

Several hundreds of LAB resides in the human large intestine and these LAB metabolized non-digested dietary carbohydrates (resistant starch, non starch polysaccharide, and other sugars like lactose, raffinose and stachyose) to variety of products such as SCFAs (e.g.:- acetic acid, propionic acid, and butyric acid), other organic acids (lactic acid , succinic acid and pyruvic acid) and gasses (H_2, H_2S , CO_2, CH_4)(Macfarlane and Macfarlane, 1997).

Dairy products, foods prepared with the use of dairy ingredients, are exclusive source of the disaccharide lactose in human diets. Before absorption, lactose is hydrolyzed by the intestinal brush border β-galactosidase(lactase) into glucose and galactose, these monosaccharides are absorbed and used as energy sources.

Rosado *et al.*, (1992) found that before fermentation, lactose content of the foods mix generally is ~6%. One example of significant bacteria – induced change that occurs during the fermentation process is the hydrolysis of 20-30% of the disaccharide lactose to its absorbable monosaccharide components glucose and galactose. In addition a portion of the glucose is converted to lactic acid. Undigested lactose remains in the intestinal lumen

and as it reaches the colon, it is fermented by colonic bacteria.Lactose content varies with the duration of the storage after fermentation, In addition, the bacterial lactose activity corresponds with the survival time of *Lactobacilli* after ingestion, then enhanced digestion of the lactose is explained partly by the improved lactase activity after food ingestion and partly by other enzymatic functions such as the activity of the lactose transport system (permease), that allows lactose to enter probiotic cell. Animal studies have suggested that LAB may induce lactase activity of the gut intestinal endothelial cells(Marteau *et al.,* 1990). Lactose (milk sugar), bacteria use it and consider it as carbon source for growth (Salminen and Salminen, 1997).

A carbohydrate source like lactose is poorly utilized by many pathogenitically significant species of the families *Enetrobacteriaceae* and *Vibronaceae* and the genera *Pseudomonas.* About 20% to 30% of the lactose in the yoghurt base are broken down to glucose and galactose and that glucose be converted to lactic acid during yoghurt fermentation. Bacterial enzymes can help break down the remaining lactose in the intestinal tract (Lonnerdal, 2003).

2.4.4.2 Whey:-

Whey is a by-product of cheese industry, also known as a residue of milk after removal of casein and most of the fat as in cheese making, also known as lacto- serum (Kulozik and Wilde, 1999). It is recognized as a value-added ingredient in many food products including dairy, meats, bakery and beverages (Brassart, 1999).

When cheese is made, most of protein, fat and minerals go into the cheese. After the cheese curd is removed, the remaining liquid whey is consisted mostly of water and lactose along with a small amount of high quality whey protein. Whey is also formed in the making of yoghurt being the thin liquid that forms on the top of the settled yoghurt.

Whey is a reliable source for a number of high quality and biologically active components like proteins, vitamins, traces milk fat, minerals (calcium, phosphorus, magnesium, zinc, sodium and potassium), and lactose as carbohydrates, (Howarth, 1996).This component capable of improving health and prevent disease (Horton, 1995).

2.4.4.2.1 Prebiotic Properities of Whey Components:-

Whey derived carbohydrate components with prebiotic activity have been found (Naidu *et al.,* 1999). Lactose in whey has been shown to support LAB, such as *Bifidobacteria* and *Lactobacilli.* Sialic acids (types of oligosaccharides) which are typically attached to proteins commonly found in whey have also been shown to have prebiotic effects (Harper, 2002). Lactose from whey is an important precursor for prebiotic products. For example galactooligosaccharides can be produced through a transgalactosylation reaction when lactose is enzymatically hydrolyzed.

2.4.4.2.2 Health Enhancing Properties of Whey Components:-

There are five beneficial areas of intestinal health modification with whey components, they are:-

a) Prebiotic Effect: this is because of carbohydrate components (lactose, sialic acids) derived from it (Naidu *et al.,* 1999).

b) Antiviral and Antimicrobial Properties of Whey Components:- Abd El-Salam *et al.*(1996), found that whey contains several unique components with broad antimicrobial properties that protect against toxins, bacteria and viruses including immunoglobulins (Igs), lactoferrin, lactoperoxidase(Lp), sphinogolipids and peptide derivative(Brody, 2000).

Significant levels of these components have been shown to survive passage through the stomach and small intestine and arrive as intact proteins in the large intestine, where they exert their biological effects (Warny *et al.,* 1999).

c) Anticancer Properties of Whey Components: - Whey is a source of specific components that animal and cell culture studies suggest have anticancer properties (Bounous, 1991). The first are the sulphur amino acids (cysteine and methionine) which are found in high levels in whey protein. Cystein and methionine are utilized in glutathione synthesis.

Glutathione is the widly distributed and is a substrate for two classes of enzymes that catalyse detoxification compounds and bind mutagens and carcinogens facilitating their elimination from the body (Parodi, 1998).

d) Cardiovascular Health: - Whey components may have positive effect on cardiovascular health (Schrezenmeir *et al.,* 2000). Whey derived peptides may protect against hypertension, inhibit platelets aggregation and lower blood cholesterol levels.

e) Immune Enhancing Properties of Whey Components:-

The human GI tract houses the largest portion of immune system. The gut associated lymphoid tissue and mucosal associated lymphoid tissue, together these tissues help to preserve and promote the integrity and function of GI tract, thus contributing to the maintenance of overall health.

2.4.4.3 Dried Whey:-

Fresh pasteurized liquid whey is rarely used due to high transportation costs and susceptibility to deterioration during storage, so one of ways of utilizing cheese whey is to condensed it by evaporation, reverse osmosis or ultrafiltration to condensed products or maximally concentrated by drying(Walzem *et al.,* 2002).

From whey, whey powder , whey protein concentrates , whey protein isolates, reduce-lactose whey and demineralized whey are produced. These dried whey produced or concentrated by methods above , are either undergo evaporation or ultrafiltration can be also used in this method.

The whey must clarified (the fat is removed) before concentrations by centrifugation, pasteurized then dried to provide a fine light cream to slightly yellow color . Dried whey has the same healthy benefits of whey.

The final product of whey powder is mostly lactose (about 68%), with some protein about 12% and ash about 8%. The typical content of dried whey is shown in the table (2-2):-

Table (2-2). Typical Nutrients Composition of Dried Whey :-(Ensminger *et al.*, 1978):

substances	%
Dry matter	93
Lactose	68
Protein	12
Minerals	
• Calcium	0.86
• Phosphorus	0.76
• Sodium	1.30
Amino acids	
• Lysine	0.94
• Threonine	0.89
• Tryptophan	0.3
Metabolizable energy, kcal/ibz	1,500

Examining Committee's Certification

We, the Examining Committee, certify that we read this thesis and have examined the student in its contents and that, according to our opinion; it is adequate as a thesis for the Degree of Master of Science, in Biotechnology.

Signature:
Name:
Scientific Degree:
Date:

(Chairman)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Title:
(Member & Supervisor)

I here by, certify upon the decision of the examining committee

Signature:
Name: Dr. Laith Aziz Al-Ani
Scientific Degree: Assist .Professor
Occupation: Dean of College of Science.

List of Contents

No.	Subject	Page No.
	Summary	I
	List of Contents	III
	List of Tables	VII
	List of Figures	IX
	List of Abbreviations	XII
Chapter One: Introduction		
	Introduction	1
Chapter Two: Literature Review		
2-1	Microbial Ecology of the Human Gut	3
2-2	Human Gastrointestinal Microflora	3
2-3	Probiotics	5
2-3.1	Probiotic Microorganisms	6
2.3.2	The History of Lactic Acid Bacteria	7
2.3.2.1	Characteristics and Requirements of Lactic Acid Bacteria	8
2.3.2.2	Inhibitory Materials Produced by LAB	9
2.3.3	Importance of Lactic Acid Bacteria in Food Industry	12
2.3.4	Importance of Lactic Acid Bacteria to Human Health	13
2.4	Prebiotics	15
2.4.1	Prebiotic Action	16
2.4.2	Synergistic Effect of Probiotic and Prebiotics	17
2.4.3	Prebiotic Digestion and Fermentation	17
2.4.4	Prebiotic Foods	18
2.4.4.1	Lactose	19
2.4.4.2	Whey	20
2.4.4.2.1	Prebiotic Properities of Whey Components	21
2.4.4.2.2	Health Enhancing Properties of Whey Components	21
2.4.4.3	Dried Whey	23
Chapter Three : Materials and Methods		
3.1	Materials	25
3.1.1	Apparatus and Equipments	25
3.1.2	Chemicals	26
3.1.3	Culture Media	27
3.1.3.1	Ready to Use (Powdered) Media	27
3.1.3.2	Laboratory- Prepared Media	27

3.1.4	Solutions and Reagents	27
3.1.4.1	Normal Saline Solution	27
3.1.4.2	Nessler's Reagent	27
3.1.4.3	Phenol Red Reagent	28
3.1.4.4	Catalase Reagent (H_2O_2)	28
3.1.4.5	Oxidase Reagent	28
3.1.5	Bacterial Isolates	28
3.1.6	Prebiotic Raw Materials	29
3.2	Methods	29
3.2.1	Sterilization	29
3.2.1.1	Moist -Heat Sterilization	29
3.2.1.2	Dry -Heat Sterilization	29
3.2.1.3	Membrane Sterilization	29
3.2.2	Media Preparation	30
3.2.2.1	Ready-to Use Medium	30
3.2.2.2	Laboratory Prepared Media	30
3.2.2.2.1	Rogoza-broth Medium(MRS)	30
3.2.2.2.2	Fermentation Media	31
3.2.2.2.3	Litmus Milk Medium	31
3.2.2.2.4	Gelatin Medium	31
3.2.2.2.5	Arganine Broth Medium MRS	31
3.2.2.2.6	Fortified MRS Broth Media	32
3.2.2.2.7	X-Gal MRS Agar	32
3.2.3	Samples Collection	32
3.2.4	Isolation of *Lactobacillus* spp	33
3.2.5	Identification of *Lactobacillus* Spp	33
3.2.5.1	Microscopic Examination	33
3.2.5.2	Biochemical Test	34
3.2.5.2.1	Catalase Test	34
3.2.5.2.2	Oxidase Test	34
3.2.5.2.3	Gelatinase Test	34
3.2.5.2.4	Acid production and Clot Formation Test	34
3.2.5.2.5	Production of Ammonia from Arginine	34

3.2.5.2.6	Carbohydrate Fermentation Test	35
3.2.5.2.7	Growth on Nutrient Agar	35
3.2.5.2.8	Growth at 45 ºC and 15 ºC	35
3.2.6	Maintaining of *Lactobacillus* Isolates	35
3.2.6.1	Daily Working Culture	36
3.2.6.2	Stock Culture	36
3.2.7	Determining Inhibitory Effect of *Lactobacillus* Isolate against Test Bacteria	36
3.2.7.1	on Solid Medium	36
3.2.7.2	in Liquid Media	36
3.2.8	Production of β- galactosidase	37
3.2.9	Determining Inhibitory Effect of *Lb.plantarum* Propagating in MRS fortified with Different Substances	37
3.2.10	Minimum Inhibitory Concentrations (MIC,s) of *Lb.plantarum* filtrate against Test Bacteria	38
3.2.11	Measurment of *Lb.plantarum* Growth Curve	39
3.2.12	Enumerating *Lb.plantarum* Cells Number before and after Addition of Prebiotic Substances	39
	Chapter Four: Results and Discussion	
4.1	Isolation of *Lactobacillus* Species	40
4.2	Identification of *Lactobacillus* Species	40
4.2.1	Cultural Characteristics	40
4.2.2	Morphological Characteristic	40
4.2.3	Biochemical Test	40
4.3	Inhibitory Activity of *Lactobacillus* isolates against Test Bacteria	43
4.3.1	on Solid Medium	43
4.3.2	in Liquid Medium	48
4.4	Production of β-galactosidase by *Lb.plantarum*	53
4.5	Inhibitory Activity of *Lb.plantarum Propagated* in MRS Fortified MRS Medium	54
4.6	Minimum Inhibitory Concentration (MIC,s)of	66

	Concentrated Filtrates of LAB	
4.7	Growth Curve Measurement of *Lp.plantarum2* Isolate Propagated in	69
4.7.1	MRS Broth	69
4.7.2	MRS Broth Fortified with Prebiotic Substances	69
4.8	*Lb.plantarum* Cells Numbers before and after Prebiotic Treatment	72

Chapter Five:Conclusions and Recommendations		
5.1	Conclusions	75
5.2	Recommendations	76
	References	77

List of Tables

No.	Subject	Page No.
2-1	Probiotic microorganisms and their safety status	7
2-2	Typical nutrients composition of dried whey	24
4-1	Biochemical Characterization of *Lactobacillus* spp. Isolated from Dairy Product	43
4-2	Inhibitory Activity of *Lactobacillus* Isolates Against Test Bacteria on Solid Medium (MRS agar) after Different Incubation Periods.	44
4-3	Inhibitory Activity of Unconcentrated Filtrate of *Lb.plantarum*2 Isolate against Test Bacteria after Propagating in MRS Broth for 24 hr.	49
4-4	Inhibition Zone Produced by *Lb.plantarum*2 Isolate Propagating in MRS Broth Fortified with Different Lactose Concentration against Test Bacteria	54
4-5	Inhibition Zone Produced By *Lb.plantarum2* Isolate Propagating in MRS Broth Fortified With Different Fresh Whey Concentration Against Test Bacteria	58
4-6	Inhibition Zone Produced by *Lb.plantarum2* Isolate Propagating in MRS Broth Fortified With Different Dried Whey Concentration Against Test Bacteria	63

4-7	Minimum Inhibitory Concentrations(MIC,s) of Concentrated Filtrates of *Lb.plantarum*2 Isolate Propagating in MRS Broth against Test Bacteria.	69
4-8,a	Minimum Inhibitory Concentrations(MIC,s) of Concentrated Filtrates of *Lb.plantarum*2 Isolate Propagating in MRS Broth Fortified with Lactose against Test Bacteria.	66
4-8,b	Minimum Inhibitory Concentrations(MIC,s) of Concentrated Filtrates of *Lb.plantarum*2 Isolate Propagating in MRS Broth Fortified with Fresh Whey against Test Bacteria.	68
4-8,c	Minimum Inhibitory Concentrations(MIC,s) of Concentrated Filtrates of *Lb.plantarum*2 Isolate Propagating in MRS Broth Fortified with Dried Whey against Test Bacteria	68
4-9	Number of *Lb.plantarum* before and after Prebiotic Treatment	68

List of Figures

No.	Subject	Page No.
4-1	Inhibitory Activity of *Lb.plantarum 2* Isolate against Test Bacteria after Propagating on Solid Medium for 24 hr.	**47**
4-2	Inhibitory Activity of Unconcentrated Filtrate of *Lb.plantarum*2 Isolate against Test Bacteria after Propagating in MRS Broth for 24 hr.	**50**
4-3	Effect of Concentrated Filtrate of *Lb. plantarum*2 Isolate when Propagated in MRS Broth	**51**
4-4	Inhibitory Activity of Three Folded Concentrated Filtrate(12.5%) of *Lb.plantarum*2 Isolate against Test Bacteria after Propagating in MRS Broth For 24 hr.	**52**
4-5	Ability of *Lb.plantarum*2 Isolate for Production of β-galactosidase (*in vitro*) on Xgal-MRS Agar Medium.	**53**
4-6	Effect of Concentrated Filtrate of *Lb. plantarum*2 Isolate when Propagated in MRS Broth Containing, Lactose 2%	**56**
4-7	Inhibitory Activity of Unconcentrated and Concentrated Filtrate of *Lb.plantarum*2 Isolate Propagated in MRS Fortified with Lactose after Incubation for 48 hr.	**57**

4-8	Effect of Concentrated Filtrate of *Lb.plantarum2*Isolatewhen Propagated in MRS Broth Containing Fresh Whey 40%v/v	**60**
4-9	Inhibitory Activity of The Three Folded Concentrated Filtrate (12.5%)of *Lb.plantarum2* Isolate Propagated in MRS Fortified with Fresh Whey after Incubation for 48 hr.	**61**
4-10	Effect of Concentrated Filtrate of *Lb. plantarum2* Isolate when Propagated in MRS Broth Containing Dried Whey 3%.	**64**
4-11	Inhibitory Activity of Three Folded Concentrated Filtrate of *Lb.plantarum*2 Isolate against Test Bacteria after Propagating in MRS Broth Fortified with Dried Whey for 24 hr.	**65**
4-12	Growth Curve of *Lb.plantarum2* Isolate in MRS Medium	**70**
4-13	Growth Curve of *Lb.plantarum2* Isolate in MRS Medium Fortified with 2% Lactose	**71**
4-14	Growth Curve of *Lb.plantarum2* Isolate in MRS Medium Fortified with 40% Fresh Whey	**71**
4-15	Growth curve of *Lb.plantarum2*	**72**

	Isolate in MRS Medium Fortified with 3% Dried Whey	

List of Abbreviations

GI	Gastrointestinal Tract
GRAS	Generally Recognized As Safe
LAB	Lactic Acid Bacteria
SCFA,s	Short Chain Fatty Acids
LDL	Low Density Lipoprotein
MRS	Man-Regosa Sharp
CFU	Colony Forming Unit
MIC	Minimal Inhibitory Concentration Correlates

CHAPTER ONE

Introduction

CHAPTER TWO

Literature Review

CHAPTER THREE

Materials and Methods

CHAPTER FOUR

Results and Discussion

CHAPTER FIVE

Conclusions and Recommendations

References

(A)

- **Abd El-Salam**, M.; El-Shibiny, S. and Buchheim, W. (1996). Characteristics and potential uses of the casein macropeptide. Int. Dairy J. 6: 327-341.

- **Al- Dulaimy**, R. K. (2005). The effect of lactic acid bacteria on bacterial causes sever acne vulgaris. M. Sc. Thesis submitted to College of Science, Al-Nahrain University.

- **Abee**, T.; Krockel, L. and Hill, C. (1995). Bacteriocins: mode of action and potentials in food preservation and control of food poisoning. Int. J. Food. Microbiol. 28(2): 169-185.

- **Agerholm-Larsen,** L.; Raben, A. and Haulrik, N. (2000). Effect of 8 week intake of probiotic products on risk factors for cardiovascular diseases. Eur. J. Clin. Nutr. 5(2): 683-684.

- **Al- Jeboury,** G. H. (2005). Probiotic effect on *Proteus mirabilis* and its adhesion property. M.S.c Thesis Submitted to the College of Science of Al-Nahrain University.

- **Aktypis**, A.; Kalantzopoulos, G.; Huis-Veld, J. and Brink, B. (1998). Purification and characterization of thermophilin T, a novel bacteriocin produced by *Streptococcus thermophilus* ACA-DC 0040. J. Appl. Micrbiol. 84: 568-576.

- **Al-Yas**, M. G. (2006). Evaluation of different methods for detecting *Helicobacter Pylori* isolated from human and the Effect of probiotics on the Bacterial Growth. Ph.D. Thesis Submitted to the College of Science of Al-Nahrain University.

- **Apella**, M.; Gonzalez, S.; Nader, M.; Romero, N. and Oliver, G. (1992). *In vitro* studies on the inhibition of the growth of *Shigella sonnei* by *Lactobacillus casei* and *Lactobacillus acidophilus*. J. Appl. Bacteriol. 73: 480-483.

- **Atlas**, R. M.; Brown, A. E. and Parks, L. C. (1995). Laboratory Manual of Experimental Microbiology. 1st ed. Mosby. Inc. Missouri.

(B)

- **Bach- Knudsen**, K. and Hessove, I. (1995). Recovery of inulin from jerusalem artichoke (Helianthus tuberosus L.) in the small intestine of man. Br. J. Nutr. 74: 101-103.

- **Bae**, S.; Fleet, G. and Heard, G. (2005). Lactic acid bacteria associated with wine grapes from several Australian vineyards. J. Apl. Microbiol. (4): 712-722.

- **Baily**, S.; Baron, E. and Finegold, S. (1990). Diagnosis Microbiology.8th ed.The C. V. Mosby Co.London.

- **Balmer**, S. E.; Hanvey, L. S. and Wharton, B. A. (1994). Diet and fecal flora in the newborn: nucleotides, Arch. Dis. Child. 70: 137-140.

- **Baron**, E. J.; Peterson, L. R. and Finegold, S. M. (1994). Diagnosis Microbiology.9th ed. Mosby-Year Book,Inc.

- **Bartram.** H.; Scheppach, W.; Gerlach, S.; Ruckdeschel, G.; Kelber, E. and Kasper, H. (1994). Dose yoghurt enriched with *Bifidobacterium longum* affect colonic microbiology and fecal metabolites in healthy subjects, Am .J. Clin. Nutr. 59: 428-432.

- **Bernet**, M.; Brassart, D.; Neeser, J. and Servin, A. (1993). Adhesion of human bifidiobacterial strains to cultural human intestinal epithelial cells and inhibition of enteropathogen-cell interactions. Appl . Environ. Microbiol . 59: 4121-41.

- **Bezirtzoglou,** E. (1997). The intestinal microflora during the first weeks of life. Anaerobe. 3: 173-177.

- **Bounous,** G. (1991). Whey proteins in cancer prevention. Cancer Lett. 57: 91-94.

- **Brassart**, D. (1999). The future of functional foods. Scientific basis and future requirements. Lebensmittel-Technology. 31: 258-266.

- **Briggs,** M. (1953). The classification of *Lactobacilli* by means of physiological test. J. Gen. Microbial. 9: 234-248.

- **Brody**, E. P. (2000). Biological activites of bovine glycomacropeptide. Br. J. Nutr. 48: 39-49.

- **Bryant,** M. P. and Burkey, L. A. (1953). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen, J. Dairy Sci. 36: 205-217.

(C)

- **Carpa**, M. L. and Ackermann, H. W. (2006). Biotechnological and nutritional control of disease. Prebiotic, Probiotics and Synbiotics. Nutr. J. 13, 73-86.

- **Chenoll**, E.; Carmen, M. and Aznar, R. (2006). *Lactobacillus* sp. Bacterocin: new lactic acid bacteria isolkated from sausage. Syst. Appl. Microbiol. 29(5): 389-395.

- **Chinda**, D.; Nakaji, S; Fukuda, S.; Sakamoto, J.; Shimoyama, T.; Nakamura, T.; Fujisawa, T.; Terada, A. and Sugawara, K. (2004). The fermentation of different dietary fibers is associated with fecal clostridia levels in men . Nutr. J. 134(8): 1881-86.

- **Choi,** S. Y. and Beuchat, L. R. (1995). Growth inhibition of *Listeria monocytogenes* by a bacteriocin of *Pediococcus acidilactici* M during fermentation of kimchi. Food Microbiol. 11: 301-307.

- **Chonan,** O. (1995). Effect of galactooligosaccharides on calcium absorption and preventing bone loss in ova riectomized rats. Biosci. Biotech Biochem. 59: 236-239.

- **Cintas**, L.; Casaus, P.; Fernandez, M. and Hernandez, P. (1998). Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A and lactocin S against spoilage and food borne pathogenic bacteria.Food Microbiol. 15, 289-298.

- **Conne**, D. E. (1993). Naturally occurring compounds. In: Antimicrobials in Foods, 2nd edition. Davidson, P. M. and Branen, A. L. (Eds). Pp.441-468. Marcel Dekker Inc., New York.

- **Conteras**, B.; Vuyst, L.; Deveresse, B.; Busanyova, K.; Raymaeckers, J.; Bosman, F.; Sablon, E.; and Vandamme, E. (1997). Isolation , purification and amino acid sequences of Lactobin A, one of the two bacteriocins produced by *Lactobacillus amylovorus* LMGP- 13138. Appl. Environ. Microbiol. 63(1): 13-20.

- **Cummings,** J. H. and Macfarlane, G. T. (1997). Colonic microflora: nutrition and health. Nutr. 13: 476-468.

- **Cummings,** J.; Rombeau, J. and Sakata, T. (1995). Physiological and clinical aspects of short chain fatty acids. Cambridge, United Kindom:Cambridge University Press.

- **Cummings,** J. H. and Macfarlane, G. T.(2002). Gastrointestinal effects of prebiotics. Br. J. Nutr. 87(2): 145-151.

(D)

- **De Man**, J.; Rogosa, M. and Sharp, M. (1960). A medium for cultivation of *Lactobacillus*. J. App. Bact. 23(1): 130-135.

- **Dunne**, C. and Shanahan, F. (2002). Role of probiotics in the treatment of intestinal infections and inflammation. Curr. Gastroenterol., 18, 40-45.

- **Duggan,** C.; Gannon, J. and Walker, A. (2002). Protective nutrients and functional foods for the gastrointestinal tract. Am. J. Clin. Nutr. 75(5): 789-808.

- **Donnet-Hughes,** A.; Rochat, F.; Serrant, P.; Aeschlimann, J. and Schiffrin, E. (1999). Modulation of nonspecific mechanisms of defense by lactic acid bacteria: effective dose. J. Dairy Sci. 82: 863-869.

- **Donohue,** D. and Salminen, S. (1996). Safely of probiotic bacteria. Asia pac. J.Clin. Nutr. 5: 25-28.

- **D'Souza**, A.; Rajkumar, C. and Cooke, J. (2002). Probiotic in prevention of antibiotic diarrhea: meta-analysis. B M J. 32 (35): 1361-74.

(E)

- **Eijsink,** V.; Skeie, M.; Middelhoven, P.; Brurberg, M. and Nes, I. (1998). Comparative studies of classII bacteriocins of lactic acid bacteria. Appl. Environ. Microbiol. 64(9): 3275-81.

- **Ellegard,** L.; Andersson, H. and Bosaeus, I. (1997). Inulin and oligofructose do not influence the absorption of cholesterol, or the excretion of cholesterol, Ca, Mg, Zn, Fe, or bile acids but increases energy excretion in ileostomy subjects. Eur. J. Clin. Nutr. 51: 1-5.

- **Elo,** S.; Saxelin, M. and Salminen, S. (1991). Attachment of *Lactobacillus casei* strain GG to human colon carcinoma cell line Caco-2: comparison with other dairy strains. Lett. Appl. Microbiol. 13: 154-156.

- **Emanuel**, V.; Adrian, V.; Ovidiu, P. and Gheorghe, C. (2005). Isolation of *Lactobacillus plantarum* strain used for obtaining a product for the preservation of fodders. Afri. J. Biotech. 4 (5): 403-408.

- **Ensminger,** M. E. and Olentine, J. R. (1978). Feeds and Nutrition-Complete. 1st ed . The Ensminger publishing companys. United State of America.

(F)

- **Farber,** J. M. (1991). Microbiology aspects of modified-atmosphere packaging technology- a review. J. Food prot. 54: 58-70.

- **Fernands**, C. F.; Chandan, R. C. and Shahani, K. M. (1992). Fermentated dairy products and health. In: The Lactic Acid Bacteria. Volume 1. The Lactic Acid Bacteria in Health and Disease (Ed.: Wood, B.J.B.). Elsevier Applied Science, London, 297-339.

- **Flickinger**, E. A.; Wolf, B. W. and Garleb, K. A. (2000). Glucose-based oligosaccharides exhibit different *in vitro* fermentation patterns and affect *in vivo* apparent digestibility and microbial in dogs. J. Nutr. 130: 1267-73.

- **Fooks,** L.; Fuller, R. and Gibson, G. (1999). Prebiotics, probiotics and human gut microbiology. Int. Dairy. J. 9: 53-61.

- **Forbes**, B. A.; Sahm, D. F. and Weissfeld, A. S. (1998). Diagnostic Microbiology. 10th ed, Mosby Inc. 870-951.

- **Fuller**, R. (1991). Probiotic in human medicine. Gut. 32: 439-442.

- **Fuller,** R. and Gibson, G. (1997). Modification of the intestinal microflora using probiotics and prebiotics. Scand J. Gastroenterol. 222: 28-31.

- **Fuller,** R. (1989). Probiotics in man and animals. J. Appl. Bacteriol. 66: 365-378.

(G)

- **Gibson,** G. R. and Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125: 1401-12.

- **Gibson,** G. and Wang, X. (1994). Regulatory effects of *Bifidobacteria* on the growth of other colonic bacteria. J. Appl. Bacteriol., 77: 412-420.

- **Gibson**, G.; Beatty, E.; Wang, X. and Cummings, J. (1995). Selective stimulation of *Bifidobacteria* in the human colony by oligofructose and inuline. Gastroenterol. 108: 975-982.

- **Greene,** J. D. and Klaenhammer J. R. (1994). Factors involved in adherence of *lactobacillus* to human Caco-2 cells. Appl. Environ. Microbiol. 60: 4487-94.

- **Grizard,** D. and Barthomeuf, C. (1999). Non-digestible oligosaccharides used as prebiotic agents: mode of production and beneficial effects on animal and human health. Reprod. Nutr. Dev. 39: 563-588.

- **Guarner**, F. and Schaafsma, G. (1998). Probiotics. Int. J.Food Microbiol. 39: 237-238.

- **Gupta**, V.; Radramma, H.; Rati, E. and Joseph, R. (1998). National quality of lactic acid fermented better gourd and fenugreek leaves. Int. J. Food Sci. Nutr. 49(2): 101-108.

(H)

- **Hammes**, W. P. and Vogel. R. F. (1995). The genus *Lactobacillus*. In :' the genera of lactic acid bacteria. Wood, B. J. and Holzapfel, W. H.(eds).

- **Harely**, P. J. and Prescott, M. L. (1996). Laboratory exercises in Microbiology. WCB/McGraw-Hill, USA.

- **Harper**, W. J. (2002). Biological properties of whey components: A review. Chicago, I11: The American Dairy Products Institute.

- **Harrigan,** W. F. and MacCance, M. E. (1976). Laboratory methods in food and dairy microbiology. Academic press, London.

- **Havenaar**, R.; Brink, B. and Huis-Veld, J. (1992). Selection of strains for probiotic use. In: probiotics, the scientific basis (ed Fuller, R.) : 209-224, London:Chpman and Hall.

- **Havenaar**, R.; Huis, I. and Veld, M. (1992). Probiotics: a general view. In: Lactic acid bacteria in health and disease. vol 1. Amsterdam: Elsvier Appl., Scie. Publishers.

- **Holzapfel**, W.; Haberer, P.; Snel, J.; Schillinger, V. and Huis-Veld, J. (1998). Overview of gut flora and probiotics. Int. J. Food Microbiol. 41: 85-101.

- **Horton**, B. S. (1995). Commercial utilization of minor milk components in the health and food industries. J. Dairy Sci. 78: 2584-89.

- **Hotchkiss**, J. H.; Chen, J. H. and Lawless, H. T. (1999). Combined effects of carbon dioxide addition and barrier films on microbial and sensory changes in pasteurized milk. J. Dairy Sci. 82: 690-695.

- **Hove**, H.; Norgaard, H. and Mortensen, P. (1999). Lactic acid bacteria and human gastrointestinal tract. Eur. J. Clin. Nutr. 53(5): 339-350.

- **Howarth**, G. S. (1996). Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. J. Nutr. 126: 2519-30.

- **Hugenholtz,** J. (1993). Citrate metabolism in lactic acid bacteria, FEMS microbial. Rev. 12: 165-178.

- **Hull,** R. R., Conway, P. L., and Evans, A. J. (1992). Probiotic foods-a new opportunity, Food Australia. 44: 112-113.

(I)

- **Ibrahim,** S. A. and Sullivan, D. J. (2000). Use of chemical mutagenesis for the isolation of food grade B-Galactosidase overproducing mutants of *Bifidobacteria*, *Lactobacilli* and *Streptococcus thermophilus*. J. Dairy Sci. 83: 923-930.

(J)

- **Jay**, J. M. (1982). Antimicrobial properties of diacetyl. Appl. Environ. Microbiol. 44: 525-532.

- **Jimenez- Diaz**, R.; Rios- Sanchez, R.; Desmazeauel, M.; Riuz-Barba, J. and Piard, J. (1993). Plantaracin S and T. Two new bacteriocins produced by *Lactobacillus plantarum* LP Colo isolated from a green olive fermentation. Appl. Environ. Microbiol. 59: 1416-24.

- **Joy**, M.; George, C.; Fahey, J. and Bryan, W. (1999). Selected indigestible oligosaccharides affect short chain fatty acids, pH and microflora in rat. J. Nutr. 127(1): 130-160.

(K)

- **Kandler**, O. and Weiss, N. (1986). Genus *Lactobacillus*. In: Bergy's Manual of Systemic Bacteriology. (Sneath, P. H. A; Mair, N. S. and Hold, J. C. eds) 2 Willian and Wilkins Co., Baltimore. M. D. USA.

- **Kilshaw**, P. J.; Heppell, L. M. and Ford, J. E. (1982). Effects of heat treatment of cow's milk and whey on the nutritional quality and antigenic properties. Arch Dis Child. 57(11): 842-847.

- **Kontula**, P.; Suihko, M.; Von-Wright, A. and Mattila-Sandholm, T. (1999). Effect of lactose derivatives on intestinal lactic acid bacteria. J. Dairy. Sci. 82: 249-256.

- **Kulozik**, U. and Wilde, J. (1999). Rapid lactic acid production at a high cell concentration in whey ultrafiltrate by *Lactobacillus helveticus*. Enzyme and Microbial Technology. 24(5): 297-302.

(L)

- **Lewus**, C.; Kaiser, A. and Montvill, T. (1991). Inhibition of food borne bacterial pathogens by bacteriocins from lactic acid bacterial isolated from meat. J. Appl. Environ. Microbial. 57: 1683-88.

- **Lilley,** D. M. and Stillwell, R. H. (1965). Probiotics: growth promoting factors produced by microorganisms. Sciense. 147-748.

- **Lin**, M.; Savaiano, D. and Susan, H. (1991). Influence of non fermented dairy products containing bacteria starter culture on lactose maldigestion in human. J. Dairy Sci. 74: 87-95.

- **Lois,** D. and McBean, M. (2003). Emerging health benefits of whey. Dairy Council Digest. 74(6): 31-36.

- **Lonnerdal**, B. (2003). Nutritional and physiologic significance of human milk proteins. Am. J. Clin. Nutr. 77(6): 1537-43.

(M)

- **Macfarland**, L. V. and Elmer, G. W. (1995). Biotherapeutic agents: past, present and future. Microecology Ther. 23: 46-73.

- **Macfarlane**, G. T. and Cummings, J. H. (1999). Probiotics and prebiotics: can regulate the activities of intestinal bacteria benefit health. West. J. Med. 171: 187-191.

- **Macfarlane**, G. and Macfarlane, S. (1997). Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. Scand. J. Gastroenterol. 32: 3-9.

- **Marteau,** P.; Vres, M.; Cellier, C. and Schrezenmeir, J. (2001). Protection from gastrointestinal diseases with the use of probiotics. Am. J. Clin Nutr. 73: 430-436.

- **Marteau,** P.; Flourie, B.; Pochart, P.; Chastange, C.; Desjeux, J. and Rambaud, J. (1990). Effect of the microbial lactase activity in yoghurt on the intestinal absorption of lactose: an *in vivo* study in lactase-deficient humans, Br. J. Nutr. 64: 71-79.

- **Meulen,** R.; Avonts, L. and Vuyst, L. (2004). Short fractions of oligofructose are preferentially metabolized by *Bifidobacterium animalis* DN-173 010. Appl. Environ. Microbiol. 70(4): 1923-30.

- **Mercenier**, A.; Pavan, S. and Pot, B. (2003). Probiotic as biotheraputic agents present knowledge and futures prospects. Current pharmaceutical Design. 8, 99-101.

- **Midolo,** P. D.; Lambert, J. R.; Hull, R. F.; Luo, O. P. and Grayson, M. L. (1995). *In vitro* inhibition of *Helicobacter pylori* NCTC 11637 by organic acids and lactic acid bacteria. J. Appl. Bacteriol. 79: 475-479.

- **Mital,** B. K. and Garg, S. K. (1995). Anticarcinogenic, hypocholesterolemic, and antagonistic activities of *lactobacillus acidophilus.* Critical Rev. Microbiol. 21(3): 174-214.

- **Molis**, C.; Flourie, B. and Ouarne, F. (1996). Digestion, excretion, and energy value of fructooligosaccharides in healthy humans. Am. J. Clin .Nutr. 64: 324-328.

- **Montes,** R. G.; Bayless, T. M.; Saavedra, J. M. and Perman, J. A. (1995). Effect of milks inoculated with *Lactobacillus acidophilus* or a yogurt starter culture in lactose-maldigesting children. J. Dairy. Sci. 78: 1657-64.

- **Moore,** M. E. and Moore, I. II. (1995). Intestinal floras of populations that have a high risk of colon cancer. Appl. Environ. Microbiol. 61: 1202-07.

- **Motlagh**, A.; Johnson, M. and Ray, B. (1992). Viability loss of foodborn pathogens by starter culture metabolites. J. Food Prot. 54: 873-878.

- **Mure,** J.; Yeow, E.; Keogh, J.; Catherine, P.; Anthony, R.; Sharp, K.; O'Dea, K. and Macrae, F. (2004). Combining wheat bran with resistant starch has more beneficial effects on fecal indexes than does wheat bran alone. Am. J. Clin. Nutr. 79(6): 1020-28.

(N)

- **Naidu,** A. S.; Bidlack W. R. and Clemens, R. A. (1999). Probiotic spectra of lactic acid bacteria. Crit. Rev. Food Sci. Nutr. 38(1): 13-26.

- **Narayanan,** N.; Roychoudhury, P. and Srivastava, K. (2004). L(+) lactic acid fermentation and its product polymerization. Biotechnology industry. 7(2):179-182.

- **Nigatu**, A. and Gashe, B. (1994). Inhibition of spoilage and food brone pathogenic by lactic acid bacteria isolated from fermenting food. Dough. Ethiop. Med. J. 32: 223-229.

(O)

- **O'Sullivan,** M. G. (1996). Metabolism of bifidogenic factors by gut flora. Int. Dairy Fed. 8: 23-30.

- **Ouwehand,** A.; Kirjavainen, P.; Gronlund, M.; Isolauri, E. and Salminen, S. (1999). Adhesion of probiotic microorganism to intestinal mucus. Int. J. Dairy. 9: 623-630.

- **Oyetayo**, V. O.; Adeluyl, F. C. and Akinyosoye, F. A (2003). Safety and protective effect of *Lactobacillus acidophilius* and *Lactobacillus casei* used as probiotic agent *in vivo*. Afri. J. Biotech. 2(11): 448-452.

- **Oyetayo**, V. and Osho, B. (2004). Assessment of probiotic properties of a strain of *Lactobacillus plantarum* isolated from fermenting corn slurry. Food, Agri. Environ. 2(1): 132-134.

(P)

- **Parodi**, P. W. (1998). A role for milk proteins in cancer prevention. Aust J. Dairy Technol. 53: 37-47.

- **Perdigon,** G.; Fuller, R. and Raya, R. (2001). Lactic acid bacteria and their effect on the immune system.Cur. Iss. Intes. Microbio. 2(1): 27-42.

- **Pereira**, A.; McCartney, L. and Gibson, R. (2003). In vitro study of probiotic potential of a bile- salt hydrolyzing *Lactobacillus fermentum* Strain and determination of its cholesterol lowering properties. Appl. Environ. Microbiol. 69(8): 4743-52.

- **Pfeiffer**, P. and Radler F. (1982). Purification and characterization of extracellular and intracellular killer toxin of *Sacchromyces cerevisiae* strain 28.J. Gen. Microbiol. 128: 2699-06.

- **Piard**, J. and Desmazeaud, M. (1991). Inhibiting factors produced by lactic acid bacteria:1. Oxygen metabolites and catabolism end-products. Lait. 71: 525-541.

- **Playford,** R. J.; Macdonald, C. E. and Johnson, W. S. (2000). Colostrum and milk- derived peptide growth factors for the treatment of gastrointestinal disorders. Am. J. Clin. Nutr. 72: 5-14

- **Playne**, M. and Crittenden, R. (1996). Commercially available oligosaccharides. IDF Bulletin. 313: 10-22.

- **Podolak**, P. K.; Zayas, J. F.; Kastner, C. L. and Fung, D. Y. (1996). Inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7 on beef by application of organic acids. J. Food prot. 59: 370-373.

- **Priebe**, H.; Harmsen, F.; Stellaard, X.; Welling, G. and Vonk, R. (2006). Colonic fermentation may play a role in lactose intolerance in humans. J. Nutr. 136(1): 58-63.

(Q)

- **Quigley,** M. E.; Hudson, G. J. and Englyst, H. N. (1999). Determination of resistant short-chain carbohydrates (non-digestible oligosaccharides) using gas-liquid chromatography. Food Chem. 65: 381-390.

(R)

- **Radke,** T. R.; Shields, R. J. and Holzgraefe, D. P. (1991). Relative efficacy of whole whey and various lactose sources in diets for early- weaned pigs. J. Anim. Sci. 69: 102-107.

- **Rastall,** R. and Maitin, V. (2002). Prebiotics and synbiotics:towards the next generation. Curr. Opin. Biotechnol. 13: 490-496.

- **Reid**, G. (1999). The Scientific basis for probiotic strains of *Lactobacillus*. Appl. Environ. Microbiol. 65(9): 3763-66.

- **Richards,** R. M. and Xing, T. P. (1995). Activity of p-aminobenzoic acid compared with other organic acids against selected bacteria. J. Appl. Bacteriol. 78: 209-215.

- **Reid**, G. and Burton, J. (2002). Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microbes Infect. 4: 319-324.

- **Rolfe,** R. D. (2002). The role of probiotic cultures in the control of gastrointestinal health. J. Nutr. 130: 396-402.

- **Rosado,** J. L.; Solomons, N. W. and Allen, L. H. (1992). Lactose digestion from unmodified, low-fat and lactos-hydrolyzed yogurt in adult lactose-maldigesters. Eur. J. Clin. Nutr. 46(1): 61-17.

- **Rossi,** M.; Corradini, C.; Amaretti, A.; Nicolini, M. and Zanoni, S. (2005). Fermentation of fructooligosaccharides and inuline by *Bifidobacteria:* a comparative study of pure and fecal cultures. Appl. Environ. Microbiol. 71: 6150-58.

- **Runar**, S. (1998). Biology of nisin: A Genetical Approach. Institute of Biotechnology and Department of Bioscince, Division of genetics. University of Helsinki (Personal Communication). FEMS. Microbiol Lett. 16: 75-79.

- **Ryan**, M.; Rea, M.; Hill, C. and Ross, R. (1996). An application in Cheddar cheese manufacture for a strain of *Lactobacillus lactis* producing a novel broad-spectrum bacteriocin, lacticin. Appl. Environ. Microbiol. 62, 612-619.

(S)

- **Salminen,** S. (1994). Healthful properties of *lactobacillus* GG. Dairy Ind. 1, 36-37.

- **Salminen,** S. (1996). Uniqueness of probiotic strains. IDF Nutr News Lett . 5: 16-18.

- **Salminen**, S. and Salminen, E. (1997). Lactose, lactic acid bacteria, intestinal microecology and mucosal protection. Scand J. Gastroenterol. 222: 45-48.

- **Salminen,** S.; Isolauri, E. and Salminen, E. (1996). Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie Leeuwenhoek .70: 347-358.

- **Sanders,** M. E. (1993). Healthful attributes of bacteria in yoghurt, Contemporary Nutr. 18: 1-2.

- **Saxelin,** M.; Elo, S. and Salminen, S. (1991). Dose response colonization of faeces after oral administration of *Lactobacillus casei* strain GG. Microbial Ecoi. Hlth. Dis. 6: 119-122.

- **Schrezenmeir**, J.; Heller, K.; McCue, M.; Llamas, C.; Lam, W.; Burow, H. and Comer, M. (2004). Benefits of oral supplementation with and without synbioitics in young children with acute bacterial infections. Clin. Pediatrics. 43(3): 239-249.

- **Schrezenmeir**, J. and Vrese, M. (2001). Probiotics, Prebiotics, and synbiotics-approaching a definition.Am. J. Clin Nutr. 73(2): 361-364.

- **Schrezenmeir,** J.; Korhonen, H. and Williams, C. (2000). Beneficial natural bioactive substances in milk and colostrum. Br. J. Nutr. 84(1):31-36.

- **Shah,** N. P. (2000). Biological properties of whey components. Br. J. Nutr. 84: 53-57.

- **Sharba**, Z. A. (2006). Isolation and characterization of *Lactobacillus acidophilius* and its ability for β-galactosidase production. M.S.c Thesis Submitted to the College of Science of Al-Nahrain University.

- **Silva**, M.; Jacobus, N.; Deneke, C. and Gorbach, S. (1987). Antimicrobial substances from a human *Lactobacillus* strain. Antimicrob. Agent and Chemoth. 31 (8): 1231-33.

- **Spanhaak**, S.; Havenaar, R. and Schaafsma, G. (1998). The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. Eur. J. Clin. Nutr. 52: 899-907.

- **Stark,** A. and Madae, Z. (1994). Dietary Fiber. In Functional Foods-Designer Foods, Pharmafoods, Nutraceuticals.2th ed. Goldberg, I., (ed). 183-201 Chapman and Hall, New York, USA.

- **Stopl**, H. and Gadkari, D. (1984). Non pathogenic members of the genus *Pseudomonas*. In: the prokaryotes. Starr, P.; Stolp, H.; Truner, H.; Balaws, A. and Schledgel, H. (Eds). and Hall, New York, USA. 1: 721-741.

(T)

- **Tannock,** G.; Munor, K.; Bibiloni, R.; Simon, M.; Hargreaves, P.; Gopal, P.; Harmsen, H. and Welling, G.(2004). Impact of consumption of oligosaccharide containing biscuits on the fecal microbiota of humans.Appl. Environ. Microbio. 70(4): 2129-36.

- **Taranto**, M.; Medici, M.; Perdigon, G.; Ruiz Holgado, A. and Valdez, G. (1998). Evidence for hypocholesteroiemic effect of *lactobacillus reuteri* in hypocholesterolemic mice. J. Dairy Sci. 81: 2336-40.

- **Tissier**, H. (1984). Texonomy and ecology of *Bifidiobacteria.* *Bifidiobacteria* Microflora. 3: 11-28.

(V)

- **Vander Meulen**, R.; Markas, L.; Verbruggh, R. and Vuyst, T. (2006). *In vitro* kinetic analysis of oligofructose consumption by bacteroides and *Bifidiobacterium* spp. Appl. Environ. Microbiol. 72: 1006-12.

- **Vaux,** A.; Morrison, M. and Hutkins, R. (2000). Displacement of *Escherichia coli* O157:H7 form rumen medium containing prebiotic sugars. Appl. Enviro. Microbio. 68(2): 519-524.
- **Vignolo**, G.; Kairus, M.; Ruiz Holgado, A. and Oliver, G. (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *lactobacillus casei* CRL 705.J. Appl. Bacteriol. 78: 5-10.

- **Voragen**, A. G. J. (1998). Technological aspects of functional food-related carbohydrates. Trends Food Sci: Technol. 9: 328-335.

(W)

- **Walker,** A. W. and Duffy, L. C. (1998). Diet and bacterial colonization: role of probiotics and prebiotics :Review. J. Nutr. Biochem. 9: 668-675.

- **Warny,** M.; Fatima, A. and Bostwick, F. (1999). Bovine immunoglobulin concentrate- *clostridium difficile* retains C difficile toxin neutralizing activity after passage through the human stomach and small intestine. Gut. 44(2): 212-217.

- **Wee,** Y. J.; Kim, j. N. and Ryu, H. W. (2006). Biotechnology production of lactic acid bacteria and its recent applications. Food Tech. Biotechnol. 44(2): 161-172.

(Y)

- **Yaeshima**, Y. (1996). Benefits of *Bifidobacteria* to human health. IDF Bulletin 313: 36-42.

(Z)

- **Zimecki**, M.; Wlaszczyk, A. and Wojiechowski, R. (2001). Health-enhancing properties of whey. Arch. Immunol. Ther. Expp. 49: 325-329.

المصادر العربيه

- العبيدي، نبراس نزار محمود(١٩٩٧). تأثير البكتريوسين المنتج من بكتريا حامض اللاكتيك على بعض انواع البكتريا المرضية. رسالة ماجستير، كلية العلوم – الجامعة المستنصرية.

- الدليمي، جيهان عبد الستار(٢٠٠٠). استخدام الكحول الاثيلي لعزل بكتريا حامض الاكتيك ودراسة تأثيرها التأزري مع خميرة الخبز ضد بعض انواع البكتريا. رسالة ماجستير، كلية العلوم- الجامعة المستنصرية.

Supervisor Certification

I certify that this thesis was prepared under my supervision at the Department of Biotechnology, College of Science, Al-Nahrain University as a partial requirements for the degree of Master of Science in Biotechnology.

Signature:

Supervisor: *Dr. Abdul W. Baqir*

Scientific Degree: *Professor*

Date:

In review of the available recommendation I forward the thesis for debate by the examining committee.

Signature:

Dr. Nabil K. Al-Ani

Title: Chairman of Biotechnology Department

Date:

الأهداء

ألى القلب الذي اتسع لكل الحنان....

والدتي

الحبيبة

ألى رمز الشموخ الذي علمني الاصرار على التفوق....

والدي

الحبيب

ألى سندي الدائم اخواتي....

سارة

وصفا

ألى من كانوا خير معين لي أخواني....

مصطفى وابراهيم

اهدي لهم ثمرة جهدي المتواضع

مروة

بسم الله الرحمن الرحيم

قُل كُلٌ يَعمَلُ علَىَ شَاكِلَتِه فَرَّبكُم اعَلمُ بمَن هُوَ اَهدّى سَبيلاً وَيَسئَلَونّكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِن أمِر رَبَّي وَمَا أُوتِيتُم مِن الَعِلمِ اِلاّ قَلِيلاً

صدق الله العظيم

سورة الاسراء الايه (٨٤-٨٥)

Summary

A total of 17 samples of dairy products were collected from Baghdad markets. Nine isolates of *Lactobacillus* spp. were obtained, and identified as (4) *Lactobacillus plantarum*, (3) *Lactobacillus fermentum*, and one of each of *Lactobacillus acidophilus* and *Lactobacillus brevis* (1 isolate). Inhibition activity of *Lactobacillus* isolates against some pathogenic bacteria was tested on solid medium for different incubation periods. After that one isolate of *Lb.plantarum* was selected due to its highest inhibitory activity. The inhibitory effect of this isolate was also tested but in MRS broth at different incubation period. Results showed that 24 hr were the most efficient period of incubation for such purpose.

Ability of the selected *Lb.plantarum* isolate to produce β-galactosidase enzyme was detected *in vitro* by using X-gal as chromogenic substrate for β-galactosidase which turn the color of colony from white to blue.

To improve ability of the isolate in exhibiting inhibitory activity, various prebiotic substances (lactose, fresh whey and dried whey) with different concentrations (1, 2, 3, 4 and 5%) w/v (for lactose and dried whey) and (10, 20, 30, 40 and 50%)v/v (for fresh whey) were used, and three incubation periods (24, 48 and 72) hr were applied with defined inoculums size .

Results testing inhibitory activity of *Lb.plantarum* with the above substances showed that *Lb.plantarum* propagated in MRS fortified with 2% lactose, 40% fresh whey and 3% dried whey with incubation period 48 hr (for each) had the best inhibitory activity compared to propagating, of *Lb.plantarum* in MRS alone.

Minimum inhibitory concentrations (MIC,s) of *Lb.plantarum* diluted concentrated filtrate were determined after propagated in MRS broth, and the same steps were repeated but when propagated in MRS broth containing the best three concentrations of prebiotics on *Lb.plantarum* activity. It was found that MIC,s value became lower after prebiotic treatment, with improving the activity against pathogenic bacteria.

The ability of prebiotic substances for improving growth of probiotic, growth curve of the *Lb.plantarum* isolate propagated in MRS broth was determined then the growth curve was evaluated but when propagated in MRS broth fortified with the three prebiotics at concentration giving best effect on *Lb.plantarum* activity. It was found that growth of the probiotic bacteria was changed.

Total viable count was performed for *Lb.plantarum* propagated in MRS broth with and without prebiotic after each incubation period (24, 48 and 72) hr. Results show that growth of probiotic bacteria was enhanced after the addition of prebiotic substances, this with concentrations of 2% lactose, 40% fresh whey and 3%dried whey.

الخلاصة

جمعت (١٧)عينة لبن من الأسواق المحلية لمدينة بغداد لغرض عزل بكتريا حامض اللاكتيك حيث تم الحصول على (٩) عزلات وعند تشخيصها وجد إنها تعود لجنس *Lactobacillus* مع اختلاف في النوع حيث تم تحديد (٤) عزلات تعود إلى *Lb.plantarum*،(٣) عزلات تعود إلى*Lb.fermentum* ،و عزلة واحده لكل من *Lb.acidophilus* و *Lb.brevis*.

و عند دراسة قدرتها على تثبيط عدد من العزلات المرضية بعد تنميتها على وسط MRS الصلب بفترات حضن مختلفة أظهرت جميع العزلات فعاليه تثبيطية جيده حيث تم اختيار أفضل عزلة أعطت أعلى فعالية تثبيطية وكانت (*Lb.plantarum*) التي تم اختبار فعاليتها التثبيطية بعد تنميتها في وسط MRS السائل بمختلف درجات الحضن(٢٤، ٤٨ و ٧٢ ساعة) وتبين إن الفعالية التثبيطية لهذه العزلة أفضل في الوسط السائل من الوسط الصلب، ضد جميع عزلات البكتريا المرضية قيد الاختبار وخصوصا بعد فترة الحضن ٢٤ ساعة.

كذلك تم التحري عن قابلية هذه العزلة لإنتاج أنزيم أل β-galactosidase وذلك بتنميتها على وسط أل MRS X-gal الحاوي على مادة أساس ملونة(chromogenic substrate) التي تتحلل بفعل إنزيم β-galactosidase فتحول المستعمرات من اللون الأبيض إلى اللون الأزرق.

لتحسين الفعالية التثبيطية للمعالجات الحيوية تم تدعيم وسط النمو بمواد مدعمة والتي شملت(اللاكتوز، الشرش و الشرش المجفف) بتراكيز مختلفه (١،٢،٣،٤و ٥ %) لكل من االلاكتوز و الشرش المجفف و (١٠،٢٠،٣٠،٤٠ و ٥٠%) للشرش مع فترات حضن مختلفة(٢٤، ٤٨ و ٧٢ ساعة) وتبين أن العزلة التي اختيرت أصبحت تمتلك فعالية تثبيطية عالية ضد جميع العزلات المرضية التي تم اختبارها وعند التركيز الآتية (لاكتوز ٢% و شرش ٤٠% و شرش مجفف٣%) خلال فترة حضن ٤٨ ساعة.

كما وتم قياس التركيز المثبط الأدنى(MIC) لراشح بكتربا حامض اللاكتيك المركز بعد نموها بوسط MRS السائل قبل وبعد اضافة تراكيز المواد المدعمة التي أعطت أعلى فعالية تثبيطية، وتبين إن قيمة ألMIC أصبحت اقل بعد الإضافة مما يعني إن نسبة تثبيط البكتريا المرضية ازدادت بعد الإضافة.

كما وتم اختبار قدرة تراكيز المواد المدعمة(التي أعطت أعلى زيادة في الفعالية التثبيطية) على زيادة نمو بكتريا أل *Lb.plantarum* و ذلك من خلال قياس منحنى النمو

وتبين إن للمواد المدعمة تأثير واضح. كما وتم قياس الأعداد الحية للل*Lb.plantarum* عند تنميتها في وسط MRS السائل بعد فترات حضن مختلفة (٢٤، ٤٨ و ٧٢ ساعة) ثم قورنت الأعداد مع تلك التي قيست ولكن بعد إضافة تراكيز المواد المدعمة التي أعطت أعلى تأثير على الفعالية التثبيطية للعزلة، وتبين إن هناك زيادة في عدد الخلايا الحية.

Acknowledgment

Praise to ***God*** *the first of all cause the glorious creator of the Universe for his mercy and kindness, and blessing upon Mohammed prophet of God and upon his familyI wish to express my deepest thanks to my supervisor* ***Dr. Abdul W. Baqir*** *for the great help and useful advises during the work.I would also to express deep thanks to* ***Dr. Hameed*** *for his continuous support. My deep gratitude and appreciation to* ***Dr****.* ***Mayssaa Ghasib*** *and* ***Hamid Gehad****It is a pleasure to thanks all staff and employers of Biotechnology department at Al-Nahrain University****.*** *Grateful thanks to my brother* ***Ibrahim*** *for his help and support all the time.* Special *thanks are also due to* ***my family*** *and every one gave me a hand to complete this work.*

Marwa

Republic of Iraq
Ministry of Higher Education
And Scientific Research
Al-Nahrain University
College of Science
Department of Biotechnology

Improvement of Inhibitory Effect of Probiotic against Some Bacterial Isolate Using Prebiotics

A Thesis
Submitted to the College of Science of Al-Nahrain University
As Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

Marwa Abbas Abdul_Razzak Kubba

B.Sc. 2004
Al- Nahrain University

December ***2006***

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الاحيائية

تحسين الفعالية التثبيطية للمعزز الحيوي ضد بعض أنواع عزلات البكتريا باستخدام مواد مدعمة للنمو

رسالة
مقدمة الى كلية العلوم – جامعة النهرين وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل
مروة عباس عبد الرزاق كبة
بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٤

Table (4-1). Biochemical test of *Lactobacillus* spp. Isolated from Dairy Product.

Isolate	NH_3 from Arginine	Growth in Litmus Milk	Gelatinase Test	Oxidase Test	Catalase Test	Growth at		Growth on Nutrient Agar	Carbohydrate fermentation test								
						15°C	45°C		arabinose	Xylose	Galactose	Manitol	Maltose	Raffinose	Lactose	Fructose	Sucrose
Lb.plantarum1	_	+	_	_	_	+	_	_	(1) +	_	(1) +	(1) +	(1) +	(2) +	(2) +	(1) +	(1) +
Lb.plantarum2	_	+	_	_	_	+	_	_	(1) +	_	(1) +	(1) +	(1) +	(2) +	(1) +	(1) +	(1) +
Lb.plantarum3	_	+	_	_	_	+	_	_	(2) +	_	(1) +	(1) +	(1) +	(1) +	(1) +	(1) +	(1) +
Lb.plantarum4	_	+	_	_	_	+	−	_	(2) +	_	(1) +	(1) +	(1) +	(1) +	(1) +	(1) +	(1) +
Lb.fermentum1	+	+	_	_	_	_	+	_	(2) +	(1) +	(1) +	(1) +	(1) +	(1) +	(3) +	(1) +	(1) +
Lb.fermentum2	+	+	_	_	_	_	+	_	(1) +	(1) +	(1) +	(1) +	(1) +	(2) +	(2) +	(1) +	(1) +
Lb.fermentum3	+	+	_	_	_	_	+	_	(1) +	(1) +	(1) +	(1) +	(1) +	(1) +	(2) +	(1) +	(1) +
Lb.acidophilus	_	+	_	_	_	_	+	_	(1) +	(1) +	(1) +	(1) +	(1) +	(1) +	(4) +	(1) +	(1) +
Lb.brevis	+	+	_	_	_	+	−	_	(1) +	(1) +	(1) +	(1) +	(1) +	(2) +	(1) +	(1) +	(1) +

+: Positive fermentation.
–: Negative fermentation.
( ): Number of days.