

Molecular Characterization of Egyptian Isolates of *Lactobacillus* and *Bifidobacterium*

Hashem S.¹; H. H. Sabit²; M. Amin³; W. Tawakkol⁴; and A. F. Shamseldin⁴

¹Microbiology Dept., College of Medicine, Assiut University, Assiut, Egypt

²Microbial Genetics Dept., College of Biotechnology, Misr University for Science and Technology, Cairo, Egypt

³Microbiology Dept., College of Pharmacy, Cairo University, Cairo, Egypt

⁴Microbiology Dept., College of Pharmacy, Misr University for Science and Technology, Cairo, Egypt

Abstract: Strains of *Lactobacillus* and *Bifidobacterium* were isolated from processed milk collected in Cairo, Egypt. *Lactobacilli* was isolated on Acetate media (SL) of Rogosa and Mitchell-Weisman. While *Bifidobacterium* was isolated on DSM medium (Difco Sporulation Medium). The isolates were characterized microscopically, morphologically and by some biochemical tests. DNA was extracted from the specified isolates using (Qiagen, Germany, Cat #51306) and species-specific primers for *Lactobacillus* and *Bifidobacterium* were designed to amplify the 16S rDNA gene as a conserved region in the bacterial DNA. Elution of the target band from the gel was performed efficiently and the 16S rDNA region was subjected to sequencing using Sequencer ABI PRISM 3730XL Analyzer. The sequencing data obtained suggested that the two studied isolates were (at the genus level) designated as *Lactobacillus* and uncultured *Bifidobacterium*. When the sequencing data was aligned on <http://www.ncbi.nlm.nih.gov>, it shows 88% homology and expected value of 7e-164 to *Lactobacillus kiranofaceins* but dendrogram tree shows more homology to *Lactobacillus plantarum* family. While the other sample showed 91% homology and expected value of 3e-113 with Uncultured *Bifidobacterium* Clone R333 16S rRNA gene. [Hashem S.; H. H. Sabit; M. Amin; W. Tawakkol; and A. F. Shamseldin. Molecular Characterization of Egyptian Isolates of *Lactobacillus* and *Bifidobacterium*. Journal of American Science 2010;6(11):959-964]. (ISSN: 1545-1003).

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1. Introduction:

Probiotics are defined as live microbial food ingredients that have a beneficial effect on human health. The concept of probiotics evolved at the turn of the 20th century from a hypothesis first proposed by Nobel Prize winning Russian scientist Elie Metchnikoff (Bibel, 1988). He suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. He believed that when consumed, the fermenting bacillus (*Lactobacillus*) positively influenced the microflora of the colon, decreasing toxic microbial activities. For human adult use, this includes fermented milk products as well as over-the-counter preparations that contain lyophilized bacteria. The microorganisms involved are usually lactic acid producers such as lactobacilli and bifidobacteria. An effective probiotic should exert a beneficial effect on the host, be nonpathogenic and nontoxic, contain a large number of viable cells, it should be capable of surviving and metabolizing in the gut, also remain viable during storage and use, having good sensory properties, and finally be isolated from the same species as its intended host (Gonzalez *et al.*, 1995).

Much attention has focused on decreasing colon cancer risk through increasing intake of dietary fiber; recently, this has included interest in the consumption of prebiotics and probiotics (Brady *et al.*, 2000). Furthermore, (Balish *et al.*, 1997) reported that the probiotic bacteria manifested different capacities to adhere to epithelial surfaces, disseminate to internal organs, affect the body weight of adult mice and the growth of neonatal mice, and stimulate immune responses. Although the probiotic species were innocuous for adults, his results suggest that caution and further studies to assess the safety of probiotic bacteria for immunodeficient hosts, especially neonates, are required.

2. Materials and methods

2.1 Isolation

Milk samples were collected from Cows at Six October governorate, Egypt. Serial dilution for the samples was carried out under aseptic conditions, 100 μ l of 10⁻⁷ dilution from each sample was transferred to a Petri dish. Warm Acetate media (SL) of Rogosa and Mitchell-Weisman agar medium and DSM agar medium were poured on each plate for *Lactobacillus* and *Bifidobacterium* respectively. The

plates were then incubated under anaerobic conditions at 37 °C for 24 hours. Single colonies were examined morphologically and microscopically using gram stain. The *Lactobacillus* colonies appeared as white small colonies around 2 mm in diameter with entire margin, while the *Bifidobacterium* were punctiform cream colonies with 0.5 mm in diameter. Both isolates were able to ferment lactose, glucose and sucrose but not mannitol. Furthermore, *Bifidobacterium* was able to ferment hexose by fructose-6-phosphate phosphoketolase (F6PPK) shunt. In addition, both isolates were subjected for catalase and indol test, and negative reaction were appeared in both samples.

2.2 DNA Extraction

MRS and DSM broth media were inoculated with *Lactobacillus* and *Bifidobacterium* respectively. 1.5 ml of the overnight culture was transferred to each eppendorf tube and were centrifuged at 8000 rpm for 1 min at 4 °C. Supernatant was discarded and 1 ml of washing buffer SET (20% sucrose, 50 mM Tris-HCL and 50 mM EDTA, pH 7.6) was added to each eppendorf. Cells were re-suspended and centrifuged and the supernatant was discarded again. 100 µl of SL (SET + 25 mg/ml Lysosyme) and 10 µl of Proteinase K (10 mg/ml) was added to the cells which is then was re-suspended by the aid of vortex. Cells were incubated in water bath at 37 °C for 2 hours. 70 µl of 10% SDS was added and re-incubated in the water bath for 10 minutes. 500 µl of TE buffer (10 mM Tris HCl and 0.2 mM EDTA) was added to each eppendorf, followed by the addition of 600 µl of Phenol:Chloroform:Isoamly mixture (25:24:1 respectively). The tubes were mixed gently, and then centrifuged at 12000 rpm for 15 minutes at 4 °C. The upper aqueous phase was transferred to a clean eppendorf tube, and equal volume of cooled Isopropanol was added and re-centrifuged for 10 minutes at 12000 rpm. Supernatant was discarded and purification was applied by addition of double volume of 70% cooled Ethanol, samples were centrifuged for more 10 minutes, and then pellet was re-suspended in 100 µl of water after removing the supernatant.

2.3 Polymerase Chain Reaction (PCR)

Extracted DNA was electrophoresed in a 1.5% agarose gel (Fisher Scientific) and was subsequently visualized with UV illumination after staining with ethidium bromide. DNA concentration was measured using Spectrophotometer apparatus Biometra then DNA was diluted to 50 ng/ml. The oligonucleotide primers used in this study were purchased from LabTechnology (Promega Corp.). Primer PAF [5' AGA GTT TGA TCC TGG CTC AG

3'] position 8-27 (using the *Escherichia coli* numbering system) and 536R [5' GTA TTA CCG CGG CTG CTG 3'] position 519- 536 were used to amplify the 5' region of the 16S rDNA gene (Yeung *et al.*, 2002). PCR was performed in Biometra PCR System. For each reaction, a 50-µl reaction mixture was prepared. The reaction mixture contained 1× buffer without MgCl₂ (Promega Corp., Madison WI), 1.5 mM MgCl₂, 20 µM dNTP, 0.1 µM primers PAF and 536R, 1.5 U Taq Polymerase (Promega Corp.), and 3 µl of DNA template. The amplification program was as follows: preheating at 94°C for 2 min, followed by 40 cycles at: 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s. After these cycles, the reaction was maintained at 72°C for 7 min and then cooled to 4°C. Five microliters of the PCR products were electrophoresed in a 1.5% agarose gel and were subsequently visualized by UV illumination after ethidium bromide staining.

2.4 Sequencing of 16S rDNA gene

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using PAF Primer [5' AGA GTT TGA TCC TGG CTC AG 3'] position 8-27 and 536R [5' GTA TTA CCG CGG CTG CTG 3'] primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

3. Results and Discussion:

In the present study, molecular identification of the isolates under study (*Bifidobacterium* and *Lactobacillus*) was performed. Significant value of these strains as an immunomodulator and stimulator of immune responses and also for the fact that they are opportunistic pathogens, especially in immunodeficient hosts as Probiotics appear to be innocuous for immunocompetent hosts and bacteria closely related to probiotic species have been associated with infections in patients. For example, *Streptococcus* spp. and *Lactobacillus* spp. have been isolated from patients with heart valve replacements who have endocarditis (Balish *et al.*, 1997). Lactic acid bacteria and bifidobacteria are increasingly being administered to pregnant women and infants with the intention of improving health. Although these organisms have a long record of safe use (Morgan *et al.*, 2010).

Amplification of conserved region 16S rDNA using PAF and 536-R primers is shown in figure (1), both the target bands for *Bifidobacterium* and *Lactobacillus* were appeared around 500 bp which was in agreement with data indicated by (Yeung *et al.*, 2002) who used the same primers with 26 different strain of *Lactobacillus* and *Bifidobacterium*. On the other hand, The PCR sequencing of a 470-bp fragment of the 16S rRNA gene, using primers plb16 and mlb16 (positions 8 to 27 and 507 to 526 in the 16S rRNA gene sequence of *Escherichia coli*, respectively) was used to identify the bifidobacteria at the species level (Rodríguez *et al.*, 2009).

Sequencing result in Figure (2) of the 518 bp DNA segment shows a high GC content in the *bifidobacterium* sample which might be studied in future for possible immune-regulation activity in mammals through out the CpG island of the foreign bacterial DNA. This might be the case as many studies (Koo and Rao, 1991) examined the effects of administration of both *bifidobacteria* (*B. pseudolongum*) and 5% neosugar [fructooligosaccharide (FOS)] to female mice given DMH resulted in 50% as many AC as in control animals at 18 and 38 weeks.

Furthermore, Figure 4 shows alignment of the base sequence "FASTA format" using the blasting tool on <http://www.ncbi.nlm.nih.gov> shows 91% homology, a high score of 407 and expected value $3e-113$ with Uncultured *Bifidobacterium* sp. Clone R333 16S ribosomal DNA gene. Figure 5 demonstrates the genetic relationship between the Egyptian *Bifidobacterium* isolate with other control

samples in a dendrogram which relies on the results of the partial 16S rDNA analysis.

On the other hand, the sequence of the partial 16S rDNA gene 673 bp for the isolated *Lactobacillus* is illustrated in Figure 3. *Lactobacillus* sequence results were aligned using BLAST tool which is illustrated in Figure 5, result shows homology of 88%, 577 score and expected value $7e-164$ with *Lactobacillus kefiranofaceins* and is illustrated by a dendrogram in Figure 6.

4. Conclusion

The study describes the molecular identification of two Egyptian isolates *Bifidobacterium* and *Lactobacillus* using species specific primers for amplification of 16S rDNA and comparing them with standard strains after alignment using blast tool on <http://www.ncbi.nlm.nih.gov>, bioinformatics analysis and dendrogram study of partial 16S rDNA gene showed a homology of 91% for the *Bifidobacterium samle* with Uncultured *Bifidobacterium* sp. Clone R333 and 88% homology with *Lactobacillus kiranofaceins* for the *Lactobacillus* isolate.

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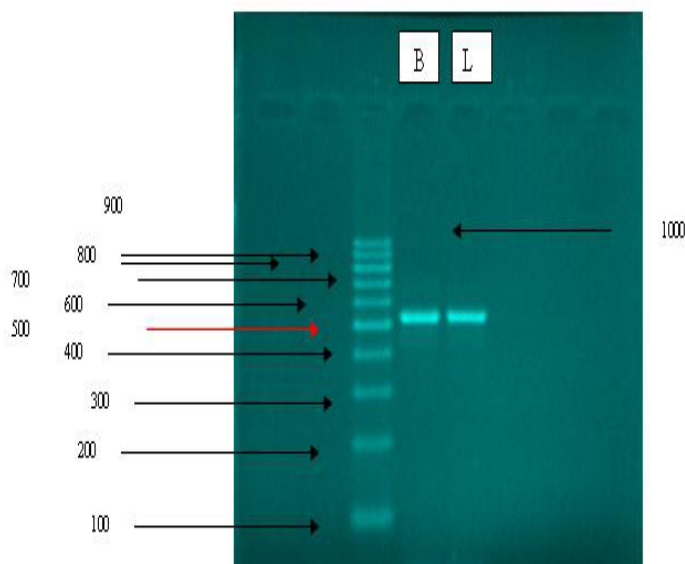


Figure 1: 16S rDNA amplification using F-PAF and 536-R primers, 16S rDNA is appeared at 518 bp and 534 bp for *Bifidobacterium* (B) and *Lactobacillus* (L) respectively.

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF429517.1	Lactobacillus kefiranofaciens ATCC 43761 16S ribosomal F	577	577	70%	7e-164	88%
DQ298155.1	Lactobacillus sp. SWM_Isolation_3 16S ribosomal RNA gen	555	555	74%	3e-157	86%
DQ141558.2	Lactobacillus plantarum strain HDRS1 16S ribosomal RNA	468	468	67%	4e-131	85%
DQ486145.1	Lactobacillus plantarum strain SFCEB2-7c 16S ribosomal RN	468	468	67%	4e-131	85%
AY230227.1	Lactobacillus plantarum strain K14 16S ribosomal RNA gen	468	468	67%	4e-131	85%
AY230221.1	Lactobacillus plantarum strain H19 16S ribosomal RNA gen	468	468	67%	4e-131	85%
AY230220.1	Lactobacillus plantarum strain H 16S ribosomal RNA gene,	468	468	67%	4e-131	85%
AB365977.1	Lactobacillus capillatus gene for 16S rRNA, partial sequenc	466	466	74%	1e-130	83%
AB112083.1	Lactobacillus plantarum gene for 16S ribosomal RNA, parti	466	466	67%	1e-130	85%
AY230225.1	Lactobacillus plantarum strain H8 16S ribosomal RNA gene	464	464	67%	5e-130	85%

Figure 4: Alignment of partial 16S rDNA gene using blast for Lactobacillus isolate.

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ518641.1	Uncultured Bifidobacterium sp. clone R333 16S ribosomal	407	407	57%	3e-113	91%
FJ518676.1	Uncultured Bifidobacterium sp. clone R330 16S ribosomal	287	287	44%	3e-77	88%
AB437351.1	Bifidobacterium psychraerophilum gene for 16S ribosomal	265	265	81%	2e-70	78%
NR_029065.1	Bifidobacterium psychraerophilum strain T16 16S ribosom	250	250	79%	5e-66	78%
AB507085.1	Bifidobacterium breve gene for 16S rRNA, partial sequenc	246	246	86%	6e-65	77%
FJ518643.1	Uncultured Bifidobacterium sp. clone R339 16S ribosomal	246	246	43%	6e-65	86%
AY735402.1	Bifidobacterium breve strain BR2 16S ribosomal RNA gene	246	246	86%	6e-65	77%
AB507110.1	Bifidobacterium sp. JCM 7013 gene for 16S rRNA, partial s	241	241	86%	3e-63	77%
EF589112.1	Bifidobacterium longum strain IDCC 4101 16S ribosomal F	241	241	82%	3e-63	77%
AY850360.1	Bifidobacterium breve strain BGM6 16S ribosomal RNA ge	241	241	86%	3e-63	77%

Figure 5: Alignment of partial 16S rDNA gene using blast for Bifidobacterium isolate.



Figure 6: Dendrogram analysis for partial 16S rDNA gene of Bifidobacterium isolate, main tree.

Corresponding author

Hashem S.

¹Microbiology Dept., College of Medicine, Assiut University, Assiut, Egypt**5. References:**

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