Original Article

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Identification of Lactobacilli from Fecal Flora of Some Iranian Infants

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Abstract

Objective: During the past 20 years identification of lactobacilli (*L*) isolated from normal flora has received great interest due to their health promoting effects. This study has aimed at characterizing the lactobacillus strains isolated from the fecal flora of Iranian infants based on phenotypic oriented methods. Moreover, the diversity of identified species among tested infants has been looked into.

Methods: Thirty two strains of lactobacilli were included in this study. The given strains were previously isolated from the fecal samples of 6 infants between 1-19 months of age. They are examined through 14 carbohydrate fermentation tests, growth ability at different temperatures and different concentrations of sodium chlorides. Cell and colony morphology were assessed as well.

Findings: The examined strains were identified as *L. acidophilus* (12 strains), *L. plantarum* (9 strains), *L. rhamnosus* (7 strains), *L. paracasei* (3 strains) and *L. fermentum* (1 strain); 2 strains remained unidentified. Accordingly *L. acidophilus* was the most predominant species among tested samples.

Conclusion: Some biochemical differences were obtained among the strains of *L. acidophilus* group and some morphological peculiarities were observed among the strains of *L. paracasei* and *L. rhamnosus* in comparison to the typical strains of *L. casei* group. These differences revealed the necessity of application of complementary molecular methods for clear identification of examined *Lactobacillus* strains.

Key Words: Lactobacilli; Fecal flora; Infants; Intestinal microflora; Probiotics

Introduction

The human intestinal microflora is complex with total counts of 10^{11}-10^{12} bacteria per gram of stool. Among this vast number of organisms, are at least 500 species, within which lactobacilli are numerically a minority[1,2]. Lactobacilli are not predominant

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among the intestinal micro flora; however, they are usually isolated throughout the gastrointestinal tract (GIT) of healthy human[3]. *Lactobacillus* strains isolated from GIT of human may live as commensal, adapted to intestine environment or they are originated from foods[3,4]. During the past twenty years studies on isolation, identification and the population level of *Lactobacillus* flora of the gastrointestinal tract have received special interest because of their health promoting effects; Such lactoflora is proved to be essential for mucosal immune stimulation and is also shown to directly correlated with competitive exclusion of pathogens in infants[3,5,6].

Human derived lactobacilli can be used as probiotics with effects especially against acute diarrhea in childhood and have immune activity properties[7,8]. Probiotics refer to viable microorganisms that promote or support a beneficial balance of the autochthonous microicrobial population of the gut[9]. It is most likely that the influence of probiotics is dependent on at least partly on the indigenous bacteria which are present in the host[4,6]. In newborn infants and small children, the extent to which lactobacilli colonize the intestine is controversial[3]. Physiological state of infants[3,8,10,12], their genetical background[13] as well as ecological differences[3,8] have been considered as the main parameters influencing the lactoflora of infants. Physiologically, changes in age up to 6 months could result in critical changes on enteric lactobacilli[3] and these changes are related to change in diet by introduction of solid foods to infants' dietary regime. From the ecological point of view, geographical differences and the degree of industrialization can account for the variations in *Lactobacillus* microbiota of infants[3,14].

Since the diversity of the indigenous lactoflora varies among individuals[6,7], the study of new strains from fecal flora of infants not only provides presumptive knowledge of beneficial microbiota of their gastrointestinal tract, it may also lead to development of novel probiotic strains[15,16]. Traditionally lacto-bacilli are characterized into three physiological groups: 1) the obligately homofermentative lactobacilli, 2) the facultatively homofermentative lactobacilli and 3) the obligately heterofermentative lactobacilli[17,18, 19].

Modern genotypic methods have proved to be the most reliable approach in exact identification of lactobacilli. However, due to the large number of species, application of sole genotypic methods is quiet difficult. Therefore, Traditional biochemical tests are still used as an important tool for identification and classification of lactobacilli[19]. This study aims at identifying some *Lactobacillus* strains, isolated from six Iranian infants. Distribution of each identified species among the tested infants is also of interest. To our knowledge, this is the first report of Iranian infants in this field of research.

Subjects & Methods

Bacterial strains and culture conditions: The 33 isolates of un-branched, Gram-positive, catalase-negative, non-spore-former rods previously characterized as lactobacilli, were included in this study. They had been isolated from 6 out of 11 healthy infants between 1 to 19 months. None of them had any medical treatment at the sampling time and they could be placed in the same social class, average educated class of people in Isfahan. No specification was considered for subjects' diet, just, due to their age, the infants younger than six months were receiving milk as solely or the main source of their diet. Others were using solid foods, regularly used by Iranian children as well as milk.

The study had the informed consent of the infants' parents. Enumeration of each isolate was performed on modified de Man-Rogosa-Sharpe medium (Sharlou, Spain) containing Vancomycin 1 mg/l^{-1} under 10% CO_2 anaerobic environment, obtained with the CO_2 generating Gas pack A system (Merck, Darmstadt, Germany)[20]. *L. rhamnosus* GG, isolated from pharma-ceutical product (Culturelle, USA) was used as reference strain

during the biochemical tests. The identification of reference strains was performed in our laboratory by biochemical methods and species-specific PCR reaction[21]. The strains were stored in MRS (Sharlou, Spain) with 30% glycerol at -80°C. Stock cultures were maintained on MRS agar slants (Sharlou) at 4°C and streaked every 4 weeks. Activated cultures were obtained from at least two successive transfers in MRS broth.

***Categorization of the isolates to fermentation groups and their identification at species level*:** The fermentation of carbohydrates was determined in MRS fermentation broth, (MRS without glucose and meat extract) containing bromocresol purple (0.05gr/l^{-1}) as a pH indicator and supplemented with 1% of the following carbohydrates: Arabinose, Cellobiose, Fructose, Galactose, Gluconate, Lactose, Maltose, Manitol, Mannose, Melezitose, Melebiose, Raffinose, Rhamnose, Ribose, Sorbitol, Sucrose, Threhalose and Xylose. Gas production from glucose and Gluconate were determined in MRS fermentation broth containing inverted Durham[22]. One percent of activated culture of each isolates was inoculated into tubes containing the above fermentation media. Tested tubes were incubated at 37°C under 5% CO_2 concentration in CO_2–air-jacketed incubator (Memmert, Germany). Following tests also were performed: Growth in MRS broth containing 4.5 % and 6.5% NaCl, growth at 15° C and 45° C in MRS broth for 5 days and at 4°C and 10° C for 12 days)[7,17,22]. All tests were repeated twice during two independent experiments. A non-inoculated of each medium was used as blank in each test. A steromicroscope (American optical, USA) was used to define colony types and cell morphology was obtained microscopically (Olympus, Japan).

Findings

The biochemical and physiological analysis of the strains revealed that they belong to three fermentative categories of lactobacilli: Obligately homofermentative (12 strains), facultatively heteofermentative (19 strains) and obligately heterofermentative (1 strain). Their morphological, biochemical and physiological characteristics are represented in table 1. Table 2 outlines the diversity of recovered species among tested infants which indicate the rich load of lactobacilli both in terms of number and species in younger infants.

The strains in *L. acidophilus* group were the most recovered, followed by *L. plantarum.* From twelve strains identified to be the strains of *L. acidophilus* group, eight strains behaved alike in all phenotypic assessments irrespective of their origins, which were three individual infants. *L*. H22, *L*. H28 and *L*.SH51 showed some differences in carbohydrate fermentation patterns with respect to the fermentation of Arabinose, Rhamnose, Cellobiose and Xylose. All 12 strains exhibited R type colony on primary isolation, but the colonical shape of the strains H28 and H29 transformed to S type after a couple of subculturing. Nine strains of *L. plantarum* were also isolated from three individual infants. Their difference in some biochemical tests could easily be related to their origin and it seems that *L. plantarum* strains derived from each infant were the identical strains. Based on the biochemical and physiological properties, seven strains were identified as *L. rhamnosus* and three strains as *L. paracasei* but their cell morphology was far different from what the literature stated for *L. casei* group[17]. They exhibited pleomorphic characteristics and became curved when they streaked on solid media. Among the rest of strains, one was identified as *L. fermentum* and the other two remained unidentified.

Discussion

Previous studies indicated that obligately homofermentative lactobacilli, typical of the human host, consist of genetically close species, such as strains of *L. acidophilus*, *L.*

Table 1- Characterization of *Lactobacillus* strains by morphologica, physiological and biochemical properties

Identified species	*L .acidophilus* N=12	*L. plantarum* N= 9	*L.para casei* N=3	*L. rhamnosus* N=7	*L. spp* N=2	*L. fermentum* N=1	*L.GG*
Gas Production from:							
Glucose	-	-	-	-	-	+	+
Gluconate	-	+	+	$1^{+}6^{W}$	+	+	+
Acid production from:							
L-Arabinose	$1^{W}11^{-}$	$5^{W}4^{+}$	-	-	+	+	-
D-Cellobiose	$1^{+}11^{-}$	+	+	+	+	-	+
Fructose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Gluconate	-	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	-
D-Mannose	+	+	+	+	+	-	+
D-Mannitol	-	+	+	$6^{+}1^{-}$	-	-	+
Melebiose	-	+	-	-	-	+	-
D-Melezitose	-	$4^{+}2^{W}3^{-}$	+	$5^{+}2^{W}$	$1^{+}1^{W}$	-	+
Raffinose	-	+	-	-	-	+	-
L- Rhamnose	$2^{+}10^{-}$	$4^{+}2^{W}3^{-}$	-	+	-	-	+
D-Ribose	-	+	+	+	+	+	+
D-Sorbitol	-	+	$1^{+}1^{W}$	$5^{+}2^{W}$	-	-	+
D-Threhalose	-	$5^{W}4^{+}$	+	+	+	-	W
Xylose	$1^{+}11^{-}$	-	-	-	-	+	-
Growth at 4C	-	-	-	-	-	-	-
Growth at10C	-	+	-	+	-	-	+
Growth at 15C	-	+	+	+	-	-	+
Growth at 45C	+	-	-	+	-	+	+
Growth in 4.5% Na Cl	$10^{+}2^{W}$	$3^{+}6^{W}$	W	$1^{+}6^{W}$	+	+	+
Growth in 6% NaCl	$10^{-}2^{+}$	$3^{+}2^{W}4^{-}$	W	W	+	W	+
Size of Colony	1-2mm	2-5mm	3-5mm	3-5mm	1-3mm	1mm≤	3-mm
Type of Colony	12X type, Rough, 2mixed type	Smooth, convex	Smooth, convex	Smooth, convex	Smooth, convex	Rough, Flat	Smooth convex

1. Obligatory homofermentative.
2. Facultative heterofermentative.
3. Obligatory heterofermentative

P.C.: Positive Control, +: positive, -: Negative, W: weak reaction

gasseri, L. crispatus, L. johnsonii and *L. delbrukeii* which might also share fermentation profile [7,19,23]. Therefore, definite identification of *L. acidophilus* by phenotypic methods is hardly possible. Comparing our results with the results of Xanthopoulos[16], eight strains of this group formed a compact unit which was phenotypically identical to the well–identified *L. acidophilus* strains isolated from infants stool by Xantaopoulos[16]. The four other strains could belong to the other aforementioned species due to their differences in biochemical and morphological traits. Although *L. acidophilus* is reported to be

Table 2- The distribution of identified *lactobacilli* strains isolated from 6 Iranian infants

1	**1**	***L. acidophilus***	L. H13, L.H110, L.H1102, L.H19,	8.29
2	**4**	***L. acidophilus***	L.H26, L.H22, L.H28. L.H24	8.92
		L. fermentum	L.H27	8
3	**4**	***L. rhamnosus***	L. 13, L. 15, L. 111,L. 3, L. 513, L. G3, L.G4	6.84
		L. paracasei	L. 14, L. 17, L.18,	6.47
		L. plantarum	L. 16, L. 162	5.3
4	**13**	***L. acidophilus***	L. Sh4, L. Sh41, L. Sh5, L. Sh51	5.44
5	**15**	***L. plantarum***	L. B4, L.B41, L.B411, L.B51	7.10
6	**19**	***L. plantarum***	L. A7, L. A71, L. A8	5.94

No*: Stool samples number
Age**: Age of the infants (month)

dominant lactobacillus in adults and infants, recent studies have revealed that *L. gasseri* is the most occurring lactobacillus in gasterointestinal tract of infants[23]. However, in this study the obligately homofermentative isolates were appeared to be biochemically different from *L. gasseri* strains in Xanthopulus[16] study with respect to fermentation of Raffinose and Melebiose. *L. plantarum* was found in great number in the stool samples of 1-2 year-old Estonian and Swedish infants[8] and Egyptian infants were found to harbor *L. plantarum* strains before 6 months of age[15]. In this study, *L. plantarum* strains were recovered from both younger and older infants with higher frequency in the latter. This confirms the results of another study where the higher population of *L. plantarum* strains in older infants' fecal flora were related to introduction of solid fermentative foods in their diet[3]. *L. rhamnosus* was reported to be the early colonizer of gastrointestinal tract of Swedish infants before six months of age[3]. Arici also reported the predominance of this species among the other lactobacilli in the stool samples of infants less than 2 years old[7].

In identification of *L. rhamnosus* and *L. casei* strains, the distinctive phenotypic characteristic stated for *L. rhamnosus* (such as fermentation of Rhamnose together with the ability to grow at 45°C) are special traits of this species which distinguished it from other lactobacilli, even closely related species like *L. casei*. Although Succi showed that *L. rhamnosus* flora of traditional cheese could represent a variety of morphotype[24], the unusual cell morphotype of *L. rhamnosus* and *L. casei* still leaves probability to doubt about their definite identification. In identification of *L. fermentum* and its differentiation from *L. reuteri*, the authors relied on the ability to the fermentation of Cellobiose[16]. It is most likely that this strain belongs to *L. cellobiosus*, however, as *L. cellobiosus* regarded the biotype of *L. fermentum*[17], which is reported as one of the most recovered species from gasterointestinal tract of infants[3,7,8,13], this strain also is assumed to be a strain of *L. fermrntum.*

With respect to colony morphology, it has been accepted that *L. acidohilus* forms irregular and fuzzy colonies termed X type[25]. Although, transformation of this type was reported in some studies as a bacterial response against environmental stresses, the altered colony was reverted to its original shape when the stressful components were eliminated from the media[26,27]. As all the examined strains underwent the equal condition during subculturing and experiments, transformation of some *L. acidophilus* strains is unexplainable, unless they belong to closely related species.

Due to limited size of the subjects exploited in this study much caution should be heeded

about the generalization. It can be suggested that more robust conclusion about the distribution of such positive lactoflora in Iranian infants could be probably drawn by larger-scale studies especially if their type of diet be more specified.

Conclusion

The majority of lactobacilli isolates examined in this study exhibited the principal phenotypic characteristics of lactobacilli isolated from infants' stools in other studies. Some biochemical differences were obtained between the strains of *L. acidophilus* group and some morphological peculiarities were observed among the strains of *L. paracasei* and *L. rhamnosus* in comparison to the typical strains of *L. casei* group. These differences revealed the necessity of application of complementary molecular methods for clear identification of examined *Lactobacillus* strains. At present, definite identification of all examined strains based on molecular methods is in process. Also, evaluation of probiotic potential of identified strains is of interest.

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