RESEARCH ARTICLE

The Relationship Between the Sources of *Lactobacillus* Isolates and their Antimicrobial Activity

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ABSTRACT

Lactobacillus genus was isolated from two different sources, healthy infants feces and some dairy products, identify isolates by traditional methods, and confirm a diagnosis by molecular detection. Susceptibility was tested to some antibiotic, then supernatants antimicrobial activity was tested against some pathogenic bacteria involved *Escherichia coli O157: H7*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella sp.*, *Salmonella typhimiurum*, and *Clostridium sp.* Morphological, Microscopically, and Biochemical test results showed that eleven isolates were *lactobacillus,* DNA was extracted from isolates, and polymerase chain reaction (PCR) reaction was performed to confirm the diagnosis using a specific primer for *16SrDNA* in *Lactobacillus* genus, Lacto F and Lacto R, all isolates gave PCR products with molecular weight 231bp. Antibiotic susceptibility test showed that *Lactobacillus* isolates of healthy infants feces have appeared as multi-resistant to more than one of antibiotics and sensitive to chloramphenicol. While *Lactobacillus* isolates of dairy products were less resistance to antibiotics, and most of them were sensitive to antibiotics. Also, results showed the varying inhibitory effect of all *Lactobacillus* isolates supernatants in the growth of pathogenic bacteria in this study; healthy infants feces isolates showed the highest inhibition zone than dairy products *Lactobacillus* isolates.

Keywords: *16SrDNA,* Antimicrobial, Dairy products, Infants, *Lactobacillus.*

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INTRODUCTION

Lactobacillus is one of the most important and mainly genus in the group of lactic acid bacteria present throughout the digestive tract, vaginal tract system, and forms an integral part of the normal intestinal flora in humans and other animals.[1] *Some of Lactobacillus* species are able to colonize certain parts of the body, such as the oral cavity, gastrointestinal and vaginal tract, as they play main role in promoting the health and competitive viability of the pathogen.[2] *Also, Lactobacillus* has a long usage history in biotechnology, particularly in the fields of manufacturing and conservation of food ingredients by using fermentation processes and production of yogurt, cheese, sauerkraut, kefir, and many other fermented foods, in addition to animal feeds.[3] *Lactobacillus* species have specific methods and are probably well known to deal with their environment.[4] *Lactobacillus* fermentation processes due to the production of organic acids, especially lactic acid, the main product of carbohydrate metabolism processes, which is generated from pyruvate, so pH reduced in the environment, which creates a wide inhibitory effect against positive and negative gram bacteria.[5] It also produces hydrogen peroxide and other biologically active compounds, which are low molecular-weight proteins or/and peptides that have an antimicrobial activity to a wide ring of pathogenic bacteria.[6] Antimicrobial activity of *Lactobacillus* may occur due to cell free culture filtrate, which containing the production of bacteriocins and extracellular agents like low molecular weight proteins and peptides present in filtrate.[7] Antimicrobial agents in *Lactobacillus* genus may be associated with isolates environment, therefore, the goal of this study, was to compare the antimicrobial activity between *Lactobacillus* isolated from healthy infants feces and other *Lactobacillus* isolated from dairy products.

MATERIALS AND METHODS

Samples Collection

Twenty-three samples of healthy infants feces from both male and female, aged between 7–30 days were collected for breastfeeding and never received any antibiotics. In addition, local dairy products samples were collected (Canon, local soft cheese) from commercial markets in sterile containers and were transported to the lab.

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Isolation and Identification of *Lactobacillus*

About 0.5 g of healthy infants feces and 1mL of dairy products were transferred to a test tube containing 9 mL of sterile MRS-L.Cysteine-HCL broth (Oxoid), incubated at 37 ° C for 24 hours., this process was repeated three times.[8] 0.1 mL of last incubation was spread on MRS – $CaCO_3$(Oxoid) and incubated anaerobically at 37ºC for 24 hours. The colonies, which are surrounded by clear zone were transferred on MRS agar (pH 5.5) for purification, step was repeated The isolates were diagnosed based on the properties of colony, gram staining, and biochemical tests, catalase production, acid and curd in litmus milk, ammonia from arganine, and growth at 15°C and 45°C in MRS broth. *Lactobacillus* genus identification was confirmed according to to.[9], as described in Bergey's Manual of Systematic Bacteriology.[10]

Molecular identification:

DNA extraction

DNA was extracted from Eleven isolates according to the protocol of i-genomics CTB DNA Extraction Mini kit (cat no.17341), which was supplied by intron Korea, to confirm the extracted DNA presence, agarose gel electrophoresis was adopted.

Primer and PCR Technique

The Identification of the *Lactobacillus* genus was confirmed by PCR technique using specific primers for the 16S rRNA gene of *Lactobacillus* genus, Lacto F 3-TGGAAACAGRTGCTAATA CCG-5 (for region V2.1-V2.2) and Lacto R 3- GTCCATTGTGGAAGATTCCC -5 (for region V2.2-V3) were designed by.[11] PCR was completed with the GeneAmp PCR System 9700 (Perkin Elmer, Wellesley, Mass.) with an initial denaturation step of 94°C for 15 minutes, then 35 cycles 94°C for 0.5 minutes, and 62°C for 1-minute. The extension step was performed at 72ºC for 1 min and a final extension at 72ºC for 6 min. Electrophoresis was completed on 1.8% agarose gel and run with a 5v/cm for 2 hours. with (3000bp) ladder, bands were visualized with a UV transilluminator

Antibiotic susceptibility of *Lactobacillus* isolates

Antibiotic susceptibility test was performed according to method of,[12] with some modified by using antibiotics tablets which included (ampicillin 10µg, chloramphenicol 30µg, cephalexin 30µg, tetracycline 30µg, gentamicin 10µg, cefotaxime 15µg. erythromycin 15µg, and amoxicillin 25µg) (Sigma, USA), *Lactobacillus* isolates were spread on MRS agar by streaking, on the agar surface antibiotic discs were placed and incubated for 24 hours at 37C° under microaerophilic condition.

Preparation of *Lactobacillus* culture supernatants:

Isolates were grown at 37 ºC for 18–20 hours in MRS broth (pH 5.5). The overnight *Lactobacilli* cultures were centrifuged at 10,000 rpm for 5 min; the supernatant was regulated to pH 5.5 ± 1 with NaOH (1N) to eliminate the effect of putative organic acids produced then filtered with Millipore 0.22 µm pore size (Pall, USA).[13]

Pathogenic microorganisms

Seven species of pathogenic bacteria were used, kindly provided from the laboratories of Biotechnologicalal and Food Science Department/College of Agriculture / Baghdad University. Gram-negative bacteria were: *Escherichia coli O157: H7*, *Pseudomonas aeruginosa*, *Salmonella typhimiurum*, *Klebsiella spp,* and *Enterococcus faecalis*, while gram-positive bacteria were: *Clostridium spp*, and *Staphylococcus aureus*. All these bacteria are pathogenic for humans and animals and also considered as important food pathogens that cause damage for some food.[14]

Antimicrobial activity of *Lactobacillus* isolates

Antimicrobial activity of isolates was performed by using isolates supernatants against some pathogenic bacteria. All selected bacteria were cultured according to the method described by R. N. Atlas, *et al.*[8] The bacteria were cultured on Muller Hinton medium (Oxide) and kept at 37ºC for 24 hours. Growth turbidity was compared with a turbidity of the standard McFarland solution by reading optical density using a spectrophotometer on wavelength 450 nm. The dilutions of bacterial culture density were adjusted to the McFarland with cells number 1.5 x 10^8 cfu/mL. To study antimicrobial activity, well diffusion was used according to a method described by[15]. Aliquots of 50 µL of the sterile *Lactobacillus* supernatant were placed in 6 mm diameter wells on Muller-Hinton-agar (Oxoid) previously seeded with the pathogenic bacteria, incubated for 18–hour of at 37°C, after that the diameters of growth inhibition zones measured, two plates were done for each *Lactobacillus* supernatant.

RESULTS AND DISSECTION:

Isolation and Identification of *Lactobacillus* genus.

From total 23 samples (nine samples of healthy infants feces and fourteen samples of local dairy products) were collected, eleven of them appeared small with entire margin, pale, round shape, soft, mucoid, convex colonies and surrounded by inhibition zone after cultured on MRS contained 1% $CaCO_3$. Isolates were gram-positive bacilli when examined microscopically and negative to catalase test, also gave negative results for gelatin test, they were able to produce clot on litmus milk medium, unable to produce ammonia from arginine, grow slowly in 15^0C while, all grew in 45^0C except one isolate, which grew slowly, all isolates were able to ferment glucose and lactose. But, unable to ferment xylose and mannitol.[9] Finally, 11 isolates (five isolates from healthy infants feces and six isolates from local dairy products) were selected for Molecular identification to confirm the diagnosis.

Molecular identification

Lacto F and Lacto R primers were designed for the detection of *lactobacilli* identification with PCR product was approximately 231 bp long region of the V2-V3 region of the 16S rDNA of *Lactobacillus* spp All eleven isolates (five isolates from healthy infants feces and six isolates from local dairy products) were displayed to molecular identification through

PCR amplification of using specific primers for *Lactobacillus* 16S rRNA. This primers amplified DNA only from bacteria belonging to the *Lactobacillus* genus and did not amplify other strains closely related to *Lactobacillus* and considered a useful tool for identifying the *Lactobacillus* genus.11

Results showed in Figure 1 that the amplified fragments size were about 231 bp. All isolates gave positive results and identified as *Lactobacillus*.

Antibiotic susceptibility of *Lactobacillus* isolates

Results in Table 1 showed that feces isolates of *Lactobacillus* appeared multi-resistant to more than one of antibiotics. *Lb.1* and *Lb.5* can be resistant to five antibiotics and *Lb.2, Lb.3* and *Lb.4* can be resistant to four antibiotics, while most of the dairy products isolates of *Lactobacillus* appeared more sensitive to most antibiotics, and several of isolates were resistant to antibiotics, which may be attributed to possessing feces isolates antibiotic resistance factors due to the large bacterial diversity of the intestine and subsequent transmission of antibiotic resistance genes which carrying on plasmids to these isolates.

Studies mentioned some genes in the *Lactobacillus* genus responsible for antibiotic resistance like genes of chloramphenicol resistance (chloramphenicol acetyltransferases CAT).[16] Erythromycin resistance genes.[17] Tetracycline resistance genes.[18] (Ammor *et al.,* 2008) Aminoglycoside resistance genes.[19] and β-lactam resistance genes (blaZ).[20]

Antimicrobial activity of *Lactobacillus* Isolates:

The results showed varying inhibitory activity for isolates supernatants on pathogenic bacteria in this study **(**Table 2), feces isolates of *Lactobacillus* showed greater inhibitory activity than dairy products isolates of *Lactobacillus,* the highest inhibition zone that observed was against *Escherichia coli O157:H7*, *Staphylococcus aureus, Salmonella typhimiurum*, and *Clostridium spp*, while weaker inhibition zone of *Lactobacillus* Isolates supernatants was observed against *Enterococcus faecalis, Klebsiella sp* and *Pseudomonas aeruginosa*. This may be due to that the isolates may have genes coding for the production of antimicrobial agents portable on plasmids, which are more likely to be transmitted between bacterial cells in intestines ecology since there is a great diversity in them at a higher rate than dairy products, bacteriocins are produced by *Lactobacillus* which have broad spectrum of inhibition pathogenic bacteria like *Escherichia coli* and *Enterococcus faecalis*.[21,22] *Lactobacillus* is containing different mechanisms underlying antibacterial activity includes production of lactic acid so lowering of pH and antibacterial compounds, like bacteriocins and non-bacteriocin compounds, and non-lactic acid molecules. Some investigations had been declared the ability of bacteriocins to inhibit pathogenic bacteria like *E. coli*, *Pseudomonas* and *Klebsiella*.[23]

The antimicrobial activity of *lactobacillus* has been confirmed for pathogenic bacteria, most of the antimicrobial

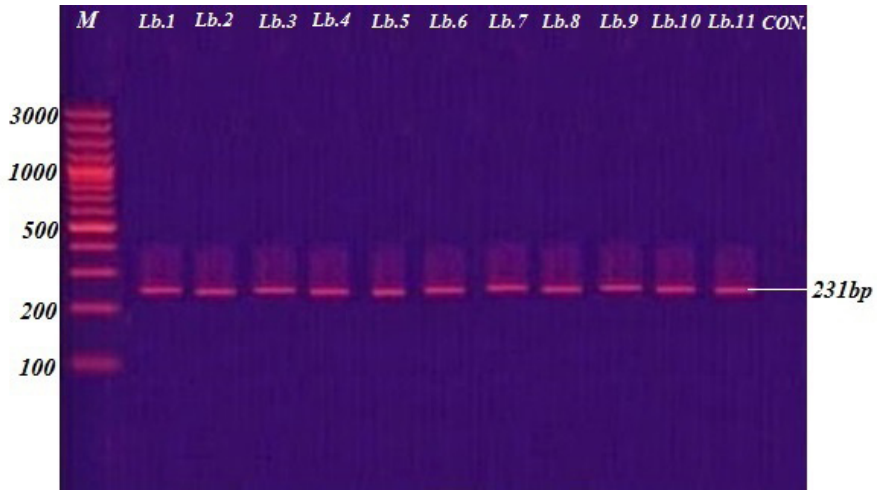

Figure 1: *Gel electrophoresis for amplification of the V2-V3 region of the 16S rDNA of Lactobacillus spp* isolates. Electrophoresis was completed on 1.8% agarose gel and run with a 5v/cm for 2 hours. With (3000 bp) ladder, Line *Lb.1 – Lb.5 Lactobacillus* isolated from infants feces, Line *Lb.6 – Lb.11 Lactobacillus* isolated from dairy products and CON: Negative control.

Table 1: Antibiotic susceptibility test of *Lactobacillus* isolates

	healthy infants feces isolates					*dairy products isolates*					
Antibiotics	*Lb.1*	*Lb.2*	*Lb.3*	*Lb.4*	*Lb.5*	*Lb.6*	*Lb.7*	*Lb.8*	*Lb.9*	*Lb.10*	*Lb.11*
Ampicillin	R	R	S	S	R	S	S	S	S	S	S
Chloramphenicol	R	S	R	R	S	R	S	S	S	S	R
Cephalexin	S	R	S	S	R	S	R	S	S	S	S
Tetracycline	R	S	R	R	R	R	S	R	S	S	S
Gentamicin	R	R	S	R	S	S	S	S	S	R	S
Cefotaxime	S	S	R	R	S	S	S	S	S	S	S
Erythromycin	R	R	S	S	R	S	R	S	R	R	S
Amoxicillin	S	S	R	S	R	S	S	S	S	S	S

R: resistant, S: sensitive

Table2: Shows antimicrobial activity of *Lactobacillus* isolates against some pathogenic bacteria

Pathogenic bacteria	*Inhibition zine mm*										
	healthy infants feces isolates					*dairy products isolates*					
	Lb.1	*Lb.2*	*Lb.3*	*Lb.4*	*Lb.5*	*Lb.6*	*Lb.7*	*Lb.8*	*Lb.9*	*Lb.10*	*Lb.11*
Escherichia coli O157:H7	14					± 0.2 18 ± 0.2 13 ± 0.2 16 ± 0.2 16 ± 0.4 10 ± 0.4	-	-	14		± 0.4 11 ± 0.4 13 ± 0.4
Pseudomonas aeruginosa	9							± 0.4 10 ± 0.2 7 ± 0.2 - 7 ± 0.2 9 ± 0.0 - 8 ± 0.2	-	10 ± 0.0	-
Staphylococcus aureus	14										± 0.0 14 ± 0.2 12 ± 0.0 14 ± 0.2 10 ± 0.0 13 ± 0.2 12 ± 0.2 10 ± 0.4 10 ± 0.2 14 ± 0.0 12 ± 0.2
Enterococcus faecalis	9										± 0.2 11 ± 0.2 11 ± 0.2 8 ± 0.2 8 ± 0.2 - 8 ± 0.0 - 9 ± 0.0 9 ± 0.4 10 ± 0.4
Klebsiella sp.	11										± 0.4 9 ± 0.2 7 ± 0.0 - 9 ± 0.2 - 7 ± 0.2 9 ± 0.2 - 9 ± 0.4 7 ± 0.0
Salmonella typhimiurum	17					± 0.0 19 ± 0.2 14 ± 0.2 17 ± 0.2 19 ± 0.0 12 ± 0.2	-	10		± 0.0 - 9 ± 0.2	-
Clostridium sp.	14 ± 0.4	16 ± 0.0	12 ±0.2	16			± 0.2 15 ± 0.2 10 ± 0.0 8 ± 0.2	-	14		± 0.0 - 9 ± 0.2

*each number represent a rate of triplicate
*- no inhibition

activity due to the production of metabolites agents such as organic acids, ethanol, hydrogen peroxide, acetone, carbon dioxide, acetaldehyde and many type of bacteriosins like reuterin , reuterecline and other.[24] Among the most important antimicrobial agents, lactic acids, hydrogen peroxide, and bacteriocines are the most common antimicrobial agents that were documented to be produced from *Lactobacillus* genus.[25] Shokryazdan *et al.,*[26] reported that *Lactobacillus* genus have shown strong antagonistic properties against a wide range of humans pathogens and can be considered the potential for prevention and treatment of infection so can play an important role to enhance health and competitive exclusion of pathogen,

CONCLUSION:

Generally, probiotics have antimicrobial properties, which differ from one to other. This study highlights the role of the probiotic environment in antigenic activity. This is likely the fact that infants feces isolates have antimicrobial factors for pathogenic bacteria is greater than that of isolates isolated from fermented dairy products, and have specific or possibly specific ways of interacting with their environment producing microbial inhibitors. These results will serve as the basis for further exploration of the role of ecosystems in their environment and their impact on host health. As well as owning for antibiotic resistance due to its presence in a mixed environment and adaptation to other species that found as well as synergistic behavior between *Lactobacillus* genus to inhibit pathogenic and food spoilage bacteria, that make stable codominance of multiple *Lactobacillus* species in an infant's intestines, so that give these species possession unique properties and competitive advantages.

REFERENCES:

1. I Petrova Mariya., Lievens, S. Elke Malik, M. Imholz, and S. Lebeer, 2015. "*Lactobacillus* species as biomarkers and agents that can promote various aspects of vaginal health". *Frontiers in Physiology*. 6. 2015, doi:10.3389 /fphys. 00081. ISSN 1664-042X.
2. FJ. Carr, D. Hill, and N. Maida 2002. "The lactic acid bacteria: A literature survey". *Crit. Rev. Microbiol.* 28: 281-370.
3. C. R. Inglin, (2015). "High-throughput screening assays for antibacterial and antifungal activities of *Lactobacillus* species". Journal of Microbiological Methods. 114, 26–29 doi:10.1016/j. mimet.2015.04.011
4. H. Mendes-SoaresSuzuki, H. Haruo, J. Roxana, and J. L.Forney, (2014). "Comparative Functional Genomics of Lactobacillus spp. Reveals Possible Mechanisms for Specialization of Vaginal Lactobacilli to Their Environment". *Journal of Bacteriology*. 196 (7): 1458–1470.
5. A. S. Naidu, (2000). "Natural Food Antimicrobial Systems" Director, Centre For Antimicrobial Research Department of Food, Nutrition & Consumer Sciences California State Polytechnic University Pomona, California, and CRC Press LLC, USA.
6. S. Dasari, R.N. Shouri, R. Wudayagiri, and L. (2014). Valluru "Antimicrobial activity of *Lactobacillus* against microbial flora of cervicovaginal infections. Asian Pac J Trop Dis. 4(1): 18–24. 2014 doi: 10.1016/S2222-1808(14)60307-8.
7. P. Ambalam, JB. Prajapati, JM. Dave, BM. Nair, A. Ljungh, and BR. Vyas (2009). " Isolation and Characterization of Antimicrobial Proteins Produced by a Potential Probiotics strain of Human Lactobacillus rhamnosus 231 and Its Effect on Selected Human Pathogens and Food Spoilage Organisms" *Microbial Ecology in Health and Disease*.; 21:211-220.
8. R. N. Atlas, A. E. Brown, and L.G. Paks, (2000). "Laboratory Manual of Experimental Microbiology" (1st ed.). Mosby. USA. 1995.
9. JF. Macfaddin "Biochemical Tests of Medical Bacteria" (3ed ed.). *Lippincott Williams and Wilkins*, USA.
10. O. Kandler, and N. Weiss, (1986). "Genus *Lactobacillus*. In: Sneath P. (Ed): BergeyÕs Manual of Systematic Bacteriology" 2. *William and Wilkins, Baltimore*,;pp. 1209- 1234.
11. R. Byun, MA. Nadkarni, KL. Chhour , FE. Martin, NA. Jacques, and N. Hunter, (2004). "Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries" *J Clin Microbiol* 42:3128 –3136. 2004 .http://dx.doi.org/10.1128/ JCM.42.7.3128-3136.
12. AW. Bauer, WM. Kirby, JC. Sheriss and M. Turc (1996). "Antibiotic susceptibility testing by standardized single method". *Am. J. Clin. Pathol.*; 45:493-496.
13. R. Georgieva, L. Yochevab, L. Tserovskab, G. Zhelezovab, N. Stefanovaa, A. Atanasovaa, A. Danguleva, G. Ivanovaa, K. Karapetkova, N. Rumyana, and E. Karaivanovaa (2015). "Antimicrobial activity and antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* spp. intended for use as starter and probiotic cultures" *Biotechnology & Biotechnological Equipment*, 29(1): 84-91.
14. RH. Abu Elnaga, S. Nagwa, and S. Mona (2014). "Bacterial aspect of Food Poisoning" *Life Sci J.*; 11(3):290-298.
15. V. Cleidson, M. Simone, F. Elza and JS. Artur. (2007). "Screening Methods to Determine Antibacterial Activity of Natural Products" *Brazilian Journal of Microbiology*, 38:369-380.
16. AS. Hummel, C. Hertel, WH. Holzapfel, and CM. Franz, (2007). "Antibiotic resistances of starter and probiotic strains of lactic acid bacteria" *Appl. Environ. Microbiol.* 73: 730–739.
17. S. Mayrhofer, C. Mair, W. Kneifel, and KJ.Domig (2011). "Susceptibility of *bifidobacteria* of animal origin to selected antimicrobial agents" *Chemother. Res. Pract.*; 989520 10.1155/2011/989520 [PMC free article] [PubMed] [Cross Ref].
18. M. Ammor, M. Gueimonde, M. Danielsen, M. Zagorec, A. Van Hoek, and C. De los Reyes-Gavilán (2008). "Two different tetracycline resistance mechanisms, plasmid-carried tet(L) and chromosomally located transposon-associated tet(M), coexist in *Lactobacillus sakei* Rits" 9. Appl. Environ. Microbiol. 74:1394–1401.
19. Rojo-Bezares B, Sáenz Y, Poeta P, Zarazaga M., Ruiz-Larrea, F. and Torres C. (2006). "Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine" *Int. J. Food Microbiol.*; 111 234–240.
20. L. Aquilanti, C. Garofalo, A. Osimani, G. Silvestri, C. Vignaroli, and F. Clementi, (2007). "Isolation and molecular characterization of antibiotic-resistant lactic acid bacteria from poultry and swine meat products" *J. Food Prot.* 70, 557–565.
21. S. Ogunbanwo, A. Sanni, and A. Onilude, 2003). "Characterization of bacteriocin produced by *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1" *African Journal of Biotechnology.* 2 (8): 219-227.
22. H. Majeed, O. Gillor, B. Kerr, and MA. Riley, "Competitive interactions in *Escherichia coli* populations: the role of bacteriocins". *ISME J.* 5:71–81. 2011

23. F. Domitille, N. Cedric, H. Marie, P. Coconnier, L. Vanessa, and L. Alain (2005). "Lactic acid-, and non-lactic acid-dependent activities of probiotic *Lactobacilli* against *Salmonella* enterica Serovar T*yphimurium*" *Applied and environmental microbiology*. 71(10): 6008–6013.
24. J. Suskovic, K. Blazenka, J. Beganovic, AL. Pavunc, K. Habjanic, S. Matosic (2010). "Antimicrobial activity—the most important property of probiotic and starter lactic acid bacteria" *Food Technology and Biotechnology*. 48(3):296–307.
25. DM. Saulnier, JK. Spinler, GR. Gibson, J. Versalovic "Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods" *Current Opinion in Biotechnology*. 20(2):135–141. 2009 [PMC free article] [PubMed].
26. P. Shokryazdan, C. Chin Sieo, P. Kalavathy, J. Boo Liang, N. Banu Alitheen, M. Jahromi, and Y. Wan Ho (2014). "Probiotic Potential of *Lactobacillus* Strains with Antimicrobial Activity against Some Human Pathogenic Strains" *Biomed Res Int.* : 927268. Published online 2014 Jul 3. doi: 10.1155/2014/927268.