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Research Article

Identification and Phylogenetic Studies of a New Probiotic *Lactobacillus* spp. Egyptian Isolate Based on 16s rRNA Gene

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Abstract

Lactobacillus represents one of the most important genera of human and animal intestinal tract. They are used in dairy and non-dairy probiotics foods to restore the intestinal microflora, which provides a health benefit. *Lactobacillus* strains were phenotypically identified and described using biochemical features and kits such as the API 50 CH system. Conventional biochemical and physiological studies have certain limitations when discriminating against large numbers of isolates with similar physiological characteristics. They are generally unreliable, and consuming money, time, and effort. There are many studies have focused on the rapid identification of molecular biology techniques for the rapid identification and detection of lactobacilli. In this study, we examined five pairs of primers on complex communities to facilitate the examination of complex micro-ecosystems. The two primers Lac1-Lac2 and Joh16SI-Joh16II showed positive results with *L. casei, L. rhamnosus*, and *L. gasseri*. Their strains were isolated from raw milk and feces of breast-fed infants. The fragments obtained from DNA samples after PCR amplification were sequenced and submitted in GenBank "NCBI" under accessions number "KY123805", "KY123789" and "KY123806". As the long-term goal of this research was to obtain patent protection for *Lactobacillus* sp. isolated from Egyptian resources and using it in food and dairy industries.

Keywords: API 50 CH; GenBank; Intestinal Microflora; *Lactobacillus*; Molecular Identification; Phylogenetic; Probiotics; 16S rRNA

Introduction

Lactobacillus spp. are microorganisms with a long-documented history of use in dairy technology and as a natural preservative in dairy products due to their ability to inhibit the growth of harmful bacteria and thus increasing the shelf life of the products [1]. In addition, The interest is enhanced by proven probiotic properties that give consumers a health benefit [2]. The genus of Lactobacillus is one of the major members of lactic acid bacteria (LAB), gram-positive, catalase-negative, rods shaped, non-spore forming bacteria and produce lactic acid as the main end product during carbohydrate fermentation [3].

The members of this genus have been isolated from different sources, e.g. milk, dairy products, sewage, plants, food products, and animal intestines. Currently, this genus contains more than 180 species [4]. However, there is a large degree of heterogeneity within the members of this genus is challenging [5].

Lactobacillus classification is traditional based on morphological and physiological criteria. These conventional criteria are often time-consuming and sometimes fail to identify the bacterial accurately. Recently, the use of biochemical characteristics in addition to Kits likes API 50 CHL system and molecular methods such as polymerase chain reaction (PCR) provided a novel approach for bacterial identification [6].

Therefore, the purpose of the inquiry under way was to isolate and classify some *Lactobacillus* spp.by classical (morphology and biochemical features included API 50 CHL system) and molecular (PCR technique) methods. An additional aim was to registering patent protection for *Lactobacillus* spp. Strains are isolated locally from Egyptian resources to increase the additive value of the Egyptian microbial diversity.

Materials and Methods

Samples collection

Forty-Nine Samples were collected from different healthy mother breast milk, fresh feces of breast-fed infants, raw milk and some dairy products. The infant ages varied from 1 to 8 months. Samples were kept in sterile glass containers, transported within two hours in the icebox to the laboratory and analyzed on the same day.

Isolation of lactobacilli culture

Serial dilution of each sample in citrate buffer was plated on MRS agar medium [7], Then incubated at 37°C for 48 hours under anaerobic conditions using GENBox anaerobic kit. Obtained colony were randomly picked, streaking twice on MRS agar plates and pre-incubated under the same previous conditions, to ensure complete purification.

Identification of isolated cultures

About 90 isolates were tested for Gram-staining [8], catalase activity [9], and cell morphology. In order to obtain a complete physiological characterization of the isolates, the following properties were also examined: the formation of acid and Co_2 from glucose [10], growth at 15 and 45°C, fermentation of glucose, indol production and gelatin liquefaction [11].

API 50 CHL system

API 50 CHL medium, intended to classify the *Lactobacillus* genus and related genera. This medium allows 49 different carbohydrates to be fermented on the API 50 CHL strip. A suspension is made with the micro-organism to be evaluated in the liquid (API 50 CHL medium), and each strip tube is then inoculated with the suspension. Through incubation, the carbohydrates are fermented into acids that cause a decrease in pH, as indicated by the indicator color change. The results constitute the biochemical profile which the identification software uses to classify the strain.

Molecular biology studies (Wet lab)

- **Genomic DNA isolation and quantification:** Seven isolates of total genomic DNA were extracted using (Gene JET Genomic DNA Purification Protocol Kit#K0721-Thermo Fisher Scientific Inc.) and eight isolates were extracted using. (ZR Fungal/Bacterial DNA MiniPrep™ Catalog No D6005 –Zymo Research CORP.). The DNA extraction procedure was performed in compliance with manual of the manufacturer. B-Total DNA was visualized on 1% agarose gel to determine DNA quality. Smeared appearance samples were considered as fragmented DNA and were excluded, DNA concentration was determined using a UV Dual-Intensity Trans Illuminator and quantified using a spectrophotometer (GENWAY630).

- S**election of Primers for Lactobacillus identification:** Lactobacillus species and group-specific primers were selected based on the sequences of 16S rRNA [12-14] (Table 1).

- **PCR amplification for 16S:** Amplification reactions were prepared in total volumes of 50 µl containing 25 µl GoTaq ® Promega, a pair of specific primers at a concentration of 0.25 µmol, 2 µl Template DNA and water nuclease-free Up to 50 µl each primers. PCR amplifications were performed in Eppendorf Master Cycler Gradient using the following PCR temperature profile: denaturation cycle of 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, annealing temperature (51°C, 53°C, 55°C, 57°C, 62°C and 65°C) for 1 minute for optimum annealing temperature, DNA extension 72°C for 90 s and final cycle 72°C for 5 minutes. and then kept at 4°C until further use.

- **Amplified fragments visualization:** Agarose gel electrophoresis 1.5% (wt/vol) was used to visualize the amplified DNA fragments, gels were stained with ethidium bromide (0.5 µg/ml) and loaded directly onto the gel, DNA ladder was also loaded onto the gel for comparison of fragment size and visualized under UV light (Dual – Intensity Trans Illuminator) and quantified using spectrophotometer (GENWAY630). The PCR product containing DNA fragments with the expected size were purified using GeneJET™ PCR Purification Kit (Thermo #K0701), then subjected to sequencing.

- **Sequencing of 16S:** The species specificity of the primers Fragments of PCR products for Lac1 - Lac2 and Joh16SI-Joh-16SII primers were sent to the University of Potsdam, Institute of Biochemistry and Biology, (Potsdam, Germany) for sequencing using an ABI 3730xl DNA sequencer. The nucleotide sequences for resulted fragments were deposited to the Genbank Database in date 14 and 16 Nov 2016 and it has been named as Strain AZ1 using Bankert tool.

Bioinformatics workflow (dry lab)

- **Primer selectivity based on *in silico* studies:** Before proceeding to wet laboratory, the *in silico* have been validated. Each primer's properties including melting temperature, percentage of GC content and suitability for PCR have been described in the report. It helps us assess potential PCR primers from the list of primers selected from Previous research for all the species being studied Lactobacillus sp. *L. acidophilus, L. johnsonii, L. rhamnosus, L. reuteri.* NCBI blast primer tool http://www.ncbi.nlm.nih.gov/tools/primer was used to test the primers as it has a wide range of parameters in this process include, nr database and *Lactobacillus*.

- **Sequences submissions and accession numbers:** The sequences of this study have been submitted to the NCBI using the Bankit tool http://www.ncbi.nlm.nih.gov/BankIt/.

- **Sequences analysis:** A search was carried out using the online tool NCBI BLASTn http:/ncbi.nlm.nih.gov/BLAST/ against the database for the collection of nucleotides (nr/nt) to find Sequence Similarity. The default parameters of BLASTN have been used.

- **Phylogenetic tree construction:** A phylogenetic tree was designed based on the 16S ribosomal RNA sequence comparisons of the PCR-amplified length polymorphism and Database sequences using blast tree construct in https:/www.ncbi.nih.gov/blast/treeview based on Fast Minimum Evolution.

Species	Primer	Nucleotide sequence	*AT (C°)	PCR product(bp)	Target Sequence	References
Lactobacillus sp.	Lac1 Lac2	F AGAGTTTGATCCTGGCTCAG R GGTTACCTTGTTACGACTT	58	1523	16S rDNA	(McOrist., *et al.* 2002)
L. acidophilus	Aci I Aci II	F AGCTGAACCAACAGATTCAC R ACTACCAGGGTATCTAATCC	62	785	16S rDNA	(Walter., *et al*. 2001)
L. johnsonii	Joh 16SI Joh 16SII	F GAGCTTGCCTAGATGATTTTA R ACTACCAGGGTATCTAATCC	57	760	16S rDNA	(Walter., *et al*. 2001)
L. rhamnosus	RhaI RhaII	F CAGACTGAAAGTCTGACGG R GCGATGCGAATTTCTATTATT	55	399	16S rDNA	(Tilsala-Timisjarvi., *et al*. 1997)
L. reuteri	ReuI ReuII	F GCCGCCTAAGGTGGGACAGAT R AACACTCAAGGATTGTCTGA	55	483	16S rDNA	(Walter., *et al*. 2001)

Table 1: The five the primer sets which used in the study.

*AT(Co): Annealing Temperature.

Results and Discussion

Phenotypic identification of bacterial isolates

Like many other bacterial species, *Lactobacillus* strains have been phenotypically identified with the use of biochemical characteristics and kits such as API 50 CH system. As shown in (Table 2), the obtained data declared that 39 out of 90 isolates were rods, gram-positive and catalase-negative strains. On the other hand, four isolates from human milk samples were able to liquefy gelatin and produce Co_2 during glucose fermentation. Moreover, all isolates from faecal and raw milk samples were also able to produce acid and gas through the fermentation of glucose. In contrast, only five isolates were failed to fermentation glucose. Thus, all these isolates were completely excluded.

Continuously, in this study, four Lactobacillus strains were identified with the use of the API 50 CHL system as belonging to L. rhamnosus and L. casei from infantile faecal samples. In this connection, similar groups of Lactobacillus strains had been earlier isolated from infantile faecal specimens from other countries [15,16], although slight variations could be observed.

In addition, this investigation was also identified by the API 50 CHL system. Also, from the present data, it was of interest to notice that 3 out of 4 tested strains identified by API 50 CHL system were isolated from infantile faecal specimens, while only one strain from raw milk samples, in contrast, no strain from colostrum can be identified by API 50 CHL system. However, the same observation

Samples	No. of isolates	Morphology		Gram staining		Catalase production		Gelatin Liquefied	Indole Produced
		Rods	Cocci	+	-	+	-		
Human milk (H)	22	16	6	22	-	2	14	+ 4 - 10	-
Faeces (F)	36	18	2	20	16	2	16	-	-
Raw Milk (RM)	20	5	3	8	12	1	4	-	-
Rayeb milk (Ray)	3	2	1	3	-	-	2	-	-
Yoghurt (Y)	2	1	1	2	-	-	1	-	-
Karish cheese (k)	7	5	-	5	2	3	2	-	-
Total	90	47	13	60	30	8	39	35	35

Table 2: The morphological and biochemical tests used for identification of some isolates.

+: Positive reaction; -: Negative Reaction.

was previously mentioned by Ozgun and Vural (2011), they concluded that the higher isolation rate of Lactobacillus spp. In infantile faecal specimens than in colostrum may be due to the sterility of colostrum. The isolated Lactobacillus were identified by API 50 CHL system as L. rhamnosus (11.8%) in addition to other species, from faeces in (Figure 1, 2 and 3).

Molecular studies

- **Amplification of 16S rRNA and visualization:** Results of PCR using 16S rRNA specific primers (Lac1- Lac2) amplified two fragment 1251bp, and 1276 bp isolated from feces *L. casei* in lan1, *L. rhamnosus* in lan2 and primer (Joh16SI-Joh16SII) amplified fragment 729 bp isolated from raw milk *L. gasser*i in lan3 (Figure 4) and this result agrees with Ven-

Figure 1: Identification of *L. acidophilus* 1 by API 50 CHL system.

Figure 2: Identification of *Lactobacillus paracasei* spp paracasei 1 by API 50 CHL system.

Figure 3: Identification of *L. rhamnosus* by API 50 CHL system.

tura., *et al.* (2001). The 16S rRNA PCR amplification was thus a useful tool for bacterial species-specific typing due to the considerable variability in size and sequence between organisms.

- **Sensitivity and Specificity of the primers***: In silico* results should be validated before proceeding in the wet lab. PCR Primer Stats receives a list of PCR primers and proceeds a report describing the characteristics of each primer, including melting temperature, percentage of GC content, and PCR suitability. It helped us evaluating the potential of the PCR primers from the list of primers selected by Walter., *et al.* (2001), McOrist., *et al.* (2002), and Tilsala and Alatossava (1997) [17] as shown in (Table 3).

- After checking the specificity and efficiency of a primer, thereafter, it is validated in the wet lab. In our study, five pairs of species-specific primers were used the results showed that four primers (Aci I- Aci II) (Joh16SI- Joh16SII) (RhaI- RhaII) (ReuI- ReuII) exhibited the Sensitivity and Specificity of primers for those strains *L. acidophilus, L. johnsonii, L. rhamnosus,* and *L. reuteri.* While (Lac-1- Lac-2) primer exhibited a lot of strains (Table 4).

- **Gene Bank submissions and accession numbers:** This is the first study detailing the discovery of *L. casei, L. rhamnosus,* and *L. gasseri* from various locations in Egypt applying molecular identification from feces and raw milk analysis and followed by being sequenced and submitted to the NCBI Genbank Database with three new accession numbers (Table 5).

- We have found this strain from feces of a breastfed infant under accession numbers (123805), (123789) under name AZ1 and strain isolated from raw milk under accession (123806) under name AZ1. This confirms that *L. casei, L. rhamnosus*, and *L. gasseri* have an excellent ability to survive passage through the gastrointestinal tract in line with the results showed in suggesting that this strain has a superior ecological fitness for adaptation in various hosts and possibly different ecological niches [18]. We found that 16S rRNA sequences permitted the biodiversity assessment of populations of *Lactobacillus* present in human intestinal and raw milk samples. The 16S rRNA sequence offers insights into the exact identification and distribution of *Lactobacillus* isolates, inter-subject variation and variations between two intestinal regions of the same person in different human subjects.

- Our results showed significant similarity between16S rRNA sequences of different *Lactobacillus* spp. Present in the Gene Bank database. BLASTn search of the non-redundant Gen Bank database yielded 100 hits for sequences similar to the strains (KY123805, KY123789 and KY123806), results showed the relatively high similarity percentage of 100% with *Lactobacillus rhamnosus* strain LRB, complete genome CP016823.1 and *Lactobacillus gasseri* strain IMAUFB062 16S ribosomal RNA gene, partial sequence JQ805680.1, 99% similarity with L*actobacillus casei* ATCC 393 DNA, complete genome JQ805680.1 (Table 6).

Figure 4: PCR amplified fragments using 16S for (A). *L. gasseri* in a 1% agarose gel Lanes1-2 (M = 1kb DNA ladder) primer (Joh16SI-Joh16SII) amplified fragment 729 bp from raw milk. (B) *L. casei* lane 1 and *L. rhamnosus* lane 2(M = 5kb DNA ladder) Lanes 1-2 Primer (Lac1 –Lac2): amplified fragment 1251 bp lane 1 and Lane2 primer: amplified fragment 1276 bp from feces.

Primers	Nucleotide sequence	Length NT	GC%	TM	AT	Product length	Speaces
Lac1	F AGAGTTTGATCCTGGCTCAG	20	50.00	56.92	58	1523	*Lactobacillus sp.*
Lac2	R GGTTACCTTGTTACGACTT	19	42.11	52.20			
Aci I	F AGCTGAACCAACAGATTCAC	20	45.00	55.96	62	785	*L. acidophilus*
Aci II	R ACTACCAGGGTATCTAATCC	20	45.00	52.57			
Joh 16SI	F GAGCTTGCCTAGATGATTTTA	21	38.10	53.40	57	760	*L. johnsonii*
Joh 16SII	R ACTACCAGGGTATCTAATCC	20	45.00	52.57			
RhaI	F CAGACTGAAAGTCTGACGG	19	52.63	55.30	55	399	*L. rhamnosus*
RhaII	R GCGATGCGAATTTCTATTATT	21	33.33	52.89			
ReuI	F GCCGCCTAAGGTGGGACAGAT	21	61.90	64.26	55	483	*L. reuteri*
ReuII	R AACACTCAAGGATTGTCTGA	20	40.00	54.20			

Table 3: *In silico* analysis of selection primer sets.

AT: Annealing Temperature; MT: Malting Temperature; GC: Guanine and Cytosine Ratio.

Primer	Nucleotide sequence	Strains *Lactobacillus spp.*
Lac1	F AGAGTTTGATCCTGGCTCAG	*L. casei*
Lac2	R GGTTACCTTGTTACGACTT	*L. rhamnosus*

Table 4: *In silico* analysis of selection primer Lac1- Lac2.

No	Source	Released Data	Locus	Length	Strain	Reference strain	Accession No
1	Feces of breast-fed infant	22-NOV-2016	16S rRNA	1251 bp	AZ1	*Lactobacillus casei*	KY123805
2	Feces of breast-fed infant	15-NOV-2016	16S rRNA	1276 bp	AZ1	*Lactobacillus rhamnosus*	KY123789
3	Raw milk" from Egyptian cows	22-NOV-2016	16S rRNA	729 bp	AZ1	*Lactobacillus gasseri*	KY123806

Table 5: Sequence features and accession numbers.

S. No	Genbank Access NO.	Species of *Lactobacillus*	Max score	Total score	Query cover	E value	Identity
1	KY123805	*Lactobacillus casei* strain AZ1 16S ribosomal RNA gene, partial sequence	2311	2311	100%	0.0	100%
2	KY123789	*Lactobacillus rhamnosus* strain AZ1 16S ribosomal RNA gene, partial sequence	2357	2357	100%	0.0	100%
3	KY123806	*Lactobacillus gasseri* strain AZ1 16S ribosomal RNA gene, partial sequence	1347	1347	100%	0.0	100%
4	AP012544.1	*Lactobacillus casei* ATCC 393 DNA, complete genome	2294	11415	100%	0.0	99%
5	CP016823.1	*Lactobacillus rhamnosus* strain LRB, complete genome	2357	2357	100%	0.0	100%
6	JQ805680.1	*Lactobacillus gasseri* strain IMAUFB062 16S ribosomal RNA gene, partial sequence	1347	1347	100%	0.0	100%

Table 6: Percentages of similarity of *Lactobacillus casei* AZ1, *rhamnosus* AZ1 and *gasseri* AZ1 and other *Lactobacillus* spp. as extracted from the Databases using BLASTn.

Phylogenetic relationship of the Genus Lactobacillus spp.

The diversity of *Lactobacillus* is reflected in the considerable phenotypic and genotypic variation in genus in addition to its ecological versatility. 16S/23S rRNA gene sequence comparative analysis showed phylogenetic relations between the lactobacilli. Collins., *et al*. (1991) originally distinguished three phylogenetic groups, the *Lactobacillus delbrueckii, Lactobacillus casei–Pediococcus*, and *Leuconostoc* group. Later, he *L. casei–Pediococcus* group was divided into the *Lactobacillus buchneri* group, the Lactobacillus reuteri group, the *L. casei* group, the *Lactobacillus Plantarum* group,

and the *Lactobacillus salivarius* group as well as the L. delbrueckii group was given a new name as the *Lactobacillus acidophilus* group [19]. Currently, the *Lactobacillus* genus phylogenetic structure includes the *Lactobacillus vitulinus–catenaformis* group, *Lactobacillus perolens* group, and the Pediococcus group [20]. Our data show that 16S-rRNA sequences enable scientists to identify the relations between closely related species. In most cases, Phylogenetic evidence shows that the strains of the same species are closer than those of other species. We have indicated that the divergence rates of (16S-rRNA) sequences can distinguish among subspecies of the same species (Figure 5, 6, and 7).

Figure 5: Phylogenetic based on 16S ribosomal RNA sequences, shown the inter and intraspecifi relationships of members of *Lactobacillus casei* strain AZ1.

Figure 6: Phylogenetic based on 16S ribosomal RNA sequences, shown the inter and intraspecific relationships of members of *Lactobacillus rhamnosus* strain AZ1.

Figure 7: Phylogenetic based on 16S ribosomal RNA sequences, shown the inter and intraspecific relationships of members of *Lactobacillus gasseri* strain AZ1.

Conclusion

The *Lactobacillus casei* group comprises 3 species. This group is homogeneous because it has facultative also heterofermentative members, their GC percentages ranges from 45 to 47 mol% and the taking place peptidoglycan type is Lys-d-Asp. Furthermore, All of them are able to form L (+)-lactic acid and to produce acetoin.

The *L. casei* group includes the most recognized species of *Lactobacillus rhamnosus, Lactobacillus paracasei, L. casei,* and *Lactobacillus zeae*. In the past, this subgroup has undergone a broad taxonomic review leading to the temporary rejection of the species name *L. Parachuting*. Such species are currently still classified in their genus as separate species [21-24]. The most common foodborne lactobacilli isolates of the *L. casei* group are *L. paracasei* and *L. rhamnosus*, especially from cheese. The *L. paracasei* and *L. casei* are also present in silage and common in the gastrointestinal tract in animals and humans.

In the *L. acidophilus* group, homofermentative lactobacilli are almost exclusive. In addition to *L. acidophilus*, several other species of industrial importance such as *L. delbrueckii, Lactobacillus crispatus*, with the subspecies *bulgaricus, lactis, delbrueckii,* and *indicus, Lactobacillus helveticus, Lactobacillus johnsonii,* and *Lactobacillus gasseri* are included. *L. delbrueckii* and *L. helveticus* perform a recognized role as starters in vegetable and dairy fermentation, respectively, although some strains of the species *L. Acidophilus*, through their positive effect on intestinal flora, has long been known to play a major role in human health and nutrition.

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Conflict of Interest

All the authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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