Isolation, Purification, and Selection Criteria of Probiotic Bacteria "Lactobacillus *Jensenii*

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Abstract

Lactobacilli isolated from stool samples of healthy infants aged 2-28 days were identified as Lactobacillus jensenii based on its morphological description on MRS agar and blood agar, Gram stain, catalase test, and results of API 20 A system.
The Lactobacilli were assessed for characteristics considered important for in vitro resistance ability to low pH (3, 4, 5, and 6), and resistance ability to different bile salt concentrations (0.5%,1%,1.5%,2%,2.5%, and 3%). The'other characteristics studied were culture and sensitivity of probiotic bacteria to different types of antimicrobial and antibiotics. The probiotic bacteria revealed inhibitory activities against several pathogenic bacteria (Staphylococus aureus, Streptococcus agalactia, Clostridium perifrenges, Pseudomonas aeroginosa, Escherichia coli, Klebsiella pneumonia, 'Salmonella typhi, Vibrio cholerae, and Shigella flexneri). Lactobacillus jensenii also exhibited its ability for attachment to epithelial cells of human intestine (in vitro).

Introduction

The human intestinal microflora is part of a dynamic ecosystem. The genus *Lactobacillus* is an important one of these bacteria. It is divided into three groups based on fermentative abilities of the species (group I: obligatory homofermentative. group II: Facultatively heterofermentative and group III: obligatory heterofermentative *Lactoba-cilli)'. Lactobacillus jensenii* belongs to subgroup I of group I, the *Lactobacillus acidophilus* is a member of subgroup II of group I, which degrade hexoses almost completely to lactic acid and do not ferment pentoses or gluconate[2].

The selection of a suitable strain of a microorganism can be regarded as the primary requirement for the use as a probiotic. The cultures of probiotic bacteria must be able to .pass the stomach-duodenum barrier in a viable state and to multiply at the site of destination in the intestine. Additionally, they must be capable of producing antagonistic metabolites against a dominating saprophytic microflora[3] and pathogenic bacteria[4] resulting in a competitive growth. These abilities are common among lactic acid bacteria (e.g *Lactobacilli & Bivido bacteria*),

In vitro test protocols can be readily adapted to examine the maintenance of a strain's ability to tolerate acidic conditions. *Lactobacillus jensenii* Survives and grows in the presence of bile and metabolizes selective substrates.

Adhesion of probiotic bacteria to human intestinal cells has been related to shortening the duration of diarrhea[5].

The objectives of this study were to isolate and identify *Lactobacillus jensenii* and to study the probiotic trials such as *in vitro* acid tolerance, bile tolerance, Culture and sensitivity, antagonism effects on several pathogenic bacteria, and' *in vitro* adherence to human enterocytes.

Material and Methods

Isolation and purification *Lactobacillus* was isolated from fecal samples of healthy infants, fed on breast milk only, aged 14-28

days.

The selective (de Man, Rogosa, Sharp (MRS) broth and agar (oxoid) and non-selective (blood agar) (BDH) were used for isolation of *Lactobacillus* and identified to the species *jensenii* by API 20 A syetem (bioM'erieuxe France).

Selection criteria

The tolerance ability of *Lactobacillus jensenii* to low pH (3,4,5,and 6) wase done according to Chatean et al method 1993 .

The tolerance ability of *Lactobacillus jensenii* to different concentration of bile salts (o.5%, 1%, 1.5%, 2%, 2.5%, 3%) was made depending on Lankapurtha and Shah method 1995[7]. O

The antimicrobial sensitivity was made according to the Cupta et al method 1995. The antimicrobial and antibiotic discs are (Amikacin, Amoxycillin, Ampicillin, Carbenecillin, Cefactor, Cefazolin, Cefuroxime, Ceftazidime, Gentamycin, Kanamycin, Nalidix acid, Rifampicin, Sparfloxacin, Vancomycin, Ceftriaxone, Augmentin, Azithromycin, Cefepime, Cefixime, Cefotaxime, Cefpodoxime, Chloromphenicol, Ciprofloxacin, Clarithromycin, Co-Trimoxozole, Erythromycin, Nitrofurantoin Norfloxazole, Roxithromycin, Sulfadiazine, and Trimethoprime.

The antagonism well assay[9] was used to measure the zone of inhibition of growth of pathogenic bacteria (e.g *Staphylococcus aureus, Streptococcus agalaclia, Clostridium perifreerichia, Pseudomobas aerogenosa, Escherichia coll, Klebsiella pneumonia, Salmonella typhi, Vibrio cholerae, and Shigella flexneri*.) by cell free extract of *Lactobacillus jensenii.*

The adhesion test was made according to Filler method 1975[10]. The method has foure steps:

a- Preparation of intestinal epithelial cells suspention.

b- Preparation of bacterial suspention.

c- Preparation of cell film from mixture of above a & b.

d- Staining of film by Lieshman stain.

Results

Description *of Lactobacillus jensenii*

Lactobacillus jensenii was anaerobic, Gram positive, rods, non-motile, non-spomlating, catalase negative. Colonies on MRS agar were round, smooth, convex, translucent or semitranslucent,and approximately 1mm in diameter. On other hand, the colonies on blood agar were round,, smooth, pin point, slightly greenish and shiny, non-haemolytic, convex and approximately 1 mm in diameter.

API 20 A system

Indol was not formed and urease is negative Acid was produced from glucose, sucurose, maltose, salicin, esculin, cellobiose mannose, and trehalose:- The manitol, lactose, xylose, arabinose, gelatin, glycerin, melezitose, raffinose, sorbitol, rhamnose, and galactose were not fermented (Table l)(fig.l)

PH and bile salts resistance abilities

Lactobacillus jensenii revealed resistance ability to different low pH and grow in acidic environment pH 3, 4, 5,and 6).In addition this bacterium has ability to resist and can grow at different concentrations of bile salts (o.5%, 1%, 1.5%, 2%, 2.5%, and 3%).

Antimicrobial sensitivity test

The results of culture and sensitivity test of *Lactobacillus jensenii* to antibacterial and antibiotic discs. The results were either sensitive (Amikacin, Amoxycillin, Ampicillin, Carbenecillin, Cefaclor, Cefazolin, Cefuroxime, Ceftazidirhe, Gentamycin, Kanamycin, Nalidixic acid, Rifampicin, Sparfloxacin, and Vancomycin) and intermediate to (Ceftriaxone) or resistance to (Augmentin, Azithromycin, Cefepime, Cefixime, Cefotaxime, Cefpodoxime, Chloromphenicol, Ciproflo-xacin, Clarithromycin, Co-Trimoxazole, Erythromycin, Nitrofurantoin, Norfloxacin, Roxithromycin, Sulfadiazine,, ' and Trimethoprime (table 2).

Table 1. Results of biochemical and others of API 20 A system for *actobacillus Jensenii* with standard bacteria.

No	Tests	Results -'	Standard*
1	Indol	-	-
2	Urease	-	-
3	Glucose	+	+
4	Manitol	-	-
5	Lactose	-	-
6	Sacurose	+	+
7	Maltose	+	+
8	Salicin	+	+
9	Xylose	-	-
10	Arabinose	-	-
11	Gelatine	-	-
12	Esculine	+	+
13	Glycerin	-	**
14	Cellobiose	+	+
'15	Mannose	+	+.,
16	Melezitose	'	-
17	Raffinose	-	£)***
18	Sorbitol	-	-
19	Rhamnose	-	-
20	Trehalose	+	D
21	Calactose	-	-
22	Spore	-	-
23	Gram stain	+	+
24	*Cocci*	-	-

* They were mentioned in Bergy's Manual systemic bacteriology. 1989[63]

** It was not mentioned in the above reference .

*** d=different.

api 20 A

bioMerieux

Origine / **Source / Herkunft / Ohgen / Prolievo :**

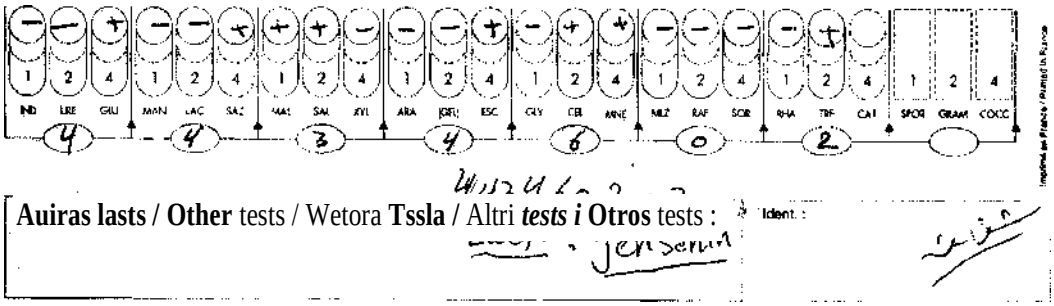

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Fig 1. API 20 A system : The read chart of *Lactobacillus jensenii*

Table 2. Results of antimicrobial sensitivity test ofLactobacillusjensenii to (31) Antibiotic discs.

No	*Antibiotic discs**	*Code*	*Disc potency tig*	*Zone diameter standard mm.*	*Zone diameter mm.*	*Results*
1	*Amikacin*	*AN*	*30*	*17*	*22*	*S*
•2	*Amoxycillin*	*AMX*	*25*	*18*	*30*	*S*
3	*Ampicillin*	*AM*	*10*	*17*	*"20*	*S*
4	*Augmentin***	*AC*	*30*	*18*	*_****	*R*
5	*Azithromycin*	*AZM*	*15*	*18*	*-*	*R*
6	*Carbencillin*	*CB*	*100*	*17*	*30*	*S*
7	*Cefaclor*	*CJ*	*30*	*18*	*30*	*S*
8	*Cefazolin*	*CZ*	*30*	*18*	*24*	*S*
9	*Cefepime*	*CFP*	*30*	*18*	*-*	*R*
10	*Ceftxime*	*CFM*	*5*	*19*	*-*	*R*
11	*Cefotaxime*	*CTX*	*30*	*23.*	*-*	*R*
12	*Cefpodoxime*	*CPD*	*30*	*21*	*-*	*R*
13	*Ceftazidime*	*CA*	*30*	*18*	*20*	*S*
14	*Ciftriaxone*	*CRO*	*30*	*21*	*20*	*I*
15	*Cefuroxime*	*cu*	*30*	*18*	*30*	*S*
16	*Chloramphenicol*	*c*	*30*	*18*	*-*	*R*
.17	*Ciprofloxacin*	*CF*	*5*	*21*	*•-*	*R*
18	*Clarithromycin*	*CLR*	*15*	*18*		*R*
19	*Co-Trimoxazole*	*CO*	*25*	*16*	*-*	*R*
20	*Erythromycin*	*E*	*15*	*23*	*-*	*R*
21	*Gentamycin*	*GM*	*10*	*15*	*20*	*S*
22	*Kanamycin*	*K*	*30*	*18*	*24*	*S*
23	*Nalidixic acid*	*NA*	*30*	*18*	*20*	*S*
24	*Nitrofurantoin*	*FT*	*300*	*17*	*-*	*R*
25	*Norfloxacin*	*NX*	*10*	*17*	*-*	*R*
26	*Rifampicin*	*RA*	*5*	*20*	*23*	*S*
27	*Roxithromycin*	*RO*	*30*	*18*	*-*	*R*
28	*Sparfloxacin*	*SC*	*5*	*19*	*'30*	*S*
29	*Sulphadiazine*	*SZ*	*300*	*18*	*-*	*R*
30	*Trimethoprim*	*TR*	*5*	*16*	*-*	*R*
31	*Vancomycin*	*VA*	*30*	*12*	*20*	*S*

**= Antibiotic discs were obtained from Alraze Co. Iraq.*

***= Trade mark (Amoxicillin+ Clavulanic).*

****= N_O inhibition zone.*

Co-Trimoxazole= Trade mark (Sulfamethoxazole + Trimethoprime)

The effect of cell free extract (treated and non-treated with NaOH) on pathogenic bacteria

Lactobacillus jensenii was assayed for it's ability to inhibit the growth of the following pathogenic bacteria (*Staphilococcus aureus, Streptococcus agalactia, Clostridium perifreges, Pseudomonas aeroginosa, Escherchia coli, Klebsiala pneumonia, Salmonella typhi, Vibrio cholera, and Shigella flexinar*).

The cell free extracts of *Lactobacilli* produced narrow inhibition zones (NIZ) (1 mm-less than 1cm.) against *Streptococcus agalactia, Pseudomonas aeroginosa, Escherchia coli, Klebsiala pneumonia, Salmonella typhi, Vibrio cholera, and Shigella flexiner.* The intermediate inhibitory zone (IIZ) (l-2cm.) seen against *Staphylococcus aureus, and Clostridium perifrenges* if the cell free extracts were treated with NaOH (IN). When they were not treated with NaOH, the antagonistic effects appeared Narrow inhibition zone for *Vibrio cholera,* the intermediate inhibitory zone showed for *Staphylococcus aureus, Streptococcus agalactia, Clostridium perifrenges, Klebsiala pneumonia.* Wide inhibition zone (WIZ) (more than 2 cm. in diameter) exhibited for *Pseudomonas aeroginosa, Escherchia coli, Salmonella typhi, and Shigella flexiner (table 3).*

Table 3. Ressults of antagonistic effects of *Lactobacillus jensenii (*cell free extract Treated and not treated with NaOH) to some pathogenic bacteria.

No	Pathogenic bacteria	*L. jensenii (* Inhibitory zone)	
		Not treated with NaOH	Treated with NaOH
1	*Staphylococcus aureus*	IIZ	IIZ
2	*Streptococcus agalactia*	IIZ	NIZ
3	*Clostridium perifrengens*	IIZ	IIZ
4	*Pseudomonas aeraginosa*	WIZ	NIZ
5	*Escherichia coll*	WIZ	NIZ
6	*Klebsiela pneumonia*	IIZ	NIZ
7	*Salmonella typhi*	WIZ	NIZ
8	*Vibrio cholera*	NIZ	NIZ
9	*Shigella flexneri*	WIZ	NIZ

NIZ= Narrow inhibitory zone (0-10 mm.)
IIZ = Intermediate inhibitory zone (10-20 mm.)
WIZ = Wide inhibitory zone (more than 20 mm.)

Adhesion test

Lactobacillus jensenii exhibited an ability to adhere the human enterocytes. The adhesion depended on the density and numbers of attached probiotic bacteria to human intestinal epithelial cells (Fig 2).

Discussion

One gram of ifant's stool were mixed with MRS broth and incubated for three days at 37c°. The precipitate in MRS broth was cultured on MRS agar and incubated for three days at 37c° under anaerobiosis. The identification of culture was based on characteristics • of *Lactobacillus* presented in Bergey's manual of determination bacteriology, carrying out morphology[1], Gram stain, catalase test, and fermentation of different carbon sources (API 20 A System). Based on these criteria, *Lactobacillus jensenii* was identified and tested for probiotic use for human.

Fig 2. Attachment *of Lactobcillus jensenii* to human intestinal epithelial cells (Lieshman stain) (1000x enlargement factor).

The most important basic criteria for selection of probiotic bacteria of human origin include acid and bile stability, culture and sensitivity, the antagonism effects, and the ability to adhere and colonize the GIT.

In present study, the probiotic bacteria *Lactobacillus jensenii* exhibited acid tolerance at low pH (3,4,5, and 6) and bile salts resistance in media containing (o.5%, 1%, 1.5%, 2%, 2.5%, and 3%) bile salts corresponding to other previous studies were done by Oh et al 2000[11] , Haeronimus et al 2000[12] and Hassoon 2005 [13].

The study showed that *Lactobacillous jensenii is resistance* to (16) antibiotics, intermediate to one antibiotic and sensitive to (14) antibiotics. Charteris et al 1998[14,13] showed that *Lactobacilli* revealed a wide range of antibiotic resistance.

The present results showed that *Lactobacillus jensenii* has ability to inhibit several pathogenic bacteria such as *Staphylococcus aureus, Strepto-coccus agalactia,'Clostridium perifren-ges, Pseu-domonas aeroginosa, Esche-rchia coli, Klebsiala pneumonia, Salmo-nella typhi, Vibrio cholera, and Shigella-flexinari).*
In vitro studies[73,15,16] have suggested that certain specific strains *of Lactobacilli* are able to inhibite the adherence of *Gardnerella vaginalis* epithelium and/or produce hydrogen peroxide (H2O2), lactic acid and/or bacteriocins, which inhibit the growth of bacteria causing bacterial vaginosis, urinary tract infection [15] and diarrhea[17].
Diane et al 2002[18] found that all four strains *of Lactobacilli (Lactobacillus jensenii, Lactobacillus crispotus, Lacto-bacillus acidophilus, and Lactobacillus gasseri)* were able to inhibit the *gonococcal* strains at low pH while *Lactobacillus jensenii and Lactobacillus crispatus* were also able to inhibit Neisseria *gonnorrhoeae* at neutral pH. None of the them were able to inhibit *Escherchia coli* strains, though *Lactobacillus jensenii* was able to inhibit *Neisseria cinerea.*
Modified *Lactobacillous jenseni* is also able to inhibit HIV-1 from infecting target cell in cervico-vaginal mucosa. The decrease in infectivity of HIV-1 with dosage controlled engineered *L.jensenii* may be a convenient contraception by using natural occurring colonies of bacteria inside the body to the block the transmission of HIV[19].
The results of this study revealed attachement of probiotie bacteria (*L. jensenii)* To the human enterocytes.
Salminen et al 1996 found that adhesion of probiotic strains to human intestinal cells and the following colonization of the human gastrointestinal tract has been

suggested as an important prerequisite for probiotic action. Adhesion verifies the potential of the strain to inhabit the intestinal tract and also to grow in intestinal condition. Adhesion also provides a contact with the mucosal surface facilitating the contact without associated lymphoid tissue mediating local and systemic immune effects. Thus, only adherent probiotics have been thought to induce immune effects and to stabilize intestinal mucosal barrier.Nesser et al 2000[21] found that the *L. jensenii* has two major carbohydrate-binding specificities. A first one for an endo-H treated yeast cell wall mannprotein carrying mainly O:- linked oligomannosides, and a second one for the gangliotri- and (gangliotetra- osylcer amides) (asialo-GMl). Similar carbohydrate binding specificities are known to be expressed on cell surface adhesion of several enteropathogens, enabling them to adher to the host gut mucosa. These findings corroborate the hypothesis that selected probiotic bacterial strains could be able to compete, with enteropathogens for the same carbohydrate receptors in the gut.

Conclusions

1- Isolation and identification of Lactobacillus jensenii by using API ID 32 A system and biochemical tests.
2- Probiotic bacteria (Lactobacillus jensenii) have selective criteria (e.g. resistance to pH and bile salts, antagonism effect on pathogenic bacteria, production of antimicrobial substances, and attachement to human enterocytes.
3- The use of probiotic bacteria (Lactobacillus jensenii) for prevention and treatment of infant's diarrhoea.

References

1. Kandler O.; and Weis N. 1989. Genus Lactobacillus, Beijerinck 1901, 212 AL. In Bergey's Manual of systemic Bacteriology, Vol.2. PP. 1209- 1234. Edited by P.H.A Sneath. N. S. Mair, M.E. Sharpe and J.G. Baitimore: Williams and Wilkingngn,s.
2. Pot B.; Ludwig W.; Kersterso k.; and Schleifer H.; 1994. Taxonomy of lactic acid Bacteria. In Bacteriocins of lactic acid bacteria: Microbiology, Genetic, and Application, PP. 13-90.. Edited byL. De Vuyst and E.J. Vandamme. London: Blackei Academic and professional.
3. Reuter G. 2001. Probiotic- Possibilities and limitations of their application in food, Animal feed, and in pharmaceutical preparations for man and animals (German). Berliner und munchener Tierayztliche Wochenschrift. 114(11-12). 410-419.
4. Forestier C., De-champs E.; Vatoux C.; Joly B. 2001. Probiotic activities of Lactobacillus casei: rhamnosus. In vitro adheranceto intestinal cells and Antimicrobial probiotics. Res-Microbiol. 152(2): 167-173.
5. Tuomola, E.; Crittenden R.; Playne M.; Isolaur E.;Salminen S. 2001. Quality Assurance criteria for probiotic bacteria Am.J. Clini. Nutr. 73(2 suppl): 393s-398s.
6. Chateau N.; Gastellanos I.; and Deschamps A.M. 1993. Distribution of pathogen Inhibition in the Lactobacillus isolates of commercial probiotic consortium. J. App. Bacteriol. 74. 36-40.
7. Lankaputhra W. G. V.; and Shah N.P. 1995. Simple methods for selective Enumeration of Lactobacillus acidophilus in yoghurt supplemented with Lactobacillus acidophilus and Bifidobacter spp. Milchwissenschaft. Vol. 51, No. 8.
8. Gupta P.K.; Mital B.K.; and Gupta R.S. 1995. Antibiotic sensitivity pattern of Various Lactobacillus acidophilus strains. Indian Journal of Experimental Biology. Vpl 33. 620.
9. Gupta P.K.; Mital B.K. and Garg S. K. 1996. Characterization of Lactobacillus Acidophilus strains for use as a dietary adjuct. International Journal of Food Microbiology. Vol. 29. No. 7.
10. Fuller. 1975. Nature of determinant responsible for the adhesion of Lactobacilli To chicken crop epithelial cell. J. Gen. Micro. 87. 245- 250.
11. Oh S., Kim S.H.; Worobo R.W. 2000. Characterization and purification of a bacteriocin produced by a potential probiotic culture, Lactobacillus acidophilus 30sc. J. Dairy. Sci. 83(12): 2747-2752
12. Hyronimus B.; Le-Marrec C.; Sassi A-H.;Deschemps A.'2000. Acid and bile Tolerance of spor-forming lactic acid bacteria.Int. J. food. Microbio.l:61(23):139.
13. Hassoon.A.F. 2005 (Isolation, characterization, and mass Production of Lactobacillus acidphlus,a.s a probiotic for

therapeutic use .ph.D these University of Tikrit/ College of medicine .
14. Charteris W.P.; Kelly P.M., Morelli; and Collins J.K. 1998.Antibiotic susceptibility of potentially probiotic Lactobacillus species. J. Food Prot. 61: 1636-1643.
15. Falagas ME.; Betsia GL.; and Athanasious S. 2007. Probiotics for the treatment of women with bacteria vaginosis. Clin. Microbiol. Infect. 13(7): 657-664.
16. Barrens R.S.; Tassone D. 2008. Use of Lactobacillus probiotics for bacterial Genitourinary infections in women: a review. Clin. Ther. 30(3):453-468.
17. Hasson A.F. ,Mehdi S.S. 2009 Acute diarrhea in children treated by lactobacillus acidophilus JABMS .Vol.lO,N0.1
18. Diane C. St.; Amant Aris E.; Valentin-Bon, and Ann E. Jesse. 2002. Inhibition of Neisseria gonorrhoeae by Lactobacillus species that are-commonly isolated from Female genital tract. Infection and Immunity. 70(12):7169-7171.
19. Theresa L.Y.; Chang , Chia- Hwachang, David A., Simpson, Qiag Xu, Patrick K. Martin, Laurel A. Lagenaur, Gray K. Schoolnik, David D. Ho, Sharon L. Hillier, Mark Holodniy, John A. Lewicki, and PETER p. Lee. 2003. Inhibition of HIV Infectivity by a neutral human isolate of Lactobacillus jensenii engineered to Express functional two-domain CD4. Proc. Natl. Acad. Sci. USA. 11672
20. Salminen S.; Lauri E.; Salminen E. 1996. Probiotics and stabilization of gut Mucosal barrier. Asia Pacific J. Clin. Nutr. 5(53-56).
22. Neeser J.R.; Granato D.; Rouvet M.; Servin A.; Tenebergs; Karlsson K.H. 2000. Lactobacillus johnsonii Lal shares carbohydrate-binding specificities with several Enteropathogenic bacteria. Glycobiology. 10(11):1193-1199.