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*Full Length Research Paper*

# Culture-dependent and culture-independent techniques to identify lactic acid bacteria in fermented products

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**Ten commercially available probiotic products were collected from the local market of Lahore, Pakistan; including dairy, freeze dried products and fruit drinks. These products were examined by using culture dependent methods, SDS-PAGE as well as by visualization of denaturing gradient gel electrophoresis (DGGE) band profiles under UV light. Identical results of the DGGE and culture-dependent analysis of the 10 probiotic products were found. A DGGE analysis of five products (Nestle yogurt, Haleeb flavored yogurt, Haleeb yoghurt, Freeze-dried product and Nestle fruit yogurt) detected *Bifidobacterium lactis, Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Bifidobacterium lactis, Lactobacillus casei, Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bacillus cereus* and *Streptococcus thermophilus* that were the same as with conventional isolation procedures. For the remaining five products (Nestle nido, Haleeb Xtra energy milk, Haleeb Labban, Haleeb chadder cheese, and freeze-dried culture), DGGE analysis was able to detect more species (*L. delbrueckii* subsp. *bulgaricus, L. rhamnosus, L. acidophilus*, *L. casei,* and *L. acidophilus)* than were recovered by culture-dependent methods. The present study has proved that the PCR-DGGE and and phenotypic identification methods may result in a very powerful tool for both qualitative and quantitative analyses of all kinds of (bacterial) fermentation products.**

**Key words:** Probiotic products, polyerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method, culture-dependent analysis.

## INTRODUCTION

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (Liaskovskii and Podgorskii, 2005). Lactic acid bacteria (LAB) are the most common typeS of microbes used. LAB has been used in the food industry for many years, because they are able to convert sugars (including lactose) and other carbohydrates into lactic acid. This not only provides the characteristic sour taste of fermented dairy foods such as yogurt, but also, by lowering the pH, and may create fewer opportunities for spoilage organisms to grow, hence creating possible health benefits by preventing gastrointestinal infections (Nichols, 2007). Strains of the genera *Lactobacillus* and *Bifidobacterium*, are the most widely used probiotic bacteria (Tannock, 2005; Ljungh, 2009). LAB is thought to have several presumably beneficial effects on immune function. They may protect against pathogens by means of competitive inhibition (by competing for growth) and there is evidence to suggest that they may improve immune function by increasing the number of IgA-producing plasma cells, increasing or improvingphagocytosis as well as increasing the proportion of T lymphocytes and natural killer cells (Reid, 2006; Ouwehand and Vesterlund, 2003). Probiotics have been shown to possess inhibitory activities

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towards the growth of pathogenic bacteria. This inhibition could be due to the production of inhibitory compounds such as bacteriocins or reuterin, hydrogen peroxide, the alteration of pH values by the production of organic acids and competitive adhesion to the epithelium (Kolida et al., 2006). Yogurt, cheese and fermented milk products are considered among primary food sources of probiotics. However, there is some debate about whether dairy product starter cultures such as *lactobacillus bulgaricus* and *Streptococcus thermophilus* should be considered probiotics. While traditional starter cultured dairy products are selected for their ability to rapidly produce desirable organoleptic qualities of cultured dairy products, the probiotic bacteria should be selected for potential to provide specific health or nutritional benefits following combustion (Gilliland and Walker, 1990). In the selection of microbial strains for probiotic use, several criteria must be considered, which include bio-safety aspects, production and processing aspects, the method of administering the probiotic, the location in the body where the microorganisms of the probiotic product must be active, survival and/or colonization in the host, and the tolerance for bile (Fuller, 1991; Gilliland and Walker, 1990). LAB have generally been considered as good probiotic organisms and the genus currently being used in probiotic preparationsare*Lactobacillus*,*Bifidobacterium*and*Streptococcus* (Jindal et al., 2011; O'Sullivan et al., 2005). Nowadays, the main focus for the identification has moved from phenotypic to genotypic methods as they yield more sensitive and accurate results, as reported for lactic acid bacteria by several authors (LICK, 2003; Callon et al., 2004). These methods were to be applied to a set of *Lactobacillus* isolates to be incorporated into probiotic feeds.

In the present study, ten commercially available probiotic products were collected from local market of Lahore, Pakistan. These products are examined by using different dehydrated cultures as well as by visualization of DGGE band profiles under UV light.

## MATERIALS AND METHODS

### Isolation of bacterial strains

Ten commercially available probiotic products were collected from local market of Lahore, Pakistan; including two freeze-dried products, seven dairy products and one fruit drink. All collected products were analysed by using a set of four isolation culture media under standardized cultivation conditions. For isolation of *Lactobacillus* and *Lactococcus* strains, De Man-Rogosa-Sharpe agar (MRSA) (catalog no. CM361; Oxoid, Basingstoke, United Kingdom) was used, whereas streptococci and enterococci were isolated on M17 medium (catalog no. CM785; Oxoid) and on kanamycin esculin azide agar base (catalog no. CM591; Oxoid), respectively. For isolation of bifidobacteria, *trans*-galactooligosaccharide medium was used; this medium contained 10 g of Trypticase soy broth (catalog no. 81-1768-0; Becton Dickinson, Sparks, Md.), 1 g of yeast extract (catalog no. L21; Oxoid), 3 g of $KH_2PO_4$ (catalog no. 1627; Vel, Leuven, Belgium), 4.8 g of $K_2HPO_4$ (catalog no. 1628; Vel), 3 g of $(NH_4)2SO_4$ (catalog no. 1.01217.1000; Merck, Darmstadt, Germany), 0.2 g of $MgSO_4 \cdot 7H_2O$ (catalog no. 1433; Vel), 0.5 g of L-cysteine hydrochloride (catalog no. C4820; Sigma, Bornem, Belgium), 15 g of sodium propionate (catalog no. P1880; Sigma), 10 g of *trans*-galactooligosaccharides (Honsha, Tokyo, Japan), and 15 g of agar (catalog no. L11; Oxoid) dissolved in 1,000 ml of distilled water. Identification of the isolates was performed by using SDS-PAGE separation of extracted cellular proteins (Temmerman et al., 2003).

### SDS-PAGE

One dimensional sodium dodecylsulfatepolyacrylamide gel electrophoresis was carried out (Laemmli, 1970). Ten percent of resolving slab gels were used (16 × 16 × 0.2 cm). Samples were prepared for electrophoresis by mixing 10 µl of extracted cells, 2.5 µl of 2-mercaptoethanol, and 7.5 µl of 0.002% bromophenol blue in 0.0625 M tris-HC1 (pH 6.8), containing 10% glycerol and 2% SDS. All protein staining were performed using Comassie Blue (Hames, 1998).

In order to verify the reliability of the DNA extraction protocol for probiotic products and to verify the identification potential of DGGE, cell suspensions of type strains were made in order to simulate the species compositions of the products. These cell suspensions were prepared by harvesting half a loop of cells with a sterile iron loop from a freshly grown pure culture on MRSA (catalog no. CM 361; Oxoid) and homogeneously suspending the cells in 10 ml of peptone physiological solution (PPS) (0.1% [wt/vol] peptone [catalog no. L37; Oxoid], 0.85% [wt/vol] NaCl.

### DNA extraction

Extraction of total bacterial DNA was followed as described by Pitcher et al. (1989) with slight modifications depending on the type of product. For dairy products, 1 ml of product was centrifuged for 10 min at 13,000 rpm in a 5804R centrifuge (Eppendorf, Hamburg, Germany); then the supernatant was removed, and the pellet was resuspended in 1 ml of Tris-EDTA (TE) buffer. Because of the large fruit content in the fruit drink, 50 ml of the drink was centrifuged for 2 min at 1,000 rpm, after which 1 ml of the top liquid was removed and centrifuged for 10 min at 13,000 rpm. After removal of the supernatant, the remaining pellet was dissolved in 1 ml of TE buffer. In the case of the capsule-type products, the content of one capsule, corresponding to approximately 100 mg, was dissolved in 10 ml of sterile PPS and softly shaken until a homogeneous suspension was obtained. One milliliter of this suspension was transferred to an Eppendorf tube and centrifuged for 10 min at 13,000 rpm, after which the supernatant was removed and the remaining pellet was suspended in 1 ml of TE buffer overnight, after which an RNA-digesting step was performed by adding 35 µl of an RNase solution (10 mg of RNase [catalog no. 34390; Serva] in 1 ml of Milli-Q water). Finally, 8 µl of the DNA solution was mixed with 2 µl of loading dye (4 g of sucrose and 2.5 mg of bromophenol blue dissolved in 6 ml of TE buffer) and electrophoresed on a 1% (wt/vol) agarose gel in 1 µL TAE buffer (catalog no. 161-0773; Bio-Rad, Hercules, Calif.) for 30 min at 100 V to verify the DNA extraction. The quality of the DNA samples was verified by spectrophotometric measurements at 260, 280 and 234 nm (Temmerman et al., 2003).

### Polymerase chain reaction

Polymerase chain reaction (PCR) was performed with a *Taq* polymerase kit (Applied Biosystems, Foster City, Calif.). The primers used in this study were those described by Muyzer et al. (1993), which amplify the V3 region of bacterial 16S rDNA. Forward

**Table 1.** Overview of probiotics strains.

| Brand name | Results of SDS-PAGE | Results of PCR-DGGE |
|---|---|---|
| Nestle Nido | *Lactococcus lactis* | *Lactococcus lactis*<br>*Lactobacillus delbrueckii* subsp. *bulgaricus* |
| Nestle yogurt | *Bifidobacterium lactis, Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *Bulgaricus, Bifidobacterium lactis*, | *Bifidobacterium lactis, Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *Bulgaricus, Bifidobacterium lactis*, |
| Haleeb yogurt | *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, | *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, |
| Haleeb Flavoured yogurt | *Lactobacillus casei* | *Lactobacillus casei* |
| Haleeb chadder cheese | *Lactobacillus plantarum* | *Lactobacillus plantarum* |
| Haleeb labban | *Lactococcus lactis*, *Enterococcus faecium*, | *Lactococcus lactis*, *Enterococcus faecium*, *Lactobacillus acidophilus*, |
| Haleeb Xtra energy milk | *Lactobacillus helveticus* | *Lactobacillus helveticus*,<br>*Lactobacillus rhamnosus* |
| Freeze dried product | *Bacillus cereus* | *Bacillus cereus* |
| Freeze dried culture | Yeast | *Lactobacillus acidophilus* |
| Nestle fruit yogurt | *Lactobacillus acidophilus*, *Streptococcus thermophilus*, | *Lactobacillus acidophilus*, *Streptococcus thermophilus*, |

primer F357-GC contained a GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3') and had the following sequence: 5'-GC clamp-TACGGGAGGCAGCAG-3'. Reverse primer 518R had the following sequence: 5'-ATTACCGCGGCTGCTGG-3' (Temmerman et al., 2003). The PCR mixtures (50 µl) contained 6 µl of 10 µl PCR buffer containing 15 mM $MgCl_2$, 2.5 µl of bovine serum albumin, 2.5 µl of a deoxynucleoside triphosphate preparation (containing each deoxynucleoside triphosphate at a concentration of 2 mM), 2 µl of each primer, 0.25 µl of *Taq* polymerase (5 U/µl ), 33.75 µl of sterile Milli-Q water, and 1 µl of a 10-fold-diluted DNA solution. The following PCR program was used: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 45 s and extension at 72°C for 1 min; and final extension at 72°C for 7 min, followed by cooling to 4°C. The PCR was verified by mixing 8 µl of PCR product with 2 µl of loading dye and electrophoresing it on a 2% (wt/vol) agarose gel for 30 min at 100 V flanked by the EZ Load 100-bp molecular ruler (catalog no. 170-8352; Bio-Rad).

#### DGGE analysis

PCR products were analyzed on DGGE gels by using a protocol based on the protocol of Muyzer and Smalla (1998) with the following modifications: The polyacrylamide gels (160 by 160 by 1 mm) consisted of 8% (vol/vol) polyacrylamide in 1 µl TAE buffer. By diluting a 100% denaturing polyacrylamide solution (containing 7 M urea and 40% formamide with a polyacrylamide solution containing no denaturing components, polyacrylamide solutions with the desired denaturing percentages were obtained. In this study, two types of denaturing gradients were used, namely, a 35 to 70% gradient. The 24-ml gradient gel was cast by using a gradient former (catalog no. 165-4120; Bio-Rad) and a pump (catalog no. 731-8142; Bio-Rad) set at a constant speed of 5 ml/min. The denaturing gels were allowed to polymerize for 3 h, after which a 5-ml nondenaturing stacking gel containing a 16-well comb was poured on top. After 1 h of polymerization, PCR samples were loaded into the wells and electrophoresis was performed for 16 h at 70 V in 1 µl TAE buffer at a constant temperature of 60°C by using the Dcode system (catalog no. 170-9081; Bio-Rad). The gel was stained with ethidium bromide (50µl of ethidium bromide in 500 ml of TAE buffer) for 1 h; this was followed by visualization of DGGE band profiles under UV light.

## RESULTS AND DISCUSSION

Results of the isolation and identification of probiotic strains from the dairy and freeze dried products tested are shown in Table 1. Four selective isolation media were selected for isolation, and this was followed by identification based on SDS-PAGE separation of whole-cell protein extracts and comparison of the species-specific patterns with a laboratory-based identification library (Temmerman, 2004). The colony counts on the media used were substantially lower in the case of the freeze-dried product; the yields were between $10^5$ and $10^7$ CFU/g of product, while the yields for the dairy products were between $10^7$ and $10^9$ CFU/ml.

For PCR-DGGE analysis, total bacterial DNA was extracted directly from the product by adding lysozyme and a number of centrifugation steps (Pitcher et al., 1989). The PCR method used in this study can amplify the V3 region of the 16S rDNA of all samples tested. For each of the 10 probiotic products, a 35 to 70% denaturant DGGE gel was used, the amplicons, an artificial mixture of type strains, the probiotic selected products and individual type strains of the species claimed on the label were loaded (Figure 1). Identification was performed on the basis of band position with those of reference pattern followed by identified strains present in newly built BN database. These identities were verified by electrophoresing the V3 amplicons of type strains or isolates originating

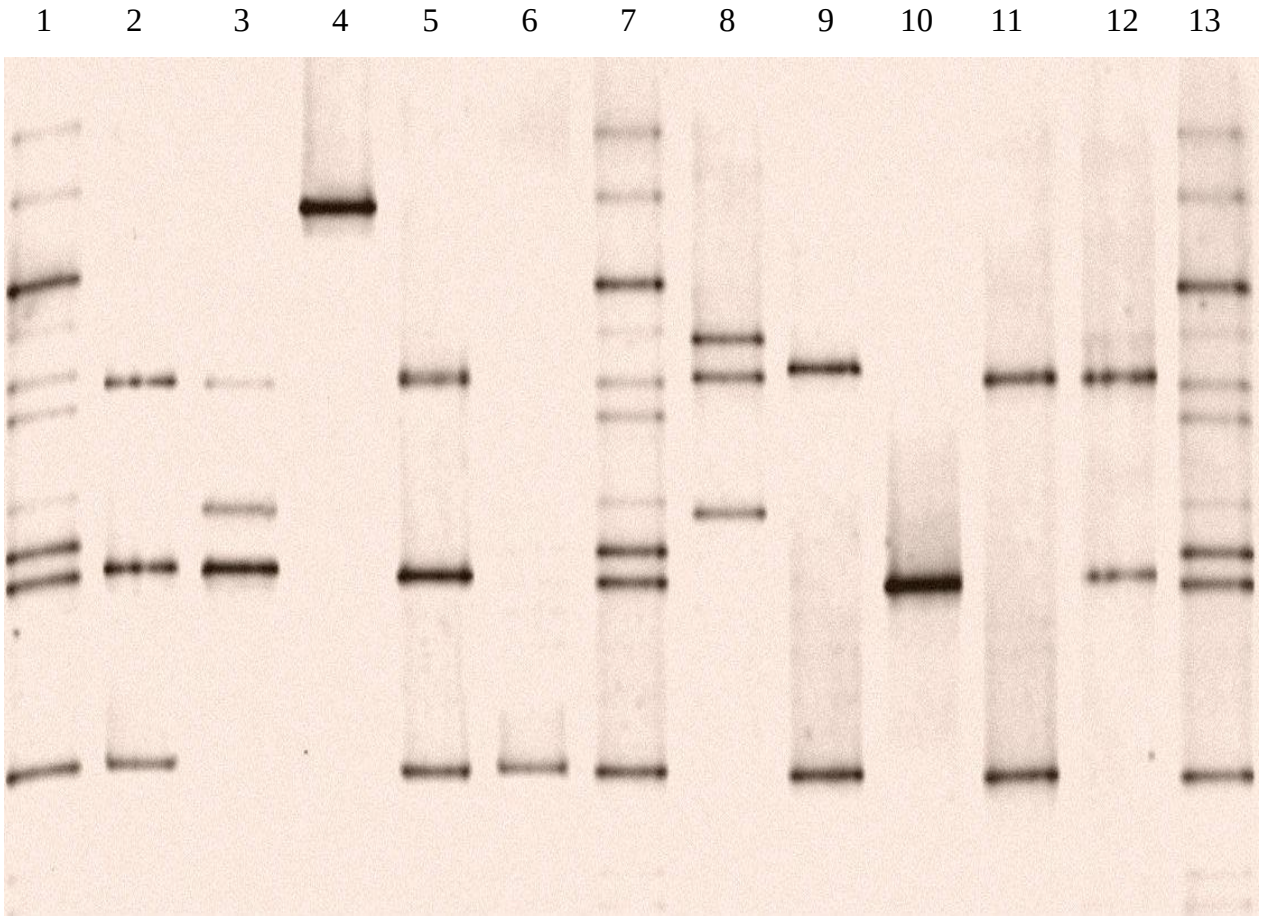


**Figure 1.** Bands of DGGE gel. Lanes 1, 7 and 13 shows the reference pattern (*Lactobacillus helveticus, Lactobacillus casei Lactobacillus rhamnosus, Lactococcus lactis, Lactobacillus acidophilus, Enterococcus faecium, Lactobacillus plantarum, Streptococcus thermophilus, Bacillus cereus,* Yeast); lane 2, Nestle nido; lane 3, Nestle yogurt; lane 4, Xtra energy milk; lane 5, Haleeb yogurt; lane 6, Haleeb flavoured yogurt; lane 8, Haleeb Labban; lane 9, Haleeb chadder cheese; lane 10, Freeze-dried product; lane 11, Freeze-dried culture; lane 12, Nestle fruit yogurt.

from the culture dependent analysis on a DGGE gel next to the probiotic product amplicons (Temmerman, 2004).

Identical results of the DGGE and culture-dependent analysis of the 10 probiotic products were found. But DGGE analysis of five products (Nestle yogurt, Haleeb flavoured yogurt, Haleeb yoghurt, freeze-dried product, and Nestle fruit yogurt) detected the same species that were detected with conventional isolation procedures. For the remaining five products (Nestle nido, Haleeb Xtra energy milk, Haleeb Labban, Haleeb chadder cheese and freeze-dried culture), DGGE analysis was able to detect more claimed species than were recovered by isolation and for freeze-dried culture; species other than those mentioned on the label which were isolated and identified.

The detection limit of the DGGE method was also determined by preparing 10-fold serial dilutions of a pure culture of *Lactobacillus rhamnosus* LMG 18243 in PPS (Temmerman et al., 2003). After 100 µl of each dilution was plated on MRSA and incubated for 48 h at 37°C aerobically, DNA was extracted from the dilution, and PCR-DGGE analysis was performed. We found that this technique produced a clear band at dilutions corresponding to concentrations down to $10^4$ CFU/ml. Two different batches of each ten product were analyzed and identical results were found in each case.

For detection and identification of the strains in probiotic products, the DGGE method was compared with a culture-dependent procedure. In DGGE method, a reliable DNA extraction and PCR analysis and identification of DGGE bands cannot be performed without gel extraction and sequencing (Ercolini et al., 2001). By using a reference pattern included in each gel and the BN software, it was possible to create a database containing all band positions for type strains representing probiotic species. Following digital normalization of the gels by comparison of the reference patterns with the standard pattern in the database, it was possible to assign an identity to each band in a band pattern representing a probiotic product. This identification based on DGGE could be confirmed by co-electrophoresing amplicons of pure cultures by protein profiling. Furthermore, multiple probiotic isolates belonging to one species produced bands

whose positions coincided with the band positions of the type strain amplicon, indicating that band patterns are species specific. However, in the case of some phylogenetically closely related species (Schleifer, 1995), the differences in band positions between two species may sometimes be too small on a 35 to 70% denaturing gel to obtain clear-cut identification. These isolated stains of probiotics can be used in improving intestinal microbial balance, which play role in inhibiting pathogens and toxin producing bacteria (Hamilton-Miller, 2003). Ashmaig et al. (2009) used another powerful tool, random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR), for the determination of the genetic relationships of the lactic acid bacteria, the results demonstrated a distinction comparative genetic clusters and their pattern was greatly related to the clustering obtained with the API 38 CHL group identification (Ashmaig et al., 2009). Today, specific health effects are being investigated and documented including alleviation of chronic intestinal inflammatory diseases (Mach, 2006), prevention and treatment of pathogen-induced diarrhea (Yan and Polk, 2012), urogenital infections (Reid, 2012) and atopic diseases (Vanderhoof, 2008). Figure 1 shows the V3 amplicons of 10 probiotic products.

## Conclusion

The PCR-DGGE analsis for the fermented and probiotic products is rapidly becoming one method of choice for exploring the microbial purity of the products. However, the challenge remains primarily in the failure to detect species that are present at lower levels. In this regard, it can be seriously questioned whether organisms present at such low levels can exert any significant probiotic effect. But the combination of culture dependent and PCR-DGGE method may result in a more reliable and powerful tool for both qualitative and quantitative analyses of all kinds (bacterial) of fermentation products.

### REFERENCES


Ashmaig A, Hasan A, El Gaali E (2009). Identification of lactic acid bacteria isolated from traditional Sudanese fermented camel's milk (Gariss). Afr. J. Microbiol. Res. 3(8):451-457.

Callon C, Millet L, Montel MC (2004). Diversity of lactic acid bacteria isolated from AOC Salers cheese. J Dairy Res 71(2):231-244

Ercolini D, Moschetti G, Blaiotta G, Coppola S (2001) The Potential of a Polyphasic PCR-DGGEApproach in Evaluating Microbial Diversity of Natural Whey Cultures for Water-Buffalo Mozzarella Cheese Production: Bias of Culture-Dependent and Culture-Independent Analyses. Syst. Appl. Microbiol. 24(4):610-617.

Fuller R (1991). Probiotics in human medicine. Gut 32(4):439-442

Gilliland S, Walker D (1990). Factors to Consider When Selecting a Culture of< i> Lactobacillus acidophilus</i> as a Dietary Adjunct to Produce a Hypocholesterolemic Effect in Humans. J. Dairy Sci. 73(4):905-911

Hames BD (1998). Gel Electrophoresis of Proteins: A Practical Approach: A Practical Approach, vol 197. Oxford University Press,

Hamilton-Miller JM (2003). The role of probiotics in the treatment and prevention of Helicobacter pylori infection. Int. J. Antimicrob. Agents 22(4):360-366

Jindal G, Pandey RK, Agarwal J, Singh M (2011). A comparative evaluation of probiotics on salivary mutans streptococci counts in Indian children. European archives of paediatric dentistry. J. Eur. Acad. Paediatrc Dentistry 12(4):211-215

Kolida S, Saulnier D, Gibson G (2006). Gastrointestinal microflora: probiotics. Adv Appl Microbiol 59:187-219

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680-685

Liaskovskii TM, Podgorskii VS (2005). [Assessment of probiotics according to the international organizations (FAO/WHO)]. Mikrobiolohichnyi zhurnal 67 (6):104-112.

LICK S (2003). Typing systems for lactobacilli. Milchwissenschaft 58(5-6):256-260

Ljungh Å (2009). Lactobacillus molecular biology: from genomics to probiotics. Caister Academic Pr,

Mach T (2006). Clinical usefulness of probiotics in inflammatory bowel diseases. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society 57(9):23-33

Muyzer G, Smalla K (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73(1):127-141

Nichols AW (2007) Probiotics and athletic performance: a systematic review. Current sports medicine reports 6(4):269-273.

O'Sullivan GC, Kelly P, O'Halloran S, Collins C, Collins JK, Dunne C, Shanahan F (2005). Probiotics: an emerging therapy. Curr Pharm Des 11(1):3-10

Ouwehand A, Vesterlund S (2003). Health aspects of probiotics. IDrugs. Invest. drugs J. 6(6):573-580.

Pitcher D, Saunders N, Owen R (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8 (4):151-156

Reid G (2006). Probiotics to prevent the need for, and augment the use of, antibiotics. Journal canadien des maladies infectieuses et de la microbiologie medicale / AMMI Canada. Canad. J. infect. diseases Med. Microbiol. 17(5):291-295

Reid G (2012). Microbiology: Categorize probiotics to speed research. Nature 485(7399):446. doi:10.1038/485446a

Schleifer KH (1995). Phylogeny of the genus Lactobacillus and related genera. Syst. Appl. Microbiol. 18:461-467.

Tannock GW (2005). Probiotics and prebiotics: scientific aspects. Caister Academic Pr,

Temmerman R (2004). Probiotics--consensus of analysis. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde 94(5):314-316.

Temmerman R, Scheirlinck I, Huys G, Swings J (2003). Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. Appl Environ Microbiol 69(1):220-226.

Vanderhoof JA (2008). Probiotics in allergy management. J. pediatric gastroenterol. Nutr. 472):38-40. doi:10.1097/01.mpg.0000338810.74933.c1.

Yan F, Polk DB (2012). Characterization of a probiotic-derived soluble protein which reveals a mechanism of preventive and treatment effects of probiotics on intestinal inflammatory diseases. Gut microbes 3(1):25-28. doi:10.4161/gmic.19245.