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# ISOLATION OF *LACTOBACILLI* FROM A COMMERCIAL POLISH KEFIR GRAIN[1]

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**Summary:** The microflora of kefir grains have been the focus of research interest for many investigators. However, the composition of kefir grains microflora remains undetermined since no systemetic method for isolations of all the bacteria from the grains has so far been established. In this study, identities of five strains of lactobacilli from a Polish kefir grain were determined by physiological and biochemical tests and were identified as *Lb. kefir, Lb. confusus* (heterofermentative), *Lb. delbrueckii* subsp. *bulgaricus, Lb.kefiranofaciens, Lb. paracasei* subsp. *casei* (homofermentative). Identities may require future conformation by using advanced techniques such as DNA profiling or protein analysis and comparisons made with the type strains that have been deposited in National Culture collections. The identities, however are consistent with the types of organisms isolated from kefir grains by other workers.

**Keywords:** Kefir grain, lactobacil.

## Bir Ticari Polonya Kefir Tanesinden Laktobasillerin İzolasyonu

**Özet:** Kefirin mikrobiyolojisi bir çok araştırmacı için ilgi odağı olmuştur. Bununla beraber, şimdiye kadar tanelerden bütün bakterilerin izolasyonu için sistematik bir metod bulunmadığından kefir tanesinin kompozisyonu tamamen belirlenmiş değildir. Bu çalışmada, Polonya kefir tanesinden izole edilen beş laktobasil suşunun tanımlanması fizyolojik ve biokimyasal testlerle yapıldı ve suşlar *Lb. kefir, Lb. confusus* (heterofermentatif), *Lb. delbrueckii* subsp. *bulgaricus, Lb.kefiranofaciens, Lb. paracasei* subsp. *casei* (homofermentatif) olarak tanımlandı. Tanımlar belki DNA profili veya protein analizleri gibi ileri teknikler kullanılarak ve ulusal kültür koleksiyonlarında depolanmış suş tipleri ile karşılaştırılarak kontrol edilebilir. Bununla beraber, tanımlar diğer araştırmacılar tarafından kefir tanelerinden izole edilen mikroorganizmaların tipleri ile uyum göstermektedir.

**Anahtar sözcükler:** Kefir tanesi, laktobasil.

## INTRODUCTION

In recent years there have been more interest in different fermented milk products known only to particular countries with a view to adapting them for commercial large-scale production in other parts of world. The best known of these in the Western world are yoghurt, cheese, acidophilus milk, kefir, koumiss and yakult. The names of the well-known fermented milks such as yoghurt, ayran/airan and probably koumys/ koumiss come from Turkish Language[1,2], so too is the name of kefir which is thought to originate from Turkish word ' key(i)f 'meaning ' good feeling ' for the sense of well being experienced after drinking it[3].

Kefir is a self-carbonated, lactic sour, fermented milk beverage and it is produced by co-incident lactic acid and alcohol fermentation. It is made from whole or skim milk and produced by adding a unique culture ' kefir grain ' to the fresh milk. Kefir grains contain a group of micro-organisms dominated by *lactobacillus*/ yeast population in the forms of grains because the organisms are embedded in a resilient insoluble polysacharide matrix called ' kefiran'[4]. Traditional kefir cannot be prepared without kefir grains. These must be recovered in gelatinous form from kefir beverage after fermentation and they cannot be re-constructed from individual microbial components.

In natural fermentations lactic acid bacteria implement competetive characteristics which allow them to produce good quality milk fermentations. Compared to other milk fermentations (e.g. yoghurt and cheese) the microbiology of kefir grain is less well understood. The kefir grains contain mixed cultures of lactic acid bacteria and yeasts. These different genera, species, strains of lactic acid bacteria together make up a dynamic, complex mixed culture. Therefore we investigated species of lactobacilli isolated and identified from a Polish kefir grain.

## MATERIALS and METHODS

Kefir grains were obtained from Biolacta-TEXEL, Olsztyn, Poland in the sterile 0.9 % NaCl solution and kept at 4°C were used for this study. 5 g of kefir

[1] Prof. Val. Marshall'ın yürütücülüğünde yapılan "Partial Characterisation of Lactobacilli Isolated from Commercial Kefir Grain" adlı doktora tezinin yayın özetidir.

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grains were incubated in 10 ml sterilised skim milk at 25°C overnight, added to 150 ml sterilised reconstituted skim milk and incubated at 25°C. The subculturing of grains continued by adding recovered grains to 150-200 ml sterilised reconstituted skim milk on a daily basis. All media (MRS, Gibson's, reconstructed milk) were prepared according to recipes. Strain purity was checked in each experiment by streaking fresh culture on to MRS plates and examining resulting colonies for uniformity (size, appearance etc.).

A sample of kefir grain was crushed aseptically into small pieceses using a stomach blender (Colworth, UK) and streak plates were prepared on lactose suplemented (2.0 %) MRS (Oxoid) agar from crushed grains and kefir beverage. The plates were incubated at 30°C for 48 hours. From these, five different colonies were chosen according to size of colonies and morphological appearance. The colonies were subcultured in lactose suplemented (2.0 %) MRS broth media at 30°C for 24 hours. All fresh isolates were labelled as P1, P2, P3, P4, P5 and stored temporarily untill identification in glass beads at -80°C[5]. Master cultures were prepared by inoculating lactose suplemented MRS (de Man, Rogosa, Sharpe) with a single glass bead of each isolate and incubating at 30°C for 48 h. Cultures were stored at 4°C for subsequent use. Experimental inocula were prepared by incubating Universal bottles containing 10 mL MRS broth under static conditions with a 2.0 % inoculum from the master culture at 30°C for 24 h. Standart inoculum for each experiment was added at 2.0 % unless otherwise stated.

Isolates were identified on the bases of Gram's reaction, colony and cell morphology, catalase activity, growth at 10 and 45°C, gas production, carbohydrate utilization profiles. Colony morphology was examined after 48 or 72 h incubation on lactose suplemented MRS agar plates at 30°C. Morphological features of Gram-stained organisms were observed under x1000 object with oil immersion. Catalase activity was investigated by adding of a few drops of 3.0 % hydrogen peroxide directly to a clump of cells of each isolate on a microscope glass slide. The vigorous evolution of gas (oxygen) bubbles is a positive result. Isolates that were rod-shaped, Gram-positive, catalase-negative, grew in lactose suplemented MRS broth reducing pH to ≤ 4.9 in 24 h were tested for ability to grow in MRS broth at 10 and 45°C. Semi-solid Gibson's media was inoculated with a dense culture of each isolate, sealed with 1 mL of 1.5 % agar and incubated at 30°C for 48 h. Displacement of the seal indicated gas production. Carbohydrate utilisation profiles were determined using the Biomerieux API 50 CHL (50410 Lactobacillus) diagnostic kit.

## RESULTS and DISCUSSION

From a sample of Polish kefir grain, isolation of lactobacilli in particular was targeted since these organisms have been found to dominate kefir fermentations[6-8]. Traditional methods were used in isolation. There are also some more advance tools such as genetic probes that could be used for same purpose. However, it does not tell you that particular bacteria at present is alive and it does not help to cultivate them.

Observation of colonies on MRS agar showed mainly two types of colonies in terms of elevation of colony, colony form and colony edge: convex and umbonate; circular and irregular; entire and undulate. Regarding colony size, each isolate was picked as representative colonies and labelled P1, P2, P3, P4 and P5 which were Gram positive, rod-shaped bacteria in pairs and in short chains with rounded ends. They were catalase negative. Isolate P5 showed Gram positive, ovoid shaped cells that exhibited evidence of budding and therefore had typical characteristics of a yeast and were confirmed on selective PDA (Potato dextrose agar) media by Gram stain. Thus P5 was removed from further examination. Repeated subculturing showed that all isolates expect P4 were pure in that colony morphology was similar. However P4 showed two distinct populations. This second colony was Gram stained and examined under light microscope which revealed Gram positive, rod-shaped bacteria in pairs and in short chains with rounded ends. It was catalase negative and therefore it was labelled as P5.Isolates that were Gram- positive rods, catalase negative, grew in MRS and showed no evidence of spores were presumed to be lactobacilli. All five isolates were further characterised by classical biochemical and physiological tests. Organisms were assessed for their ability to grow at 10, 30 and 45°C, in addition the media pH was recorded after 24 h incubation. Morphological, physiological and biochemical data showed that kefir isolates can be divided into two groups. Group I contained 2 strains (P1 and P3). Colonies were entire (circular) and smooth. They were convex (raised) and opaque (white). Both isolates were unable to grow at 10°C. P1

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did not grow at 45°C whereas P3 showed evidence of a weak growth. When glucose is converted to lactic acid under homofermentative metabolism, a low pH can be expected but if fermented to products other than lactic acid (e.g. ethanol, acetic acid) as in heterofermentative metabolism, then media pH may be less acidic. Growth in MRS by P1 and P3 was indicative of heterofermentative metabolism (pH above 4.0) and this was confirmed by the presence of gas in Gibson's medium. Group II contained another 3 strains (P2, P4 and P5). Colonies P4 and P5 were entire (circular), convex and rough while P2 was irregular, umbonate. All were opaque (white). All isolates did not show significant evidence of growth at 10 and 45°C. Growth of isolate P5 in MRS was indicative of homofermentative metabolism (pH below 4.0) while P2 and P4 were not. However, the absence of gas was observed in Gibson's medium. Therefore, they were confirmed as homofermentative.

Fermentation profiles of broth-grown culture were determined by the API 50CHL system. The comparison of selected differential carbohydrates for kefir isolates based on data available by Kandler and Weiss[9], Marshall and Tamime[10]. Carbohydrate fermentation of organisms showed that none of isolates were able to ferment glycerol, erythritol, D-arabinose, L-xylose, adonitol, b methyl-xyloside, dulcitol, inositol, sorbitol, a methyl-D-mannoside, insulin, melezitose, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, 2 keto-gluconate, 5 keto-gluconate. Isolates P1, P2, P4 fermented only a few carbohydrates where as P3 and P5 fermented a considerable number of sugars. While it is not uncommon for homofermentative species of lactic acid bacteria to utilise few sugars, heterofermentative species are regarded as more versatile. It is noteworthy therefore that, isolate P1 ferments only seven sugars. Each isolate was able to metabolize the disaccharides, lactose and maltose, plus ribose, D-fructose and D-glucose. It is also interesting to note that all isolates were able to utilise the five-carbon sugar, ribose, the metabolism of which requires a phosphoketolase, an enzyme associated with heterofermentation 11 after ribose-5-phosphate is converted to ribulose-5-phosphate. Isolate P2 was unable to utilize galactose of the API gallery. API identification software (BioMerieux) showed that the strain destinations for P1, P2, P3, P4 and P5 were identified with the score of between 0.89 to 0.94 as *Lb. kefir, Lb. delbrueckii* subsp. *bulcaricus, Lb.confusus, Lb. kefiranofaciens* and *Lb. paracasei* subsp. casei respectively. The identities were consistent with the types of organisms isolated from kefir grains by other workers[7,12-14].

Kefir grains used for making kefir are a complex ecosystem which is stable, but not always the same. Different grains show different compositions, but at least a part of composition is stable in that many of the organisms isolated are common to all types. Libudzisz et al.[15] and Anulo et al.[6] isolated *Lb. brevis, Lb. kefir, Lb. acidophilus* and *Lb. casei* ssp. *rhamnosus* in their Polish and Spanish kefir grains with the exception of *Lb. casei* ssp. *alactosus, Lb. helveticus ssp. jogurti, Lb. delbrueckii* ssp. *lactis* in Polish kefir grain and *Lb. casei* ssp. *pseudoplantarum, Lb. fermentum, Lb. viridescens, Lb. gasseri* in Spanish kefir grain. Simova et al.[16] isolated *Lb. brevis, Lb. delbrueckii* subsp. *bulgaricus, Lb. casei* subsp. pseudoplantarum from Bulgarian kefir grains.These differences may be explained by the lack of asepsis during manufacture of kefir.

Although the kefir as a natural starter culture has been studied by many authors[6,17-22] studies show that the taxonomic relationships among bacterial species in kefir have not been fully investigated completely. *Lactobacillus brevis* was isolated by La Riviere et al.[4], Rossi and Rossi[23], Hirota et al.[19] reported the presence of both *Lactobacillus brevis* and Lactobacillus buchneri in Danish grains. Vayssier[24] isolated homo-fermentative *Lactobacillus casei* ssp. alactosus and hetero-fermentative *Lactobacillus cellobiosus* and reported the presence of *Lactobacillus rhamnosus* and *Lactobacillus helveticus* ssp. *jogurti* and *Lactobacillus casei* ssp. *alactosus* and *Lb. cellobiosus* in Canadian and French grains. Moreover, Ottagalli et al.[18] demonstrated the presence of the *Lactobacillus acidophilus* in addition to and *Lactobacillus brevis* in Bulgarian, Russian, Yugoslavian grains. However Vescovo et al.[25] showed that lactic acid bacteria isolated from kefir exhibited a high DNA/DNA homology to each other, but not with strains of *Lactobacillus brevis*. The taxonomic position of the most frequently isolated lactic acid bacteria in the kefir was reinvestigated[12] and it is now accepted that the main heterofermentative rod isolated from kefir is that described as *Lactobacillus kefir* ssp. nov. by Toba et al.[26]. In addition, Fujisawa et al.[14] isolated and identified a capsule-forming homo-fermentative bacterium, *Lactobacillus kefiranofaciens* from kefir grains and a new *lactobacillus* strain

tentatively named *Lactobacillus* ssp. KBP-167B was reported as a homofermentative isolate[27]. At present, *Lactobacillus kefirgranum*, a homofermentative species and *Lactobacillus parakefir*, a heterofermentative species, are proposed as two new species of genus Lactobacillus[21]. The former organism, however, was found to be similar to species belonging to the *Lb. acidophilus* group with respect to G+C content and physiological characteristics. Furthermore, *Lb. kefirgranum* did not grow in the presence of 4% NaCl. Lb. *parakefir* was similar to *Lb. brevis* and *Lb. kefir* in its pattern of acid production from carbohydrates, but differed from these taxa by producing the L-(+) isomer of lactic acid and by not fermenting gluconate.

In fact, although many micro-organisms have been islotaed from the kefir grains it is not clear which ones are truly kefir species and which ones are opportunistic and transient. The studies shows that the taxonomic relationships among bacterial species in kefir is imperfect. Moreover, some organisms have been difficult to culture, so there may still be kefir organisms are to be identified and characterised. With new biotecnology tools (e.g. genetic probes and total protein analysisi) it should soon be possible to more precisely define and name these bacteria.

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