Probiotic Lactobacilli Fermented Dairy Products

Subjects: Microbiology

Contributor: Hamid B. Ghoddusi

A selection of 36 commercial probiotic fermented dairy products from UK and Europe markets were evaluated for the numbers, types, and viability of Lactobacillus strains against the stated information on their packages. A comparative study was carried out on selectivity of MRS-Clindamycin, MRS-Sorbitol, and MRS-IM Maltose, to select the right medium for enumeration of probiotic Lactobacillus.

Keywords: probiotic ; lactobacilli ; fermented dairy product ; identification ; enumeration ; rep-PCR

1. Introduction

Certain dairy products are vehicles by which consumers receive adequate counts of probiotic lactobacilli [1]. Probiotic effects are dependent on the number of viable microbial cells that reach the human gut [2]. Therefore, their viability in the product is considered as an important prerequisite for achieving health effects.

There are various reports regarding the adequate number of probiotic microorganisms in different products in order to ensure the probiotic effects. The recommended quantity of probiotic lactobacilli that needs to be consumed for a health benefit varies in different studies [3]. Some of the suggested minimum levels of viable cells in dairy products are 10^5 CFU/g [4], 10^6 CFU/g [5][6], and 10^7 CFU/g [7]. It is not simple to keep a high number of viable probiotic bacteria in fermented milk throughout the shelf life, because their viability in the product matrix is influenced by numerous factors. Such parameters include temperature of storage condition, hydrogen peroxide (H_2O_2), which might be produced by other existing bacteria, dissolved oxygen content due to process conditions, pH of the final product and, finally, strain variation, which may be considered the most important factor for the survival of probiotic cultures in the final product [8].

Probiotic lactobacilli are incorporated alone or in combination with other commercial cultures into specific dairy products. Interactions between microorganisms in cocultured products cause difficulties in enumeration. *Lactobacillus acidophilus, Lactobacillus casei,* and *Bifidobacterium lactis* are the most frequently used strains in commercial probiotic products [9].

In the past few decades, many selective/differential media have been developed for accurate enumeration of *Lactobacillus* spp. in fermented milks. However, due to presence of closely related species of *Lactobacillus* spp. in probiotic products, the differential enumeration seems challenging and relies directly on differences in colonial morphology [10].

There are also various instructions regarding the probiotic enumeration, but only a few are official protocols for lactobacilli, for example, ISO (2006). Enumeration in cocultured products is more complicated than in products made with single culture. In mixed cultures, inhibitory agents are needed to suppress the interfering species in order to recover the target lactobacilli. However, one real concern is that some culture media that contain antibiotics might also restrict the growth of target lactobacilli, and the counts may not be representative of the real number of viable cells present in the product [11]. On the other hand, some antibiotics cannot inhibit the growth of all nontarget bacteria [12]. Several reports have revealed the misidentification of a number of strains belonging to some lactobacilli [13][14].

The probiotic ability is often strain dependent and, therefore, accurate detection and identification of probiotic lactobacilli is required. Characteristics including phenotype, physiological and biochemical features, and sequence comparisons of 16S rRNA gene have been suggested to make the identification of *Lactobacillus* species more reliable [15]. There are, however, taxonomic dispute and ambiguity among some lactobacilli due to the differences at nucleotide level in the 16S rRNA gene [16]. It is therefore hard to differentiate between some species and strains of lactobacilli [17], and some closely related groups of lactobacilli species are indistinguishable based on phenotype. Molecular identification methods, on the other hand, have proven to be consistent, rapid, reliable, and reproducible, compared to phenotypic methods. For example, species-specific oligonucleotide probes have been employed to identify various *Lactobacillus* species [18]. Most genetic probes have been designed based on 16S rRNA or 23S rRNA genes [19].

In general, there are some ambiguities in differentiation of specific lactobacilli. According to the study by Singh et al. (2009), there are similarities at nucleotide level in the 16S rRNA gene in some lactobacilli, such as *Lb. acidophilus*, *Lb. casei*, *Lb. plantarum,* and *Lb. delbrueckii*, making it hard to distinguish them in a mixed culture. It has been reported that sometimes *Lb. gasseri* and *Lb. johnsonii* are difficult to differentiate from each other, even by molecular methods [20]. *Lactobacillus plantarum* and *Lb. pentosus* have greater than 99% similarity with only 0.3% difference in their 16S rRNA sequences [21]; however, some alternative molecular markers have been used for discrimination among these species.

Recent research into the relatedness of species in the *Lb. acidophilus* group has used sequence analyses of genes such as 16S rRNA, *rpoA*, *pheS* [22], *groEL* [23], and *tuf* [24].

The aim of the work described in this research was to isolate, enumerate, and identify *Lactobacillus* spp. in commercial probiotic dairy products from the UK and European supermarkets using genotyping methods. In addition, accuracy of the label descriptions for fermented milk products was assessed.

The study was carried out before the introduction of the new taxonomy for the *Lactobacillaceae* family in April 2020, and all the original old bacterial names kept unchanged.

2. Discussion

The use of food as a carrier for probiotic organisms is of considerable interest to food manufacturers due to the claimed health-associated benefits of probiotics. However, maintaining high numbers of viable probiotics in fermented milks is not easy, and a large quantity of probiotic cultures is needed to compensate for the likely losses of probiotics during the shelf life [25]. Procedures for enumeration of lactobacilli have not been properly defined. Such a situation causes difficulties in quality control of the probiotic products containing *lactobacillus* species using the conventional enumeration technique. The suitability of various media to selectively enumerate lactobacilli has been examined in different studies. Although there are several elective/selective media for isolation of lactobacilli, the levels of recovery of the lactobacilli are discordant with each other.

Oberg et al. (2011) reported that while MRS-Sorbitol is a medium designed for *Lb. acidophilus* in which sorbitol is the sole sugar, *Lb. casei* can also grow on the medium, although only at elevated incubation temperature (42 °C). At this temperature, the MRS-Sorbitol medium gave higher bacterial counts compared to the *Lb. casei* specific medium (*Lactobacillus casei* agar), indicating that it could be used to obtain the total LAB count at different temperature [26]. However, in our study, colonies of target strains were recovered at 37 °C on MRS-Sorbitol agar. Due to the high recovery, no other recovery temperatures were employed.

MRS-Sorbitol demonstrated higher viable counts than MRS-Clindamycin, suggesting that MRS-Sorbitol might allow the growth of additional LAB. Shah (2000) stated that MRS-Sorbitol agar could not be used for selective enumeration of *Lb. casei* and *Lb. acidophilus* in products containing both bacteria.

This study also reports that MRS-IM Maltose is not an ideal choice for selective enumeration of lactobacilli since the recovery was low compared with other MRS variants.

MRS-Clindamycin has been proposed for enumeration of lactobacilli in different studies [10][11]. Furthermore, the International Organization for Standardization (ISO) (2006) recommended MRS-Clindamycin agar for the enumeration of *Lb. acidophilus* in dairy products in the presence of other probiotics including other lactobacilli, streptococci, and bifidobacteria [11]. Simplicity of medium preparation and availability of the antibiotic supplement led to its consideration as the preferred medium compared to the other selective media. Moreover, for *Lb. casei* to grow on MRS-Sorbitol, the incubation temperature should be raised to 42 °C, therefore it is impossible to have differentiation on one medium and at one incubation temperature [26]. Hence, in our research, MRS-Clindamycin was considered as a reliable medium to selectively enumerate *Lactobacillus spp.* in fermented dairy products. Having said that, the selectivity of MRS-Clindamycin may not be 100%, as *S. thermophilus,* which is difficult to distinguish morphologically from *Lactobacillus* spp., was also isolated and identified in sample no. 23. This was not further investigated.

Our research shows that on the purchase and the expiry dates, respectively, 86% and 61% of tested samples contained the minimum recommended therapeutic level of $\log_{10}$ 6–7 CFU/g, concordant with the findings of the others [25]. Other researchers have also reported commercially probiotic dairy products with inadequate amounts of viable probiotics [27][28][29], which in some cases may be attributable to disruption of the cold chain [30]. In this study, during cold storage, the number of *Lactobacillus* spp. in some samples decreased considerably. The most important contributing factors for loss of cell viability are decreasing pH during storage, presence of dissolved oxygen, and presence of preservatives in the final

products [8]. In this study, the pH decline between the purchase and expiry date was in some cases noticeable. It could be due to continued fermentation process by LAB even in low temperatures (post-acidification). However, no correlation was found between pH decline of samples and their probiotic counts.

The presence of dissolved oxygen might be the other important reason for drop in viability of cell count in fermented milk [31]. The majority of tested products in this study were stirred yoghurts, in which air could have been incorporated when the yoghurt was mixed with the fruit compote. In addition, some of the commercial fruit products contain preservatives to control contamination and this might affect the viability of the probiotic cells [32].

Based on results obtained in this research, which confirmed lower counts of probiotic cultures approaching the end of shelf life, and supported by the study of Jayamanne and Adams (2006), it is recommended that probiotic fermented products need to be consumed earlier than the expiry date to ingest maximal numbers of probiotic bacteria.

Although there are no universally established standards for microbial content and health claims for probiotic products, the manufacturers should at least clearly express the genus, species, and strain of the probiotic microorganism(s) and also the minimum viable count of each probiotic strain at the end of shelf life [3][33]. To ensure that the consumers benefit from commercial probiotic products, it is necessary to confirm the identity of the claimed organisms at species/strain level and that they are present in the product in appropriate numbers before consumption. Some of the tested products in this study presented inadequate information on the labels. Microbial investigations of probiotic products by others have indicated that the number and identity of recovered species do not always correspond to those stated on the labels of products [34] [35].

Identification of probiotic species used in carrier products should be verified in support of claimed health benefits. To obtain accurate and reliable identification of the probiotic species, molecular techniques should be applied. It has been suggested that DNA profiling by PCR-based methods are the best means for identification of probiotic bacteria at strain level [9][36]. Many misidentifications of probiotic microorganisms may be due to the use of solely phenotypic methods for taxonomic characterization [37].

The rep-PCR fingerprinting profile revealed relative genetic differences between the tested isolates. In this study, 85 isolates from fermented milks were grouped based on their DNA patterns by rep-PCR, and 20 isolates out of 85 were selected for identification by sequence analysis of 16S rRNA. Amplification of the 16S rRNA gene often provides a rapid and reliable tool for bacterial identification without the need for phenotypic characterization. However, 16S rRNA sequencing cannot discriminate between closely related species. Thus, sequencing of alternative genes, such as *rpoA,* with more discriminatory power has been proposed [38][39].

In this research, amplification and sequencing of the *rpoA* gene did not provide enhanced discriminatory information for the tested isolates compared to the use of 16S rRNA gene sequences. Sequencing of other genes, such as *rpoB* and *pheS,* would enhance discriminatory potential, enabling differentiation of strains with close genetic profiles. Anyogu et al. (2014) stated that sequencing of the *pheS*, *rpoA,* and *rpoB* genes along with 16S rRNA gene sequencing provides a better identification of LAB and *Bacillus* isolate.

Even though more media have been suggested in recent years for the enumeration of probiotic lactobacilli in fermented dairy products, none seems to be suitable for all lactobacilli or at least for *Lb. acidophilus*/*Lb. casei* (which are the two most frequently used lactobacilli in the products marketed in the UK/EU), or at the same time be able to act as a differential medium for these two species. Therefore, in this study we examined and compared a limited number of media.

3. Conclusions

Evaluation of MRS-IM Maltose, MRS-Sorbitol, and MRS-Clindamycin as selective media for enumeration of probiotic *Lactobacillus* spp. in commercial fermented milks indicated that MRS-IM Maltose and MRS-Sorbitol were not the best choices for enumerating lactobacilli in fermented dairy products. Instead, the advantage of MRS-Clindamycin was its simplicity and ease of preparation, as well as being differential for *Lb. acidophilus* and *Lb. casei*. Our study of commercial probiotic dairy products in the UK/European market has shown that the most frequent species used in the probiotic products was *Lb. acidophilus* followed by *Lb. casei*. Some other strains were identified which are not popular in fermented dairy products. Commercial use of other useful probiotics, such as *Lb. helveticus*, *Lb. plantarum,* and *Lb. fermentum,* is recommended for dairy producers to provide more diversity amongst probiotic products. Although 16s and *rpoA* gene sequences have been extensively used to classify *Lactobacillus* strains, identification of lactobacilli at species and/or subspecies level using these gene sequences is proven to be difficult. Therefore, analysis of other gene sequences might

be helpful as alternative genomic markers to the aforementioned gene sequencing techniques, and may have a higher discriminatory power for reliable identification of *Lactobacillus* spp.

References

1. Vinderola, C.G.; Reinheimer, J.A. Enumeration of Lactobacillus casei in the presence of L. acidophilus, bifidobacteria and lactic starter bacteria in fermented dairy products. Int. Dairy J. 2000, 10, 271–275.
2. Leahy, S.C.; Higgins, D.G.; Fitzgerald, G.F.; Sinderen, D. Getting better with bifidobacteria. J. Appl. Microbiol. 2005, 98, 1303–1315.
3. Guarner, F.; Khan, A.G.; Garisch, J.; Eliakim, R.; Gangl, A.; Thomson, A.; Krabshuis, J.; Le Mair, T.; Kaufmann, P.; De Paula, J.A.; et al. World Gastroenterology Organisation Practice Guideline: Probiotics and Prebiotics—May 2008: Guideline. S. Afr. Gastroenterol. Rev. 2008, 6, 14–25.
4. Shah, N.P.; Lankaputhra, W.E.V.; Britz, M.L.; Kyle, W.S.A. Survival of Lactobacillus acidophilus and Bifidobacterium bifidum in commercial yoghurt during refrigerated storage. Int. Dairy J. 1995, 5, 515–521.
5. Ghoddusi, H.B.; Robinson, R.K. Enumeration of starter cultures in fermented milks. J. Dairy Res. 1996, 63, 151–181.
6. Talwalkar, A.; Kailasapathy, K. Comparison of selective and differential media for the accurate enumeration of strains of Lactobacillus acidophilus, Bifidobacterium spp. and Lactobacillus casei complex from commercial yoghurts. Int. Dairy J. 2004, 14, 142–149.
7. Korbekandi, H.; Mortazavian, A.M.; Iravani, S. Technology and stability of probiotic in fermented milks. In Probiotic and Prebiotic Foods: Technology, Stability and Benefits to the Human Health; Shah, N.P., Ed.; Nova Science Publishers: New York, NY, USA, 2011; pp. 131–169.
8. Vinderola, C.G.; Costa, G.A.; Regenhardt, S.; Reinheimer, J.A. Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. Int. Dairy J. 2002, 12, 579–589.
9. Tabasco, R.; Paarup, T.; Janer, C.; Peláez, C.; Requena, T. Selective enumeration and identification of mixed cultures of Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, L. acidophilus, L. paracasei subsp. paracasei and Bifidobacterium lactis in fermented milk. Int. Dairy J. 2007, 17, 1107–1114.
10. Van de Casteele, S.; Vanheuverzwijn, T.; Ruyssen, T.; Van Assche, P.; Swings, J.; Huys, G. Evaluation of culture media for selective enumeration of probiotic strains of lactobacilli and bifidobacteria in combination with yoghurt or cheese starters. Int. Dairy J. 2006, 16, 1470–1476.
11. Ashraf, R.; Shah, N.P. Selective and differential enumerations of Lactobacillusdelbrueckii subsp. bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus casei and Bifidobacterium spp. in yoghurt-a review. Int. J. Food Microbiol. 2011, 149, 194–208.
12. Novik, G.I.; Astapovich, N.I.; Ryabaya, N.E. Production of hydrolases by lactic acid bacteria and bifidobacteria and their antibiotic resistance. Appl. Biochem. Microbiol. 2007, 43, 164–172.
13. Yeung, P.S.M.; Sanders, M.E.; Kitts, C.L.; Cano, R.; Tong, P.S. Species-specific identification of commercial probiotic strains. J. Dairy Sci. 2002, 85, 1039–1051.
14. Bull, M.; Plummer, S.; Marchesi, J.; Mahenthiralingam, E. The life history of Lactobacillus acidophilus as a probiotic: A tale of revisionary taxonomy, misidentification and commercial success. FEMS Microbiol. Lett. 2013, 349, 77–87.
15. Kwon, H.S.; Yang, E.H.; Yeon, S.W.; Kang, B.H.; Kim, T.Y. Rapid identification of probiotic Lactobacillus species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol. Lett. 2004, 239, 267–275.
16. Singh, S.; Goswami, P.; Singh, R.; Heller, K.J. Application of molecular identification tools for Lactobacillus, with a focus on discrimination between closely related species: A review. LWT Food Sci. Technol. 2009, 42, 448–457.
17. Song, Y.L.; Kato, N.; Matsumiya, Y.; Liu, C.X.; Kato, H.; Watanabe, K. Identification of Lactobacillus species of human origin by a commercial kit, API 50CHL. J. Assoc. Rapid Methods Autom. Microbiol. 1999, 10, 77–82.
18. Park, S.H.; Itoh, K. Species-specific oligonucleotide probes for the detection and identification of Lactobacillus isolated from mouse faeces. J. Appl. Microbiol. 2005, 99, 51–57.
19. Sghir, A.; Antonopoulos, D.; Mackie, R.I. Design and evaluation of a Lactobacillus group-specific ribosomal RNA-targeted hybridization probe and its application to the study of intestinal microecology in pigs. Syst. Appl. Microbiol. 1998, 21, 291–296.
20. Walter, J.; Tannock, G.W.; Tilsala-Timisjarvi, A.; Rodtong, S.; Loach, D.M.; Munro, K. Detection and identification of gastrointestinal Lactobacillus species by DGGE and species-specific PCR primers. Appl. Environ. Microbiol. 2000, 66,

297–303.

21. Quere, F.; Deschamps, A.; Urdaci, M.C. DNA probe and PCR-specific reaction for Lactobacillus plantarum. J. Appl. Microbiol. 1997, 82, 783–790.
22. Naser, S.M.; Dawyndt, P.; Hoste, B.; Gevers, D.; Vandemeulebroecke, K.; Cleenwerck, I.; Vancanneyt, M.; Swings, J. Identification of lactobacilli by pheS and rpoA gene sequence analyses. Int. J. Syst. Evol. Microbiol. 2007, 57, 2777–2789.
23. Claesson, M.J.; van Sinderen, D.; O'Toole, P.W. Lactobacillus phylogenomics-towards a reclassification of the genus. Int. J. Syst. Evol. Microbiol. 2008, 58, 2945–2954.
24. Ventura, M.; Canchaya, C.; Meylan, V.; Klaenhammer, T.R.; Zink, R. Analysis, characterization, and loci of the tuf genes in Lactobacillus and Bifidobacterium species and their direct application for species identification. Appl. Environ. Microbiol. 2003, 69, 6908–6922.
25. Wang, J.; Guo, Z.; Zhang, Q.; Yan, L.; Chen, W.; Liu, X.M.; Zhang, H.P. Fermentation characteristics and transit tolerance of probiotic Lactobacillus casei Zhang in soymilk and bovine milk during storage. J. Dairy Sci. 2009, 92, 2468–2476.
26. Oberg, C.J.; Moyes, L.V.; Domek, M.J.; Brothersen, C.; McMahon, D.J. Survival of probiotic adjunct cultures in cheese and challenges in their enumeration using selective media. J. Dairy Sci. 2011, 94, 2220–2230.
27. Iwana, H.; Masuda, H.; Fujisawa, T.; Suzuki, H.; Mitsuoka, T. Isolation and identification of Bifidobacterium spp. in commercial yoghurt sold in Europe. Bifidobact. Microflora 1993, 12, 39–45.
28. Schillinger, U.; Guigas, C.; Holzapfel, W.H. In vitro adherence and other properties of lactobacilli used in probiotic yoghurt-like products. Int. Dairy J. 2005, 15, 1289–1297.
29. Gilliland, S.; Reilly, S.; Kim, G.; Kim, H. Viability during storage of selected probiotic lactobacilli and bifidobacteria in a yogurt-like product. J. Food Sci. 2002, 67, 3091–3095.
30. Godward, G.; Kailasapathy, K. Viability and survival of free, encapsulated and co-encapsulated probiotic bacteria in yoghurt. Milchwissenschaft 2003, 58, 396–399.
31. Shah, N.P. Probiotic bacteria: Selective enumeration and survival in dairy foods. J. Dairy Sci. 2000, 83, 894–907.
32. Kailasapathy, K. Chemical composition, physical and functional properties of milk and milk ingredients. In Dairy Processing and Quality Assurance; Chandan, R.C., Ed.; John Wiley & Sons Inc.: Ames, IO, USA, 2008; pp. 75–103.
33. Reid, G.; Zalai, C.; Gardiner, G. Urogenital lactobacilli probiotics, reliability and regulatory issues. J. Dairy Sci. 2001, 84, E164–E169.
34. Hamilton-Miller, J.M.T.; Shah, S. Deficiencies in microbiological quality and labelling of probiotic supplements. Int. J. Food Microbiol. 2002, 72, 175–176.
35. Temmerman, R.; Pot, B.; Huys, G.; Swings, J. Identification and Antibiotic Susceptibility of Bacterial Isolates from Probiotic Products. Int. J. Food Microbiol. 2003, 81, 1–10.
36. Gueimonde, M.; Delgado, S.; Mayo, B.; Ruas-Madiedo, P.; Margolles, A.; Gavilan, C. Viability and diversity of probiotic Lactobacillus and Bifidobacterium populations included in commercial fermented milks. Food Res. Int. 2004, 37, 839–850.
37. Ouwehand, A.C.; Vankerckhoven, V.; Goosens, H.; Huys, G.; Swings, J.; Vancanneyt, M.; Lahteenmaki, A. The safety of probiotics in foods in Europe and its legislation. In Probiotics in Food Safety and Human Health; Goktepe, I., Juneja, V.K., Ahmedna, M., Eds.; Taylor and Francis Group: Boca Raton, FL, USA, 2006; p. 416.
38. Koo, H.N.; Hong, S.H.; Seo, H.G.; Yoo, T.S.; Lee, K.N.; Kim, N.S.; Kim, C.H.; Kim, H.M. Inulin Stimulates NO Synthesis Via Activation of PKC-α and Protein Tyrosine Kinase, Resulting in the Activation of NF-κb by IFN-γ-Primed Raw 264.7 Cells. J. Nutr. Biochem. 2003, 14, 598–605.
39. Santos, S.R.; Ochman, H. Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. Environ. Microbiol. 2004, 6, 754–759.