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METHODS TO ASSESS LACTIC ACID BACTERIA DIVERSITY AND COMPATIBILITY IN FOOD

REVIEW

M. Geria and A. Caridi*

Department of AGRARIA, "Mediterranea" University of Reggio Calabria, Via Feo di Vito, I-89122 Reggio Calabria. Italy

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Food microflora is a complex and mutable ecosystem where the effects of microbial culture addition are still not entirely foreseeable due to microbial diversity. Starter, probiotic, and adjunct microorganisms are widely selected and used in food to improve quality and safety; they may be formulated as monostrain or multistrain cultures. Lactic acid bacteria are included among the main groups deemed useful for these aims. Compatibility tests can constitute an effective way to assess interactions among lactic acid bacteria. Food microflora composition is generally examined using both culture-dependent and culture-independent methods. The existing limits of each method can be overcome by combining them, so that they give more information on microbial complexity. Since mixed cultures of starter, probiotic, or adjunct lactic acid bacteria provide more beneficial effects than single cultures, future research should be guided by compatibility tests to show the most suitable and beneficial mixed cultures.

Keywords: food, lactic acid bacteria, adjunct cultures, probiotic cultures, starter cultures, microbial diversity, compatibility test

Lactic acid bacteria (LAB) are widely selected and used to guarantee food quality and safety; they play an important role in the improvement of required characteristics (Holzapfel, 2007). It is necessary to avoid combinations of strains which display mutually inhibitory properties (Kailasapathy & Chin, 2000). On the contrary, it may be very useful to combine LAB strains based on their compatibility and/or coexistence tests: bacteriocin production by *Lactobacillus plantarum* can be increased significantly under co-culture with certain LAB strains (Man et al., 2012). It is interesting to note that bacteriocins have unconsciously been consumed for centuries with food normally containing bacteriocinogenic strains of LAB (Cleveland et al., 2001). Even if LAB are usually responsible for the fermentative processing and preservation of many foods, LAB dominance is not always complete. In table olive production, a starter strain of *Lactobacillus plantarum* was found to be compatible with the great majority of natural lactic populations, enhancing the dominance of the lactic microflora over the other microorganisms, leading to an improvement in the lactic fermentation process and good quality results in table olives, particularly as regards the level of bloater spoilage (Lamzira et al., 2005). Effectively, it may be very useful to investigate the effects of starter cultures of selected LAB on LAB communities (Jung et al., 2012). The functionality of a multistrain starter culture could be more effective and consistent compared to a monostrain one, owing to a greater chance of effectively and fully performing the fermentation process. However, most research on LAB selection has been aimed at monostrain cultures, due to the easier

* To whom correspondence should be addressed
Phone: +39 0965 680578; fax: +39 0965 680578; e-mail: acaridi@unirc.it

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study design and patent application. The development of methods suitable to determine compatibility among LAB will improve the possibilities of combining monostrain into multistrain cultures.

1. Compatibility tests for starter, probiotic, and adjunct cultures

1.1. General remarks

Natural multispecies starter cultures are currently used to produce food. The sourdough microflora contains established and compatible associations of different LAB, above all *Lactobacillus* spp., and yeasts (De Vuyst & Neysens, 2005). Although produced in non-aseptic environments, such associations may continue over very long periods of time. This enduring coexistence is partly due to their equal growth rates, determined by a multitude of ecological factors. Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Jensen et al., 2012). Although a quality parameter in probiotic LAB is the ability to monitor them, there are only a few reports that have described the enumeration of probiotic LAB – such as *Lactobacillus acidophilus*, *Lactobacillus casei,* and *Lactobacillus rhamnosus* – in the presence of non-probiotic LAB – such as *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* (Champagne et al., 1997; Ravula & Shah, 1998; Bogovič Matijašić & Rogelj, 1999; Wang et al., 2010). The selective media developed to perform this enumeration have been reviewed (Cogan et al., 2007). Multistrain probiotic cultures usually show enhanced efficacy with a variety of strain-specific probiotic properties. A comparison of functionality and efficacy between monostrain and multistrain probiotics has been carried out, showing that: (a) usage of multistrain probiotics should be developed and supported; (b) strains and species should be previously tested for their compatibility or for their synergistic action (Timmerman et al., 2004). Selected non-starter LAB adjunct cultures may be used in food production to: (a) accelerate ripening, (b) produce desirable flavour, or (c) protect against foodborne pathogens by competition for specific compounds, production of antimicrobials, and nutrient depletion. Clearly, this practice requires an evaluation of multispecies interaction among adjuncts, starters, and pathogens. LAB can also produce antimicrobial substances, such as organic acids, diacetyl, hydrogen peroxide, and bacteriocins, thus inhibiting food spoilers and pathogenic bacteria (Szigeti, 2001; Pálmai & Buchanan, 2002; Pálmai & Kiskó, 2003), as well as strains of the same or closely related species (Perin et al., 2012). So in the design of every adjunct LAB selection study, one of the screening procedures is the evaluation of the strains' antimicrobial activity. However, in multistrain cultures, the isolates showing antagonism might cause loss of viability of the other strains, leading to a diminished efficacy. Growth of LAB itself determines inhibition of unwanted microorganisms; however, their ability to produce bacteriocins is of basic importance in biological strategies of food preservation (biopreservation) which extends shelf-life and improves food safety using microorganisms and/or their metabolites (Ross et al., 2002). Bacteriocinogenic adjunct cultures able to antagonize undesirable bacteria with no effect toward useful starter and non-starter LAB would be an ideal food preservative. Both direct – using the microorganism, and indirect – using the bacteriocin-compatibility tests have been performed.

1.2. Direct tests

A simple coexistence assay has been performed using a cross-streak method. The isolates, identified as *Lactobacillus salivarius* and *Lactobacillus reuteri,* were streaked perpendicularly across each other on MRS agar plates. The plates were cultured at 37 °C for 48 h anaerobically to observe their antagonism against each other and to determine whether they are compatible in vitro. However, whether the strains' in vivo compatibility corresponds with their in vitro behaviour should be confirmed (Guo et al., 2010). The production of bacteriocin-like compounds by sourdough LAB has important effects on the starter microflora. To increase knowledge of LAB interactions during sourdough fermentation, compatibility tests have been performed using 77 strains from different species of *Lactobacillus* isolated from sourdoughs showing antagonistic activity against other sourdough lactobacilli (Corsetti et al., 1996). An agar-spot deferred method and a well-diffusion assay were used (Schillinger & Lücke, 1989). Antimicrobial activity has been detected in 47% of *Lactococcus,* 21% of *Lactobacillus,* and 41% of *Enterococcus* among 755 wild LAB isolated from Egyptian dairy products. To accelerate the screening procedures to obtain starter, adjunct, and protective culture strains, LAB of identical genus were examined by internal interaction: so each strain was applied as an inhibitor microorganism while another was taken as indicator microorganism (Ayad et al., 2004). Compatibility among adjunct dairy LAB identified as *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus brevis* and non-starter dairy microflora of the species *Enterococcus durans, Enterococcus faecium,* and *Pediococcus* spp. was evaluated using the agar-spot method (Spelhaug & Harlander, 1989) and measuring the inhibition zones by Autodesk AutoCAD 2007 (Geria, 2008). To screen LAB for associative growth in mixed cultivation, a tube containing 4 ml of 10% skim milk was inoculated with 1% of 2 different strains in pure and mixed culture: the size of the inoculum in the mixed culture was therefore at the 0.5% level for each strain. The cultures were incubated at 30 °C or 37 °C for 24 h, at which time the pH value of the culture was determined. Any interaction occurring in mixed cultures is reflected by acid production. The acidification of growth medium in batch culture is a good reflection of bacterial growth, which is why pH measurement is sometimes used to track growth (Kimoto-Nira et al., 2012). Another screening assay to evaluate the compatibility between different LAB can be performed by using the plate diffusion technique (Maldonado et al., 2012). Aliquots (35 µl) of cell-free supernatant obtained from the early stationary phase of the third subculture of the microorganisms grown in MRS broth were placed into holes (4 mm diameter) of MRS 1% agar plates with 10^9 CFU ml^{-1}, 10^7 CFU ml^{-1}, and 10^5 CFU ml^{-1} with the indicator lactobacilli strains. The plates were incubated for 2 h at room temperature and then 48 h at 37 °C, when the inhibition was assessed.

1.3. Indirect tests

Compatibility among 31 selected strains of dairy LAB has been indirectly determined using the agar well diffusion assay (Casla et al., 1996); the following LAB species were tested for their antagonistic activity one against another: *Lactococcus lactis* subsp. *lactis, L. lactis* subsp. *cremoris, L. lactis* subsp. *lactis* var. *diacetilactis, Leuconostoc mesenteroides* subsp. *dextranicum, L. mesenteroides* subsp. *mesenteroides, Lactobacillus plantarum, Lactobacillus brevis,* and *Lactobacillus casei* subsp. *casei* (Herreros et al., 2005). Enterococci are used in many different applications as adjunct cultures; the inhibitory effect of *Enterococcus faecalis* (Villani et al., 1993) and *Enterococcus faecium* (Cocolin et al., 2007) cell-free culture supernatant against LAB has been tested by using the agar well diffusion assay as described

by Tagg and co-workers (1976) and by Schillinger and Lücke (1987), respectively. The following LAB were examined: *Carnobacterium divergens, Carnobacterium piscicola, Enterococcus casseviflavus, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus leichmanni, Lactobacillus plantarum, Lactobacillus sakei, Lactobacillus salivarius, Lactococcus lactis, Leuconostoc cremoris, Leuconostoc mesenteroides, Pediococcus pentosaceus, Streptococcus thermophilus,* and *Weissella paramesenteroides* (Villani et al., 1993; Cocolin et al., 2007). The antimicrobial activity of kefir is ascribed to metabolites or bacteriocins produced by LAB. A strain of *Lactobacillus plantarum,* isolated from kefir, produced a bacteriocin active against a few pathogens, but also against *Lb. casei, Lb. salivarius,* and *Lb. curvatus,* as detected using the cell-free supernatant adjusted to pH 6.0 (Powell et al., 2007).

2. Study of LAB community dynamics as a possible system to evaluate LAB compatibility

The dynamic changes of LAB microflora in food can be assessed by culturing methods that allow the identification of different LAB species (Xiong et al., 2012); however, these methods are time-consuming, expensive, and sometimes still provide uncertain results. The trend is towards culture-independent methods, characterised by rapidity and reliability. The main motivation for their use is the lack of knowledge of the real conditions under which most bacteria grow in their natural habitat and the difficulty of developing media for cultivation which accurately resemble these conditions (Ercolini, 2004). Most of these methods are based on direct analysis of DNA extracted from food, amplification of genes encoding 16S rRNA and analysis of polymerase chain reaction (PCR) products using a genetic fingerprinting technique. Denaturing Gradient Gel Electrophoresis (DGGE) is the most commonly used culture-independent fingerprinting technique; this electrophoretic method is capable of detecting differences between DNA fragments of the same size but with different sequences. These fragments are separated using a denaturing gradient gel – containing a mixture of urea and formamide – based on their differential melting profile. The technique was profitably used to identify LAB in mixed culture, thus monitoring LAB dynamics during fermentation of pozol (Ampe et al., 1999), sour cassava starch (Ampe et al., 2001), soy sauce (Tanaka et al., 2012), sausage (Kesmen et al., 2012), sourdough (Meroth et al., 2003), malt whisky (van Beek & Priest, 2002), probiotic products (Temmerman et al., 2003) and, primarily, dairy products (Aponte et al., 2008). Temporal Temperature Gel Electrophoresis (TTGE), or Temperature Gradient Gel Electrophoresis (TGGE), is also able to separate DNA fragments of the same length but with different sequences. In this case separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear temperature gradient. The TTGE technique is usually employed to identify LAB microflora in dairy products (Falentin et al., 2012). Pulsed Field Gel Electrophoresis (PFGE) employs an alternating field of electrophoresis to allow the separation of the large DNA fragments obtained from restriction digests with rare-cutting enzymes, with increasing pulse times throughout the run, and the resulting fingerprint profiles can be explored for culture identification. As such, the technique can be more time-consuming than other fingerprinting strategies. However, the profile generated by PFGE represents whole genome and this technique has a good discriminatory power. PFGE was used for

differentiation at strain level of LAB isolates during storage of black olives to monitor the structure and succession of the community (Doulgeraki et al., 2012). Terminal Restriction Fragment Length Polymorphism (TRFLP or sometimes T-RFLP) is a molecular biology technique to profile microbial communities according to the position of a restriction site closest to a labelled end of an amplified gene. The method, which allows separation of large genomic fragments, is based on digesting a mixture of PCR amplified variants of a single gene using one or more restriction enzymes and detecting the size of each of the individual resulting terminal fragments using a DNA sequencer. The TRFLP method is used to study LAB microflora in dairy products (Samelis et al., 2011) and to detect and differentiate the species of LAB in wine and beer (Bokulich & Mills, 2012). Length Heterogeneity PCR (LH-PCR) analysis is similar to the T-RFLP method. The difference is that LH-PCR analysis distinguishes different organisms based on natural variations in the length of the 16S rRNA sequences. The major advantage of LH-PCR over other methods of analysis is that it is efficient, reliable and highly reproducible: it is theoretically possible to obtain an estimate of both qualitative and quantitative composition of dominant populations within a microbial community (Pogačić et al., 2010). The LH-PCR method is commonly used to study LAB microflora in dairy products (Martin-Platero et al., 2009). Single-Strand Conformation Polymorphism (SSCP), or Single-Strand Chain Polymorphism, studies conformational difference of single-stranded nucleotide sequences of identical length using either an acrylamide gel-based or a capillary-based automated sequencer for the separation of denatured PCR products. This allows the sequences to be distinguished by means of gel electrophoresis, which separates the different conformations. The SSCP technique has a low detection threshold and high resolution and the DNA samples can be analysed automatically; it allows the description of dairy LAB community (Samelis et al., 2011). Repetitive-sequence-based PCR (rep-PCR) fingerprinting is a relatively simple, rapid, and sensitive method for discriminating between closely related strains, also employed for the primary differentiation and grouping of the LAB isolates. This technique is used first to classify and then to type the strains. Unknown LAB strains isolated from food samples can be initially screened and grouped by using rep-PCR fingerprinting for cost-efficient speciation and typing (Kesmen et al., 2012). The Rep-PCR method is used to differentiate – at species, subspecies and potentially strain level – a wide range of LAB isolated from dairy products (Feutry et al., 2012) or fermented sausages (Kesmen et al., 2012). Pyrosequencing, an automated high-throughput sequencing technique that involves the synthesis of single-stranded deoxyribonucleic acid and the detection of the light generated by pyrophosphate released in a coupled reaction with luciferase, was recently proposed to study food fermentations (Jung et al., 2011). This technique allows the rapid and accurate sequencing of nucleotide sequences that can then be used to analyse the population structure, gene content and metabolic potential of the microbial communities in an ecosystem. Pyrosequencing was used to study the microbial diversity and community dynamics of the LAB populations of kefir grains (Leite et al., 2012). Fluorescence in situ hybridization (FISH) with 16S rRNA gene probes is a culture-independent molecular method enabling microbial identification and physical detection of microorganisms in a food matrix. It also provides information about the distribution of microbial populations in environmental samples. In food microbiology, FISH is used for the identification of bacteria in situ, without the need of isolation. It is a 'non-PCR-based' molecular technique that uses a fluorescently labelled 16S rRNA bacterial domain probe to allow observation of colonies of microbial cells distributed in a food matrix, such as cheese (Pogačić et al., 2010). Culture-independent molecular approaches still fail to

completely identify the microbiota. Therefore, it is worthwhile devoting effort to improving the detection limits of culture-independent methods. The study of gene expression and translation into proteins within natural environments are two emerging fields in microbial ecology that hold special promise in the study of bacterial function (Pogačić et al., 2010). Moreover, massive parallel sequencing, metagenomics, and metatranscriptomics will allow us to study microbial diversity in greater depth (Cardenas & Tiedje, 2008). The approach of combining 16S rRNA gene profiles and the profiles of functional genes may enable the structure of microbiota to be related to the function in the ecosystem. Functional diversity, which is closely related to the complexity of the food microbiota, plays a crucial role in flavour compound development; therefore, structure-function studies should provide new insights into the role of the complex LAB community (Irlinger & Mounier, 2009). A combination of culture-dependent and culture-independent methods often allows a better investigation of LAB succession, connection, and interaction, giving more complete information on the microbial complexity (Aponte et al., 2008; Samelis et al., 2011; Falentin et al., 2012; Feutry et al., 2012; Kesmen et al., 2012).

3. Conclusion

Direct compatibility tests represent a simple and interesting way to predict interaction among LAB in food. On the other hand, culture-independent methods are more effective to understand LAB composition and dynamic changes of LAB microflora. Although highly sensitive and reliable, both categories of method have limits that may be overcome by combining them, thus giving more information on microbial complexity. Based on the discussion, a polyphasic approach with the combination of culture dependent and culture-independent methods may be the best strategy to study microbial communities. Certainly, multistrain cultures are more effective and consistent compared to monostrain ones (Settanni & Moschetti, 2010). Since mixed cultures of starter, probiotic, or adjunct lactic acid bacteria provide more beneficial effects than single cultures, future research should be guided by compatibility tests to show the most suitable and beneficial mixed cultures.

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