IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM VINEGAR FLIES AND MERLOT GRAPES

By

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at the University of Stellenbosch

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December 2005

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Summary

Thirty lactic acid bacteria were isolated from the intestinal tract of *Drosophila simulans* Stuvervant and nine lactic acid bacteria from Merlot grapes collected from the same winery in the Stellenbosch region, South Africa.

The isolates were grouped according to morphological, biochemical and physiological characteristics. Isolates selected from each group were identified to species level by PCR with species-specific primers, PCR-based DGGE and 16S rDNA sequencing. The majority of isolates from the intestinal tract of *Drosophila simulans* Stuvervant belonged to the species *Lactobacillus plantarum*, but *Lactobacillus paracasei, Lactobacillus sanfranciscensis, Leuconostoc mesenteroides* subsp. *mesenteroides, Lactococcus lactis* subsp. *lactis, Enterococcus faecalis* and *Pediococcus pentosaceus* were also identified. As far as we could determine, this is the first report on the isolation of *L. paracasei, L. sanfranciscensis, L. mesenteroides* subsp. *mesenteroides, L. lactis* subsp. *lactis, E. faecalis* and *P. pentosaceus* from vinegar flies. *Lactobacillus plantarum* has previously been isolated from Merlot grapes.

The genotypic relatedness among isolates of *L. plantarum* isolated from the intestinal tract of vinegar flies and from Merlot grapes were determined by RAPD-PCR. The isolates were grouped into four genotypically well-separated clusters. Thirteen isolates from grape must and five from flies yielded identical RAPD-PCR banding patterns and grouped into one cluster, suggesting that they are descendants from the same strain. This suggests that *L. plantarum* has the ability to use vinegar flies as a vector.

Opsomming

Dertig melksuurbakterieë is vanuit die dermkanaal van *Drosophila simulans* Stuvervant geïsoleer en nege melksuurbakterieë vanuit Merlot-druiwe. Die druiwe is afkomstig van dieselfde wynkelder in die Stellenbosch-area van Suid-Afrika.

Die isolate is volgens morfologiese, biochemiese en fisiologiese eienskappe gegroepeer. Verteenwoordigende isolate vanuit die fenotipiese groepe is tot spesievlak met behulp van lukraak ge-amplifiseerde polimorfe-DNA (RAPD) polimerase ketting-reaksie (PKR), PKR met spesie-spesifieke inleiers, PKR-gebaseerde denaturerende gradient-jel elektroforese (DGGE) en 16S rDNA sekwensering geïdentifiseer.

Die meerderheid isolate uit die ingewande van *Drosophila simulans* Stuvervant is as *Lactobacillu*s *plantarum* geklassifiseer. Stamme van *Lactobacillus paracasei, Lactobacillus sanfranciscensis, Leuconostoc mesenteroides* subsp. *mesenteroides, Lactococcus lactis* subsp. *lactis, Enterococcus faecalis* en *Pediococcus pentosaceus* is ook geïdentifiseer. Sover bekend, is dit die eerste keer dat *L. paracasei, L. sanfranciscensis, L. mesenteroides* subsp. *mesenteroides, L. lactis* subsp. *lactis, E. faecalis* en *P. pentosaceus* uit asynvlieë geïsoleer is. *Lactobacillu*s *plantarum* is voorheen uit Merlot-druiwe geïsoleer.

Die genotipiese ooreenkoms tussen die stamme van *L. plantarum* wat uit die asynvlieë en Merlot-druiwe geïsoleer is, is deur middel van RAPD-PKR bepaal. Hiervolgens is die stamme in vier genotipies goed-gedefinieerde groepe geplaas. Dertien isolate vanuit druiwemos en vyf vanuit asynvlieë het identiese RAPD-PKR bandpatrone vertoon en het in een groep gesorteer. Hierdie resultate dui daarop dat die stamme heel moontlik uit een voorouer ontstaan het en dat asynvlieë heel moontlik as vektor vir *L. plantarum* dien.

Acknowledgments

I wish to express my gratitude to:

Prof. L.M.T. Dicks, Department of Microbiology, University of Stellenbosch;

Dr. C.A. Van Reenen, Department of Microbiology, University of Stellenbosch;

Dr. S.D. Todorov, Department of Microbiology, University of Stellenbosch;

Dr. R.C. Witthuhn, Department of Food Science, University of Stellenbosch;

Dr. M. Du Toit, Institute for Wine Biotechnology, University of Stellenbosch;

Prof. W.H. Holzapfel, Federal Research Centre for Nutrition, Institute of Hygiene and Toxicology, Karlsruhe, Germany; and

the National Research Foundation (NRF), South Africa, for funding the research.

Contents

1. Introduction

Lactic acid bacteria (LAB) comprise a wide range of genera, including a considerable number of species. The genera currently regarded as LAB include *Bifidobacterium Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Carnobacterium*, *Aerococcus*, *Alloiococcus*, *Dolosigranulum*, *Globicatella*, *Vagococcus*, *Melissococcus*, *Lactosphaera*, *Oenococcus*; *Enterococcus*, *Tertragenococcus* and *Weissella* (Klein et al., 1998; Euzéby, 2005). Lactic acid bacteria produce lactic acid from hexoses and are widely used in the food and beverage industries (Holzapfel et al., 2001). The occurrence of LAB in nature is related to their high demand for nutrients. They have been isolated from various fermented foods, including plant and meat products (Kandler and Weiss, 1986) and the intestinal tracts and mucus membranes of humans and animals (Holzapfel et al., 1998). Species such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactococcus lactis*, *Enterococcus faecium*, *Enterococcus faecalis* and *Bifidobacterium* spp. are used in probiotic products (Hammes and Vogel, 1995).

A number of lactic acid bacterial species have been isolated from grapes and the wine environment. Most belong to the genera *Pediococcus*, *Lactobacillus*, *Leuconostoc* and *Oenococcus*. During winemaking LAB carry out a secondary fermentation called the malolactic fermentation (MLF). MLF can be beneficial or detrimental depending on the wine style (Davis et al., 1985; Du Plessis et al., 2004).

The intestinal tract of insects is a rich source of nutrients and contains indigenous LAB populations (Dillon and Dillon, 2004). Lactobacilli, lactococci, leuconostocs, enterococci, streptococci and bifidobacteria have been isolated from insects (Rada et al., l997; Tholen, 1997; Bauer et al., 2000; Reesen et al., 2003; Kacaniova et al., 2004; Pidiyar et al., 2004). In insects, LAB assist in the decomposition and detoxification of non-digested food. LAB also protects insects from the invasion of intestinal pathogens (similar to probiotic strains in humans and animals), produce vitamins or form complex interactions with the immune system of the host (Basset et al., 2000; Dillon and Dillon, 2004; Kacaniova et al., 2004).

Vinegar flies (genus *Drosophila*) are a common agricultural pest, causing extensive damage to fruit orchards. The flies lay their eggs on healthy fruit which is used by the developing larvae as a source of nutrition (Demerec, 1950; Doane, 1967). *Drosophila* spread yeast in wineries (Demerec, 1950) and it has been speculated they may contaminate fermentation processes (Kvasnikov et al., 1971).

Little research has been done on the microbiota of vinegar flies. *Lactobacillus plantarum* and enterococci were isolated from vinegar flies by Kvasnikov et al. (1971). Their identification was based on physiological and biochemical characteristics, including sugar fermentation profiles which are often not reliable (Van Reenen and Dicks, 1996). Little research has been conducted on wine associated microorganisms and their association with vinegar flies.

The present study was undertaken to identify the LAB population present in the vinegar fly gut by using PCR with species-specific primers, PCR-based DGGE, 16S rDNA sequencing and RAPD-PCR. The possibility that the insect can act as a vector for LAB was also investigated. Analysis of RAPD-PCR banding patterns was carried out on LAB isolated from grapes collected from the same vineyards where the vinegar flies were collected.

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2. Taxonomy of lactic acid bacteria with special emphasis on species found in the insect gut

2.1 The lactic acid bacteria

Introduction

LAB comprise a wide range of genera, including a considerable number of species. It is generally accepted that LAB are Gram-positive, catalase negative, without cytochromes, non-motile, asporogenic, micro-aerophilic to strictly anaerobic, and grow at low pH (Stiles and Holzapfel, 1997). They are nutritionally fastidious and require carbohydrates, amino acids, peptides, nucleic acid derivatives and vitamins (Aquirre and Collins, 1993). Some species produce catalase in media containing blood (Aguirre and Collins, 1993) or pseudocatalase when grown in the presence of low sugar concentrations (De Vuyst and Vandamme, 1994). Endospore-forming lactic acid-producing bacteria are classified in the genera *Bacillus* and *Sporolactobacillus*.

With the exception of bifidobacteria, all LAB belong to the Gram-positive phylum with a G+C (guanine plus cytosine) content of less than 50% (Schleifer and Ludwig, 1995). The physiological and biochemical properties of bifidobacteria are similar to that of LAB and they share common ecological niches, including the gastro-intestinal tract of humans and animals (Klein et al., 1998).

Lactic acid bacteria are strictly fermentative and have a complex metabolism. They require specific carbohydrates, amino acids, peptides, fatty acids, esters, salts and vitamins. Due to their fastidious growth, they have adapted their metabolism and have active transport systems (Stiles and Holzapfel, 1997).

Phylogenetic relatedness

Phylogenetically LAB are members of the *Clostridium-Bacillus* subdivision of Gram-positive eubacteria. Lactobacilli and streptococci, together with related facultatively anaerobic taxa, evolved as individual lines of descent about 1.5–2 billion years ago when

the earth changed from an anaerobic to an aerobic environment (Stiles and Holzapfel, 1997).

The genus *Lactobacillus* is intermixed with strains of the genera *Pediococcus* and *Leuconostoc*. From a physiological point of view, the division of *Lactobacillus* spp. into three groups, namely Thermobacterium (Group I), Streptobacterium (Group II) and Betabacterium (Group III) does not correspond to their phylogenetic groupings (Stiles and Holzapfel, 1997). On the other hand, the phenotypically defined genus *Streptococcus* is not a phylogenetically coherent genus and species are grouped into at least three moderately related genera, i.e. *Streptococcus*, *Lactococcus* and *Enterococcus* (Schleifer and Kilpper-Bälz, 1985). The genus *Bifidobacterium*, frequently grouped with the lactobacilli, is the most ancient group of the *Actinomycetes* subdivision of the Gram-positive eubacteria. The propionibacteria, microbacteria and brevibacteria also belong to the *Actinomycetes* subdivision, but are off-shoots of non-lactic acid bacteria (Stiles and Holzapfel, 1997).

Taxonomy in the past

Traditionally, lactic acid bacteria have been classified on the basis of phenotypic properties, e.g., morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, and carbohydrate metabolism. Orla-Jensen subdivided LAB in 1919 into the genera *Betabacterium*, *Thermobacterium*, *Streptobacterium*, *Streptococcus*, *Betacoccus*, *Tetracoccus* and *Microbacterium* based on morphological and phenotypic characteristics (Stiles and Holzapfel, 1997).

Studies based on comparative 16S rRNA sequencing showed that some taxa generated on the basis of phenotypic features do not correspond with suggested phylogenetic groupings. Thus, some species are not readily distinguishable by phenotypic characteristics. Consequently, modern molecular techniques, including polymerase chain reaction-based, and other genotyping methods, have become increasingly important in the identification of species and in the differentiation of strains.

Habitat

Lactic acid bacteria prefer growing in nutritionally rich habitats with a pH range between 4.5 and 6.4 and at mesophilic to slightly thermophilic temperatures (Kandler and Weiss 1986). They are widespread in nature and are found in soil, water, fermented food and beverages, manure, sewage, silage and in the gastro-intestinal tract. They occur naturally on grapes and their ability to grow in grape juice and wine has been well documented (Davis et al., 1985). The species commonly associated with grapes or the winemaking process belongs to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Wibowo et al., 1985; Van Vuuren and Dicks, 1993; Dicks et al., 1995).

Large populations of lactic acid bacteria inhabit the proximal region of the digestive tracts of pigs, fowl, and rodents (Stiles and Holzapfel, 1997). Some gastrointestinal strains of lactic acid bacteria adhere to and colonise the surface of stratified squamous epithelium in the oesophagus, crop, or stomach (De Vuyst and Degeest 1999). Other LAB colonise the gastrointestinal lumen (Stiles and Holzapfel, 1997; Reesen et al., 2003; Holzapfel et al., 2001).

The intestinal tract of insects is a rich source of nutrients and supports the growth of a number of microorganisms (Bignell, 1984). However, only a few studies have been published on the presence of lactic acid bacteria in insect gut. It is assumed that many insect species derive their microbiota from the surrounding environment such as the phylloplane of food plants or the skin of the animal host (Bignell, 1984). The honeybee's normal microflora is acquired by consuming pollen, and through contact with older bees in the colony (Rada et al., 1997). The digestive tract of adult honeybees contains lactobacilli, bacilli, bifidobacteria (Rada et al., l997) and enterococci (Kacaniova et al., 2004). *Melissococcus pluton* is closely associated with the brood of honey bees (Baily, 1984). *Enterococcus faecalis* and *Lactococcus lactis* subsp. *lactis* have been isolated from the hindgut of termites (Bauer et al., 2000; Tholen et al., 1997), *Streptococcus* spp. from the gut of desert locusts (Hunt and Charnley, 1981) and crickets (Ulrich et al.,

1981), *Lactococcus* spp. from the gut of mosquitos (Pidiyar et al., 2004), and *Lactococcus* spp., *Lactobacillus* spp. and *Leuconostoc* spp. from the intestinal tract of wasps (Reesen et al., 2003). *Lactobacillus plantarum*, enterococci and "hetero-enzymatic" cocci were isolated from the vinegar fly by Kvasnikov et al. (1971).

Role of lactic acid bacteria in the gut

The major role of LAB in the gut of mammals is to ferment non-digestible dietary residue and endogenous mucus produced by the epithelium (Roberfroid et al., 1995). Through microbial metabolism, short-chain fatty acids, vitamin K and ions are produced that are readily absorbed (Guarner and Malagelada, 2003). The gut microbiota also serves as a vital modulator of the immune system (Tlaskalova-Hogenova et al., 2004) where Toll-like receptors (TLR) have recently been recognized as important signaling devices for the recognition of commensal microflora (Rakoff-Nahoum et al., 2004).

Lactic acid bacteria also play a role in the competitive exclusion of pathogens, and stimulation/modulation of mucosal immunity. Strains used as probiotics usually belong to the genera *Lactobacillus*, *Enterococcus* and *Bifidobacteria*. Several strains of *Lactobacillus* spp. have been included in animal feed (Holzapfel et al., 1998; 2001) and may be developed as delivery vehicles for digestive enzymes and vaccine antigens (Pouwels et al., 1998; Steidler et al., 1995). Their innate acid tolerances, ability to survive gastric passage, and safety record during human consumption are key features that can be exploited to effectively deliver therapeutic compounds to targeted locations and tissues.

The role of bacteria in the insect gut is similar to the role they perform in mammals (Savage, 1977). Intestinal microbes may contribute to food digestion, produce essential vitamins for the host, and keep out potentially harmful microbes. Nutritional contributions may take several forms: improved ability to live on suboptimal diets, improved digestion efficiency, acquisition of digestive enzymes, and provision of vitamins. These nutritional contributions are well established for endosymbionts such as *Buchnera* spp. (Douglas, 1998), but in many cases the indigenous gut bacterial

community could provide similar benefits. Plant material is low in nitrogen, specific amino acids, sterols, and B vitamins, and in many cases microorganisms synthesize these components (Cruden and Markovetz, 1987; Douglas, 1998). Microorganisms detoxify plant allelochemicals such as flavonoids, tannins, and alkaloids (Douglas, 1992).

Aphids feeding on plants with phloem sap that contains a low concentration of essential amino acids rely on bacterial endosymbionts to provide the required amino acids (Douglas, 1998). Kacaniova et al. (2004) ascribed the role of lactic acid bacteria in the gut of bees as decomposition and detoxification of non-digested food. The bacteria in the hindgut of the house cricket *Acheta domesticus* increases the metabolism of soluble plant polysaccharides (Kaufman and Klug, 1991). Spirochetes provide the carbon, nitrogen, and energy requirements of termite nutrition via acetogenesis and nitrogen fixation (Brune et al., 1995; Breznak, 2002). Microbial nitrogen fixation accounts for 60% of the nitrogen in some termite colonies (Tayasu et al., 1994).

An important function of the indigenous intestinal microbiota in humans and domesticated animals is their ability to withstand colonisation of the gut by non-indigenous species, including pathogens to prevent enteric infections (Berg 1996). Bacteria in the insect gut may also act in a similar manner. The gut microbiota of silkworm larvae provides a buffering action to prevent proliferation of pathogenic streptococci and *Serratia piscatorum* (Kodama and Nakasuji, 1971). Germ-free locusts reared in isolation on irradiated diet were more susceptible to fungal infection than locusts reared on a conventional diet. A cocktail of phenolic compounds detected in the gut fluid or frass of conventional locusts were absent from the axenic locusts and were therefore implicated as the antifungal agents (Charnley et al., 1985).

Production of chemicals by gut microbiota can influence insect behaviour. Nolte et al. (1973) isolated a bacterial-derived pheromone called locustol from the locust *Locusta migratoria migratorioides*. Guaiacol and phenol produced by gut bacteria in locust are released through fecal pellets. These compounds function as components of a cohesion pheromone (Obeng-Ofori et al., 1994; Dillon et al., 2000; Dillon and Charnley, 2002).

The insect gut provides an excellent environment for gene transfer between bacteria. Transconjugation between bacterial strains allow for rapid adaptation of microbial communities (Dillon and Dillon, 2004). Studies with *E. coli* containing antibiotic-resistant plasmids have indicated that horizontal gene transfer to *Yersinia pestis* occurred in the flea midgut after only 3 days of coinfection. Ninety-five percent of co-infected fleas harboured antibiotic-resistant *Y. pestis* transconjugants after 4 weeks (Hinnebusch et al., 2002).

Importance of lactic acid bacteria in the industry

LAB possess GRAS (Generally Regarded as Safe) status, although some pathogenic *Streptococcus* and *Enterococcus* species have been described (Holzapfel et al., 2001). In the food industry, lactic acid bacteria yield stable and safe end-products with unique organoleptic and sensorial qualities and are therefore added as starter cultures to basic food products such as milk, meat, vegetables and cereals. Lactic acid bacteria are particularly suitable as antagonistic micro-organisms in foods, since they are capable of inhibiting other potential pathogenic food-borne bacteria by the production of organic acids (e.g. lactic acid), hydrogen peroxide, bacteriocins and other antimicrobial proteins (Aquirre and Collins, 1993; De Vuyst and Vandamme, 1994). Lactic acid bacteria also produce an abundant variety of homo- and heteropolysaccharides (Aquirre and Collins, 1993) that may improve the textural properties of food such as fermented milk (De Vuyst and Degeest, 1999).

During winemaking, LAB carry out MLF. Most of the LAB isolated from the wine environment have the ability to conduct malolactic fermentation. MLF can be beneficial or detrimental, depending on the wine style (Davis et al., 1985). MLF de-acidifies wine by conversion of L-malate (L-malic acid) to L-lactate (L-lactic acid) and is favored in high-acid wines produced in cool-climate regions. This process is less desired in warm-climate regions where already low-acid wines are further de-acidified by MLF. There is a need to control the MLF to enhance the positive attributes and reduce potential negative

impacts on the particular wine. This is done through inoculation of starter cultures in order to perform MLF (Davis et al., 1985). Lactic acid bacteria are being used in the production of industrial chemical and biological products, including biopolymers (*Leuconostoc* spp.), bulk enzymes (*Lactobacillus brevis*), ethanol, aminopeptidases (*Lactococcus lactis*) and lactic acid (*Lactobacillus casei*, *Lactobacillus delbrueckii* and *Lactobacillus brevis*) (Gold et al., 1996; Hofvendahl and Hahn-Hagerdal, 2000). They also play an important role in the spoilage of processed and fermented foods. Examples include the souring and off-flavours in meat and dairy products. Species of *Pediococcus*, *Leuconostoc* and *Lactobacillus* are involved in the spoilage of wine, beer and fruit juices. These organisms cause cloudiness and often produce off-flavours and polymers (Aquirre and Collins, 1993).

The Genus *Lactobacillus*

The genus *Lactobacillus* is part of the *lactobacillus-leuconostoc-pediococcus–streptococcus* supercluster of the clostridia sub-branch of Gram-positive bacteria with *Lactobacillus delbreuckii* as the type species (Kandler and Weiss, 1986). All species are catalase-and cytochrome-negative. Growth temperatures range between 2°C and 53°C, with the optimum between 30°C and 40°C (Kandler and Weiss, 1986). Cell size range from 0.7-1.1 x 2.0-4.0 micrometer. The genomic G+ C content ranges from 32 to 54%.

The genus *Lactobacillus* is divided into three phenotypic groups: (A) obligately homofermentative, (B) facultatively heterofermentative and (C) obligately heterofermentative (Hammes and Vogel, 1995).

Group A comprises the obligately homofermentative species which lacks the enzymes glucose 6-phosphate-dehydrogenase (G-6-PDH) and 6-phosphogluconate-dehydrogenase (6-P-GDH). These lactobacilli cannot ferment pentoses or gluconate (Pot et al., 1994), but ferment hexoses such as glucose almost exclusively to lactic acid. Group A can be subdivided into two groups on the basis of DNA-DNA homology. Subgroup 1 consists of *L. delbrueckii* and its subspecies, *L. delbrueckii* subsp. *delbrueckii*, *L. delbrueckii* subsp.

leichmanni, *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, with a DNA homology of 80% and higher. Subgroup 2 consists of the *L. acidophilus* group. Species within this group cannot be differentiated according to physiological characteristics (sugar fermentation, growth behaviour, etc), but can be distinguished based on DNA homology (Gasser and Janvier, 1980).

Group B contains the facultatively heterofermentative species and ferments hexoses to lactic acid. These organisms produce gas from gluconate but not from glucose. In contrast with the obligately homofermentative group, the species in group II have both dehydrogenase enzymes (G-6-PDH and 6-P-GDH). Pentoses are fermented to lactic and acetic acid via an inducible pentose phosphoketolase pathway (Pot et al., 1994). Group B consist of three genotypic complexes of species and subspecies (Kandler and Weiss, 1986). Subgroup 1 consists of *L. plantarum*, *L. pentosus* and *L. paraplantarum* (Curk et al., 1996), with a DNA homology ranging from 80% to 100%. Subgroup 2 consists of *L. zeae*, *L. casei*, *L. paracasei* and *L. rhamnosus*. The latter three species are used as human and animal probiotics.

Historically, the *L. casei* group comprised of only one species, *L. casei*, which was divided into the subspecies *casei*, *alactosus*, *pseudoplantarum*, *tolerans* and *rhamnosus*. Collins et al. (1989b) reclassified the *L. casei* group to *L. paracasei* and *L. rhamnosus*. *Lactobacillus casei* subsp. *casei* was transferred to the species *L casei* without any subspecies. *Lactobacillus paracasei* comprises two subspecies, viz. *L. paracasei* subsp. *paracasei*, which includes the former *L. casei* subsp. *alactosus* and *L. casei* subsp. *pseudoplantarum*, and *L. paracasei* subsp. *tolerans*, originally *L. casei* subsp. *tolerans*. *Lactobacillus rhamnosus* consists only of the strains of the former subspecies *rhamnosus*. The cell wall of *L. rhamnosus* contains rhamnose and *L. rhamnosus* ferments rhamnose. *Lactobacillus casei* and *L. paracasei* could not be differentiated biochemically and the taxonomic position of. *L paracasei* remains unclear. Dellaglio et al. (1991) disagreed with the classification of the *L. casei*-group and requested an opinion on the designation of the type strain of *L. casei*. Dicks et al. (1996) proposed a rejection of the name *L.*

paracasei and the inclusion of all strains in the species *L. casei*, with ATCC334 designated as the type. The authors transferred ATCC 393 to a revived species, *L. zeae*.

Group C contains the obligately heterofermentative species that lack the FDP-aldolase enzyme. These bacteria ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. Gas is produced from glucose. Lactic and acetic acids are produced from pentose via the pentose phosphoketolase pathway (Pot et al., 1994).Group C include: *Lactobacillus bifermentans*, *Lactobacillus buchneri*, *Lactobacillus brevis*, *Lactobacillus collinoides*, *Lactobacillus confuses*, *Lactobacillus fermentum*, *Lactobacillus fructovorans*, *Lactobacillus fructosus*, *Lactobacillus halotolerans*, *Lactobacillus hilgardii*, *Lactobacillus kandleri*, *Lactobacillus kefir*, *Lactobacillus malefermentans*, *Lactobacillus oris*, *Lactobacillus panis*, *Lactobacillus parabuchneri*, *Lactobacillus parakefir*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus sanfranciscensis*, *Lactobacillus suebicus*, *Lactobacillus vaccinostercus*, *Lactobacillus viridescens* and *Lactobacillus vaginalis* (Hammes and Hertel, 2005).

Given the diversity of metabolic properties exhibited by members of the *Lactobacillus* genus they are found in a number of fermented food products. In these products the lactobacilli contribute to their preservation, nutrient availability and flavour. Lactobacilli are used as starters in the fermentation of pickles, olives and sauerkraut (McKay and Baldwin, 1990; Salminen et al., 1998). A number of dairy products are produced using *Lactobacillus* either alone or in combination with other lactic acid bacteria. Acidophilus milk is produced with *L. acidophilus*. *Lactobacillus bulgaricus*, in combination with *Streptococcus thermophilus,* is used to produce yoghurt. A balance between these two starters can affect product quality (Salminen et al., 1998).

Lactobacillus species play an essential role in bread making and a number of unique strains have been identified in products, most notably sourdough bread. Typical species of lactobacilli identified in sourdough bread include *L. acidophilus*, *L. farciminis*, *L. delbrueckii* subsp. *delbrueckii*, *L. casei*, *L. plantarum*, *L. rhamnosus*, *L. brevis*, *L. sanfranciscensis* and *L. fermentum*. The exact composition of most sourdough breads is

not known and attempts to blend starters to mimic a particular product are sometimes less than satisfactory. Traditional sourdough fermentations are carried out by 'back-slopping', a process where a remaining fraction of a fermentation batch is used to start the next fermentation. Any contaminating microflora are out competed by the indigenous lactobacilli. The number of lactic acid bacteria in the dough can reach 10^7 cfu g^{-1} (Salminen et al., 1998).

An important property of *Lactobacillus* spp. is their ability to produce bacteriocins (Table 1). Bacteriocins probably evolved to provide the producing organism with a selective advantage in a complex microbial niche. Incorporation of *Lactobacillus* spp. as starters or the inclusion of a purified or semi-purified bacteriocin preparation as an ingredient in food provides a margin of safety in preventing the growth of pathogens (Salminen et al., 1998).

Table 1. Selected bacteriocins produced by *Lactobacillus* species (Axelsson, 1998; Chen and Hoover, 2003)

Bacteriocin	Producer	Sensitive strains
Lactacin B	*L. acidophilus*	*L. delbrueckii*, *L. helveticus*
Lactacin F	*L. acidophilus*	*L. fermentum*, *S. aureus*, *E. faecalis*
Brevicin 37	*L. brevis*	*P. damnosus*, *O. oeni*
Lacticin A	*L. delbrueckii*	*L. delbrueckii* subsp. *Lactis*
Helveticin J	*L. helveticus*	*L. helveticus*, *L. delbrueckii* subsp. *bulgaricus*
Sakacin A	*L. sakei*	*Carnobacterium piscicola*, *L. monocytogenes*
Plantaricin A	*L. plantarum*	*Lactococcus lactis*, *E. faecalis*
Gassericin A	*L. gasseri*	*L. acidophilus*, *L. brevis*
Plantaricin 423	*L. plantarum*	*O. oeni, Listeria monocytogenes L. brevis*
Plantaricin D	*L. plantarum*	*L. sake*, *Listeria monocytogenes,*
Sakacin P	*L. sake*	*P. damnosus, L. monocytogenes*

A great deal of attention has been directed toward the role of lactobacilli as probiotics. Strains which have been examined for their probiotic effects include *L. acidophilus* LA1, *L. acidophilus* NCFB 1748, *Lactobacillus* GG, *L. casei* Shirota and *L. gasseri* ADH. The

benefits of adding probiotic lactobacilli to the diet include immune enhancement, lowering of faecal enzyme activity, prevention of intestinal disorders and reduction of viral diarrhea. Most probiotic strains colonise the intestinal tract, thereby excluding colonisation by pathogens (Stiles and Holzapfel, 1997). Their ability to colonise the GI tract has also directed research to the use of lactobacilli as delivery-vehicles for therapeutic compounds such as immunomodulators, antibodies, enzymes and vaccines (Marteau and Rambaud, 1993; Hols et al., 1997).

Lactobacillus plantarum

Lactobacillus plantarum is one of the most naturally abundant and widely distributed lactic acid bacteria. Some strains of *L. plantarum* are found as natural commensals of the gastrointestinal tract (GI tract), the oral cavity and the female urogenital tract of animals and humans. *Lactobacillus plantarum* survives passage though the stomach and persists for 6 days in the human GI tract (Holzapfel et al., 1998).

Lactobacillus plantarum is also commercially important and is included in several mixed starter cultures for the production of fermented meat, vegetables, grass silage and certain dairy products (De Vuyst and Vandamme, 1994). As a malolactic bacterium *L. plantarum* is responsible for the decrease of wine acidity and improvement of wine taste and flavour. As a spoilage agent *L. plantarum* can cause increasing volatile acidity and, in some cases, the degradation of tartaric acid leading to a deprecation of quality (Lonvaud-Funel 1999). Some strains are marketed as probiotics. *Lactobacillus plantarum* 299v is marketed as a probiotic that may confer various health benefits to the consumer (Adawi et al., 2001). *Lactobacillus plantarum* 423 is another strain that has probiotic properties and also produces a class II antimicrobial peptide that might play an important role in food preservation (Van Reenen et al., 1998). The ability of *L. plantarum* to persist in the human GI tract has stimulated research aimed at the use of *L. plantarum* as a delivery vehicle for therapeutic compounds (Adawi et al., 2001).

The ecological flexibility of *L. plantarum* is reflected by its relatively large genome size, large number of proteins involved in transport functions, and high metabolic potential. Kleerebezem et al. (2003) sequenced the entire genome of *L. plantarum* strain WCFS1, and found it to be considerably larger (3.3 Mb) than other LAB isolates with a genome size between 1.8 and 2.6 Mb (Chevalier et al., 1994; Kleerebezem et al., 2003).

Lactobacillus plantarum is found on plants and plant-derived materials where amino acids and peptides are not readily available; therefore *L. plantarum* needs to metabolise many different substrates. The *L. plantarum* genome encodes 342 proteins involved in carbohydrate transport and metabolism. *L. plantarum* contains genes for the complete Embden–Meyerhoff–Parnas (EMP) pathway and a number of enzymes involved in the degradation of pentoses and hexoses (Kleerebezem et al., 2003). As a homofermentative bacteria capable of malolactic fermentation *L. plantarum* in wine can degrade arginine via the ADI pathway and not via the arginase/urease pathway as in heterofermentative LAB. Arginine is quantitatively one of the most important amino acids in grape musts and wine (Lonvaud-Funel 1999).

The *L. plantarum* genome encodes 268 proteins predicted to be involved in the metabolism and transport of amino acids. Enzymes required for the biosynthesis of all amino acids, with the exception of leucine, isoleucine and valine are encoded on the genome. The *L. plantarum* genome encodes a high number (90) of proteins predicted to be involved in the transport and metabolism of vitamins and cofactors. All enzymes necessary for the biosynthesis of folate are present in *L. plantarum*, thus *L. plantarum* is capable of synthesizing its own folate (Boekhorst et al., 2004).

The chromosome of *L. plantarum* encodes an excess of 200 extra-cellular proteins, many of which are bound to the cell envelope. Some of these extra-cellular proteins play a role in adhesion or binding to other cells or proteins, including mucus-binding and fibronectin-binding. Extra-cellular proteins also promote intercellular adhesion leading to cell clumping (Kleerebezem et al., 2003).

A large proportion of the genes encoding sugar transport and utilization, as well as genes encoding extracellular functions, appear to be clustered in a 600-kb region near the origin of replication. Many of these genes display deviation of nucleotide composition, consistent with a foreign origin. These findings suggest that these genes, which provide an important part of the interaction of *L. plantarum* with its environment, form a lifestyle adaptation region in the chromosome (Chevalier et al., 1994; Kleerebezem et al., 2003). *Lactobacillus plantarum* has a relatively small percentage of its genes involved in core functions such as replication and translation which would indicate *L. plantarum* has experienced relatively little genome decay (Boekhorst et al., 2004).

The genus *Leuconostoc*

Leuconostocs are almost spherical, sometimes lenticular, and resemble short bacilli with rounded ends. They are approximately 0.5–0.7 μm × 0.7–1.2 μm in size and are arranged in pairs or chains. In nutrient media during active growth, they may convert to short chains. Under more stressful conditions, the chains are longer (Garvie, 1986a). Most strains grow between 20°C and 30°C. The medium pH decreases from 6.5 to 4.4 towards stationary growth. Like the other LAB, leuconostocs have a high demand for growth factors and need complex media (Reiter and Oram, 1982; Garvie, 1986a).

Leuconostoc spp. are physiologically closely related to heterofermentative lactobacilli, but are differentiated from other LAB by their morphology and the exclusive production of D-lactate from D-glucose (Axelsson, 1998). Sequencing of 16S rDNA, 23S rDNA and of the *rpo*C gene (encoding the small subunit of DNA-dependent RNA polymerase) have placed *Leuconostoc* spp. into a phylogenetically defined group (*Leuconostoc 'sensu stricto*'), distinct from heterofermentative *Lactobacillus* spp., *Weissella* spp. and O*enococcus oeni* (Garvie, 1986a). The *Leuconostoc* genus comprises *L. mesenteroides*, with the subspecies *mesenteroides*, *dextranicum* and *cremoris*. Other species include: *Leuconostoc amelibiosum*, *Leuconostoc argentinum*, *Leuconostoc carnosum*; *Leuconostoc citreum*, *Leuconostoc dextranicum*, *Leuconostoc durionis*, *Leuconostoc fallax*, *Leuconostoc ficulneum*, *Leuconostoc fructosum*, *Leuconostoc gasicomitatum*,

Leuconostoc gelidum, *Leuconostoc kimchii*, *Leuconostoc inhae*, *Leuconostoc lactis* and *Leuconostoc pseudomesenteroides* (Euzéby, 2005).

Leuconostoc strains are used in several industrial fermenting processes for the production of food and beverages, but they are highly undesirable in some products. They can improve or decrease quality according to the strain and to the conditions (Sutherland, 1996). *Leuconostoc* species together with *Lactococcus*, *Streptococcus* and *Lactobacillus* spp. are used in the production of fermented milk, butter and cheese. They have poor acidifying abilities and are mainly selected for their capacity to produce typical aroma compounds such as ethanol, acetoin and diacetyl. The balance between diacetyl, which is the most aromatic, and the other products is very dependent on the pH of the medium, temperature and redox potential, probably much more than on the strain itself. The sensory quality of fermented milk also depends on the viscosity. Slime is formed from the synthesis of polysaccharides. Besides other ropy strains, *Leuc*. *mesenteroides* subsp. *mesenteroides* and *dextranicum* strains synthesize dextrans from saccharose. This inducible and unstable property must be controlled when preparing starters. Excessive ropiness may also lower the quality of yoghurts (Sutherland, 1996).

Like other LAB, leuconostocs preserve food by producing antagonistic compounds, or when competing with the indigenous microflora by exhausting most of the available nutrients. They exhibit antagonistic activities against closely related bacteria and potential pathogenic microorganisms. In chilled beef stored under vacuum, off-flavours and discoloration by *L. sakei* and *Carnobacterium maltaromicus* are prevented by seeding with an antagonistic strain of *L. gelidium* (Borch et al., 1996). This strain produces the bacteriocin leucocin A. Similarly, two strains of *L. carnosum* and *L. mesenteroides* subsp. *dextranicum* isolated from meat produce bacteriocins active against LAB and *Listeria* spp. The bacteriocin-coding genes are homologous to the corresponding N-terminal coding region of leucocin A. Moreover, mesentericin (Y 105) from *L. mesenteroides*, although from a different source, differs from leucocin A by only two amino acids and inhibits *Lactobacillus*, *Carnobacterium* and *Listeria* spp. This

suggests that bacteriocins closely related to leucocin A may occur in several other *Leuconostoc* spp. (Klaenhammer, 1993).

The useful property of *L. mesenteroides* subsp. *mesenteroides* or subsp. *dextranicum* to produce dextran in some cases becomes a real spoilage factor in others. The biodeterioration of sugar cane includes souring and dextran formation which can lead to a 4–9% loss of recoverable sugar. High viscosity also induces significant processing problems such as retardation of crystallization and reduced yields. Similar problems occur in the sugar beet industry (Tallgren et al., 1999). In the rum industry leuconostocs also forms dextrans during fermentation. If their population is high enough, they inhibit yeasts and can even stop alcoholic fermentation (Tallgren et al., 1999).

The biochemical and pharmaceutical industry have been conducting the commercial production of dextrans and levans by *L. mesenteroides* for more than 50 years (Broker, 1977; Alsop, 1983; Sutherland, 1996). Dextrans are used in the manufacture of blood plasma extenders, heparin substitutes for anticoagulant therapy, cosmetics, and other products (Alsop, 1983; Sutherland, 1996). Another use of dextrans is the manufacture of Sephadex gels or beads. These gels are used for fractionation and purification of biopolymers, including, human serum albumin, blood clotting factors, immunoglobulin G and haptoglobulin. Insulin producers use Sephadex gel to remove proinsulin and protease impurities in the final stages of purification of porcine or bovine insulins (Sutherland, 1996).

The genus *Pediococcus*

Pediococci are facultatively anaerobic cocci, 0.6-1.0 mm in diameter. A distinctive characteristic of pediococci is the formation of tetrads via cell division in two perpendicular directions in a single plane (Simpson and Taguchi, 1995). The genus consists of eight species, viz. *P. acidilactici*, *P. pentosaceus*, *P. parvulus*, *P. dextrinicus*, *P. damnosus*, *P. inopinatus*, *P. halophilus*, and *P. urinaeequi* (Dellaglio et al. 1981; Garvie, 1986b; Kim et al. 1992). Phylogenetically, the genera *Pediococcus* and

Lactobacillus form a super-cluster divided into two sub-clusters. Species of *Pediococcus* fall within the *Lactobacillus casei – Pediococcus* sub-cluster.

Pediococci have a strictly fermentative metabolism with lactic acid as the major metabolic end product (Garvie, 1986b; Axelsson, 1998). Lactic acid is produced from hexose sugars via the Embden-Meyerhof pathway and from pentoses by the 6 phosphogluconate/phosphoketolase pathway (Axelsson, 1998). Strains of *P. pentosaceus* have been reported to contain between three and five resident plasmids (Graham and McKay, 1985). Plasmid-linked traits include the ability to ferment raffinose, melibiose, and sucrose, and the production of bacteriocins. Plasmids can be conjugally transferred between *Pediococcus, Enterococcus*, *Streptococcus* and *Lactococcus* (Gonzalez and Kunka, 1983).

Pediococci, especially *P. pentosaceus* and *P. acidilactici*, can be isolated from a variety of plant material and fruit. *Pediococcus pentosaceus* have been isolated from the gastrointestinal tract of poultry (Juven, et al., 1991), ducks (Kurzak et al., 1998), and other animals, including insects (Vanbelle et al., 1990; Tannock, 1997; Hudson et al., 2000). *Pediococcus pentosaceus* is used as a starter culture in sausage fermentations, cucumber and green bean fermentations, soya milk fermentations, and silage (Simpson and Taguchi, 1995). *Pediococcus pentosaceus* and *P. acidilactici* are found in most cheese varieties during ripening (Beresford et al., 2001). In the brewing industry *P. damnosus* is a contaminant of pitching yeast (Stiles and Holzapfel, 1997).

Pediocins, inhibitory to a range of food pathogens, have been isolated from *P. pentosaceus* and *P. damnosus* (Daeschel and Klaenhammer, 1985; Gonzalez and Kunka, 1986). Pediocin is mostly inactive against spores, but inhibits *Listeria monocytogenes*. In Europe, pediocin is used in the form of a dried powder or in a culture liquid to extend the shelf life of salads and salad dressings, and to serve as an anti-listerial agent in products such as cream, cottage cheese and meats products (Montville and Winkowski, 1997). Several commercial probiotic feeds containing *P. pentosaceus* are available (Vanbelle et al., 1990).

The genus *Lactococcus*

Lactococci are spherical homofermentative bacteria and produces exclusively L(+)-lactic acid from D(-)-glucose. They grow between 5°C and 40°C, with optimum growth at 30°C (Schleifer and Ludwig, 1995). Under anaerobic conditions, lactococci have a fermentative metabolism that enables the transformation of various types of carbohydrates to lactic acid and trace amounts of acetate, ethanol, formate, and 2,3-butanediol (Condon, 1987). Under aerobic conditions a mixture of lactate and acetate is produced. The ability of *L. lactis* to grow under aerobic conditions is associated with the presence of NADH oxidase which contributes to the regeneration of NAD^+ during the metabolism of carbohydrates (Condon, 1987; Duwat et al., 2001).

Schleifer et al. (1985) generated the genus *Lactococcus* by separating the mesophilic lactic streptococci from the true streptococci (genus *Streptococcus*) and the enterococci (genus *Enterococcus*). The genus *Lactococcus* comprises the species *Lactococcus lactis* subsp. *lactis*, *Lactocccus lactis* subsp. *diacetylactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus garvieae*, *Lactococcus plantarum*, *Lactococcus rafinolactis* and *Lactococcus piscium* (Pot et al., 1994). Lactococci are commonly found in nature, on plant and animal surfaces and in the intestine of fish and insects (Shannon et al., 2001; Reesen et al., 2003). Lactococci are not considered to be natural inhabitants of the human gastrointestinal tract (Stiles and Holzapfel, 1997).

Many of the functions important for successful fermentations in lactococci are linked to plasmid DNA (McKay and Baldwin, 1990). Plasmids are commonly exchanged between strains via conjugation and with the chromosome by Insertion Sequence (IS) elements (Dunny and McKay, 1999). IS elements are segments of DNA in bacteria that can move from one position to another. This causes insertional mutations. When IS elements transpose, promoters within IS elements themselves may alter expression of nearby genes (Dunny and McKay, 1999). These exchanges and rearrangements mediate rapid strain

adaptation and evolution and add to the instability of important metabolic functions in food fermentations (Beimfohr et al., 1997).

Lactococci are widely used in the dairy industry for the production of cheese and buttermilk. *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are the most important lactic acid bacteria used in the dairy industry (Salema et al., 1991; Stiles and Holzapfel, 1997). The DNA sequence divergence between the subspecies *lactis* and *cremoris* is estimated to be between 20 and 30%. *Lactococcus lactis* subsp. *cremoris* can be distinguished from *L. lactis* subsp. *lactis* by its inability to produce acid from maltose and ribose, growth at 40^{o}C and in the presence of 4% (w/v) NaCl (Schleifer et al., 1985).

Lactocccus lactis is the most extensively characterized LAB and has been used to produce heterologous proteins of biotechnological and medical interest, such as enzymes and antigens (Bolotin et al., 2001). *Lactocccus lactis* also has potential as a live vaccine (Langella and Le Loir, 1999).

Bolotin et al. (2001) sequenced the complete genome of *Lactococcus lactis* subsp. *lactis* IL1403. The genome is 2.4 Mb in size and revealed a number of unexpected findings, such as the genes encoding the biosynthetic pathways for all 20 amino acids, albeit not all of which are functional, a complete set of late competence genes, complete prophages, and partial components for aerobic metabolism. The presence of numerous pseudogenes suggests *Lactococcus lactis* subsp. *lactis* is undergoing a regressive evolution process towards a specialised bacterium dedicated to growth in milk. Evolution has shaped the *L. lactis* genome by selection for optimal growth in this well-defined ecological niche. *Lactococcus lactis* has maintained a well-developed nitrogen metabolism while its sugar catabolism has strongly degenerated. *Lactococcus lactis* shares its ecological niche with other LAB such as *L. bulgaricus*, resulting in specific metabolic cooperation, which is either revealed by the maintenance of dedicated pathways (e.g. folate and formate production) or by the loss of key metabolic functions provided by the symbiotic partner (e.g. casein hydrolysis) (Bolotin et al., 2001).

Small genomic islands acquired by lateral gene transfer are present in *L lactis* subsp. *lactis*. These regions encode a number of important industrial phenotypic traits such as polysaccharide biosynthesis, bacteriocin production, restriction-modification systems and oxygen tolerance. The restricted ecological niche and its corresponding adaptive evolution provide *L. lactis* subsp. *lactis* with the ability to grow under favorable conditions (Bolotin et al., 2001).

It is interesting to note that small genomes have also evolved in pathogens, such as mycoplasmas and chlamydias, as well as in mutualistic symbionts, such as those found in insects. The smallest genome currently known for any cellular organism is 450 kb in a *Buchnera* sp. (Gill et al., 2002). Reductions in genome size results from a decreased selection to maintain gene functionality. This reduction is greater than any increase due to horizontal transfer and gene duplication leading to an overall reduction in genome size, a process known as deletional bias. Deletional bias itself has been proposed as defense against the invasion of IS elements and phages (Lawrence et al., 2001).

Lactococcus lactis which produces nisin was the first bacteriocin that received GRAS status (Federal Register, 1988). Nisin is used to inhibit listerial growth and biofilm formation. The spores of *Clostridium botulinum* become more sensitive to heat treatment when nisin is applied to a product. Nisin has been added to a variety of food products including milk, cheese and other dairy products, canned foods, mayonnaise and baby foods. In cheese spreads, it is used as an antibotulinal agent while in the dairy industry teats are dipped in nisin to prevent mastitis (Montville and Winkowski, 1997).

The genus *Enterococcus*

Enterococci are facultative anaerobe bacteria that occur singly, in pairs or short chains. Growth occurs between 10°C and 45°C, with an optimum at 35°C. Enterococci can grow in broth containing 6.5% NaCl or at pH of 9.6 and are able to survive 60°C for 30 min (Collins et al., 1989a).

The genus *Enterococcus* was first described in 1899 (Stiles and Holzapfel, 1997) and subsequently divided into four groups; 'enterococci' (or faecal streptococci), dairy streptococci, the Viridans group and the pyogenous streptococci by Sherman (1937). The groups 'viridans' and 'enterococci' have been reclassified to oral and faecal streptococci respectively (Jones, 1978).

Based on 16S rDNA sequence data, the genus *Streptococcus* '*sensu lato'* was split into *Streptococcus* '*sensu stricto'*, the genera *Enterococcus* and the genera *Lactococcus* (including the 'lactis'-group) (Schleifer and Kilpper-Balz, 1984). Currently, 26 species have been validly published and at least three more species are proposed for validation (Euzéby, 2005). Recently described species have considerable differences in their physiological and biochemical behaviour compared to typical enterococci.

The *Enterococcus faecalis* group comprises *E. faecalis*, *Enterococcus haemoperoxidus* and *Enterococcus moraviensis*. The *Enterococcus faecium* group comprises *E. faecium*, *Enterococcus durans*, *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus porcinus* and *Enterococcus villoru*. The *Enterococcus avium* group comprises *E. avium*, *Enterococcus pseudoavium*, *Enterococcus malodoratus* and *Enterococcus raffinosus*. The *Enterococcus casseliflavus* group comprises *E. casseliflavus*, *Enterococcus gallinarum* and *Enterococcus flavescent*. The *Enterococcus cecorum* group comprises *E. cecorum* and *Enterococcus columbae*. The *Enterococcus dispar* group comprises *E. dispar* and *Enterococcus asini*, and the *Enterococcus saccharolyticus* group comprises *E. saccharolyticus* and *Enterococcus sulfureus*. Other species are *Enterococcus gilvus*, *Enterococcus pallens* and *Enterococcus ratti*. *Enterococcus solitarius* is validly published but, based on molecular data, belongs to the genus *Tetragenococcus* (Franz et al., 1999).

E. faecium is mainly used as an animal probiotic and *E. faecalis* as a human probiotic. *Enterococcus faecium* differs from *E. faecalis* in its growth requirements and metabolism. It requires folic acid for growth and is unable to derive energy from pyruvate, citrate, malate, gluconate and serine (Nusser, 1991; Devriese et al., 1993).

Some enterococci may cause disease, especially in patients with underlying disease. Infections caused by the genus *Enterococcus* (most notably *E. faecalis*, which accounts for around 80% of all hospital infections) include urinary tract infections, bacteremia, intra-abdominal infections, and endocarditis (Huycke et al., 1998).

This dualistic nature of enterococci gives rise to concern about their use and safety as probiotics and starter cultures in the food industry. Enterococci can acquire resistance against ampicillin. They can also acquire resistance against glycopeptide antibiotics (e.g., vancomycin and teicoplanin), which are used to treat infections of multiresistant enterococci (Leclercq and Courvalin, 1996). The potential spread of antibiotic resistant enterococci in the environment is an unwanted consequence in the use of antibiotics.

The genus *Bifidobacterium*

Bifidobacteria were originally isolated and described in the period 1899–1900 (Sgorbati et al., 1995). They were originally isolated from human faeces and were quickly associated with a healthy GI tract due to their abundance in breast-fed infants compared to bottle-fed infants (Sgorbati et al., 1995). They belong to the Actinomycetales branch of the high G+C Gram-positive bacteria (Klein et al., 1998; Ventura et al., 2004). This branch also includes the *Corynebacterium*, *Mycobacterium* and *Streptomycetales* families (Stiles and Holzapfel, 1997). Bifidobacteria are rods of variable appearance, usually somewhat curved and clubbed. In unfavourable growth conditions they show branching and pleomorphism (Poupard et al., 1973). Bifidobacteria are facultatively anaerobic (Simpson et al., 2004). The sensitivity to oxygen differs between species and between different strains within a species (Shimamura et al., 1992; Ahn et al., 2001; Talwalkar and Kailasapathy, 2003). *Bifidobacterium psychraerophilum*, isolated from pig caecum, tolerates high levels of oxygen and grows under aerobic conditions (Simpson et al., 2004).

Most human strains of bifidobacteria grow optimally at 36 to 38°C, whereas animal strains appear to have a slightly higher optimum growth temperature of 41 to 43°C. The

exception is *Bifidobacterium thermacidophilum,* which exhibits a maximal growth temperature of 49°C (Dong et al., 2000) and a *B. psychraerophilum* which grows at temperatures as low as 4°C (Simpson et al., 2004). Bifidobacteria are acid-tolerant with an optimum growth pH between pH 6.5 and pH 7.0. Strains of *Bifidobacterium lactis* and *Bifidobacterium animalis* can survive exposure at pH 3.5 (Saavedra et al., 1994). Bifidobacterium strains do not survive pH 8.5 (Biavati and Mattarelli, 2001). The cell walls of bifidobacteria have a typical Gram-positive structure, consisting of a thick peptidoglycan envelope containing polysaccharides, proteins and teichoic acids (Gomes and Malcata, 1999). The amino acid composition of the basic tetrapeptides of murein can differ among species and even among strains of the same species and can, in some cases, be used for their differentiation (Lauer and Kandler, 1980).

Bifidobacteria are saccharolytic organisms and have the ability to ferment glucose, galactose and fructose. Differences in their ability to ferment other carbohydrates and alcohols occur between species (Sgorbati et al., 1995; Gomes and Malcata, 1999; Ventura et al., 2004). Glucose is fermented via the fructose-6-phosphate shunt to acetic and lactic acid. Fructose-6-phosphate Phosphoketolase (F6PPK) is a key enzyme and its presence is the most common diagnostic test for this genus, as it is not present in other Gram-positive intestinal bacteria (Sgorbati et al., 1995).

2.2 Taxonomic methods

Introduction

Taxonomist gathers organisms into defined groups, provides appropriate nomenclature for the different groups and are involved in the identification of previously unknown microorganisms. Before the introduction of molecular biology techniques, taxonomic studies were hampered by a lack of clear concepts on the identity of micro-organisms and lack of methodologies to analyse complex communities. Detection and identification were almost completely established on culture-based methods and the species concept

was based on phenotypic rather than genotypic characteristics. Since gene expression is often influenced by environmental factors, such as substrate supply, pH, temperature and redox potential, the phenotype of an organism is less stable than its genotype. As a consequence, bacterial taxonomy contains many controversies (Holzapfel et al., 2001).

A molecular approach to taxonomy has activated the interest in evolution, the origin of life and opened up the opportunity to analyse complex communities on the basis of DNA sequence diversity. By simply retrieving DNA sequences from the environment and comparing these with known sequences from the database, it became clear that most of these DNA sequences were new (Amann et al., 1995). New molecular technologies are also increasingly used for analysis of the complex intestinal ecosystem of mammals, birds and insects. They contribute to a better understanding of the interaction between host and microbes in the intestinal tract.

Typing systems

Microbial typing data is mandatory for the definition of species. Typing systems are used to define specific characteristics of the object under study. The procedures are specific for different phenotypic or genetic parameters and can be general (i.e., applicable to any microbial species), species or genus specific. For example, plasmid profiling is adequate only for organisms possessing these extrachromosomal elements. Ideally a typing system should have a high degree of reproducibility. In addition, the procedure should not be too costly or complicated and should be easily accessible (Holzapfel et al., 2001).

Phenotypic methods

Phenotypic methods include the examination of cell and colony characteristics (form, colour and dimension). Cell wall composition (especially for bifidobacteria), cellular fatty acid composition and the structure of isoprenoid quinines are also used to characterize bacteria. Physiological features include the organism's ability to grow at different temperatures, pH levels, salt concentrations, in the presence of different

chemicals (e.g. antimicrobial agents) and the metabolism of different compounds (Schleifer and Kandler, 1972; Vandamme et al., 1996). Some of the phenotypic characteristics used to distinguish lactic acid bacteria are shown in Table 2. The fermentation pattern of carbohydrates can be used for strain identification at species level. Lactic acid bacteria often contain plasmids coding for key enzymes involved in biochemical pathways. Due to the instability of plasmids, especially in the absence of selective pressure, some tests which are usually positive can turn negative (Holzapfel et al., 2001).

Table 2. Phenotypic characteristics for differentiation of selected genera of lactic acid bacteria (Axelsson, 1998)

Character	*Carno*	*Lactob*	*Aeroc*	*Enteroc*	*Lacto/Vago*	*Leuco/Oenoc.*	*Pedio*	*Strepto*	*Tetragen*	*Weissella*
Tetrad formation	–	–	+	–	–	–	+	–	+	–
$C0_2$ from glucose[b]	–	+/–	–	–	–	+	–	–	–	+
Growth at 10°C	+	+/–	+	+	+	+	+/–	–	+	+
Growth at 45°C	–	+/–	–	+	–	–	+/–	+/–	–	–
Growth at 6.5% NaCl	ND	+/–	+	+	–	+/–	+/–	–	+	+/–
Growth at 18% NaCl	–	–	–	–	–	–	–	–	+	–
Growth at pH 4.4	ND	+/–	–	+	+/–	+/–	+	–	–	+/–
Growth at pH 9.6	–	–	+	+	–	–	–	–	+	–
Lactic acid[c]	L	D,L,DL[d]	L	L	L	D	L,DL[d]	L	L	D, DL[d]

[a]+, positive; -, negative; ND, not determined

[b]Test for homo- or heterofermentation of glucose; negative and positive denotes homofermentative and heterofermentative respectfully.

[c]Configuration of lactic acid produced from glucose.

[d]Production of D-, L- or DL-lactic acid varies among species.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE groups bacteria by comparing their whole cell protein patterns obtained by highly standardised SDS-PAGE. Digitally processed electrophoretic patterns of representative strains can be stored in computer files to identify other unknown isolates (Vandamme et al., 1996). Comparison of the protein fingerprints gives a reliable measure of taxonomic relatedness (Vandamme et al., 1996). A disadvantage of this technique is

that it is time consuming. Standardised and reproducible experimental conditions are also required.

Genotypic methods

All molecular genetic methods for distinguishing organism subtypes are based on differences in their DNA sequences. Classification of LAB is becoming more dependent on genotypic methods to eliminate overlapping phenotypic characteristics among genera. Genotypic methods include (i) DNA-base composition, (ii) DNA hybridisation studies, (iii) 16S and 23S rDNA sequence analysis and (iv) RAPD (random amplified polymorphic DNA) PCR (Pot et al., 1994). Nucleic acid probes have been developed for several species of lactic acid bacteria often in combination with priming methods (Drake et al., 1996; Tilsala-Timisjarvi and Alatossava, 1997).

Denaturing gradient gel electrophoresis (DGGE)/temperature gradient gel electrophoresis (TGGE)

DGGE and TGGE are gel-electrophoretic separation procedures for double stranded DNA of equal size, but with different base-pair composition or sequence (Muyzer and Smalla, 1998). DGGE and TGGE are sensitive enough to separate DNA on the basis of single point mutations (Sheffield et al., 1989). Both techniques are gaining increased popularity in microbial ecology for analysing the diversity of total bacterial communities. In PCR-DGGE, DNA is extracted from biological samples and the 16S rDNA genes are amplified using the appropriate primer pair. One of the primer pairs has a G+C "clamp" attached to the 5' end that prevents the two DNA strands from completely dissociating, even under strong denaturing conditions. This approach allows amplification of unknown bacterial species. The mixture of PCR products, all approximately of the same length, is subsequently separated on a polyacrylamide gel containing a linear gradient of DNA denaturants. Sequence differences in the double stranded DNA influence the melting behavior of the PCR amplicons, therefore PCR amplicons with different sequences will migrate to different positions in the gel. This results in separation of amplicons, and the

pattern of separated bands illustrates the bacterial diversity in the sample. The intensity of an individual band is a semi-quantitative measure for the relative abundance of this sequence in the population (Muyzer and Smalla, 1998).

TGGE and DGGE of 16S rDNA amplicons are exceptional tools to study the species composition of unknown samples. Since individual bands can be excised and sequenced after electrophoresis, the identity of the bacteria present in the sample can be determined without cultivation. By inter-sample comparison, dominant shifts in population composition can be monitored and bacterial population dynamics can be studied in more detail (Muyzer and Smalla, 1998).

Other applications of these techniques include identifying 16S rDNA sequence heterogeneity (Nubel et al., 1996), monitoring specific physiological groups, facilitating isolation and determining PCR biases (Muyzer, 1999). As an alternative to comparing DGGE profiles by eye, similarity indices may be calculated by computer analysis of scanned fingerprints or using Shannon-Weaver indices which allows a more subjective analysis of data (Nubel et al., 1996).

Random Amplified Polymorphic DNA (RAPD)-PCR

The RAPD-PCR assay, also referred to as arbitrary primed PCR, was first described by Welsh and McClelland (1990). RAPD assays are based on the use of short random sequence primers, 9 to 10 bases in length, which hybridise with sufficient affinity to chromosomal DNA sequences at low annealing temperatures. They can then be used to initiate amplification of regions of the bacterial genome. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product, with a molecular length corresponding to the distance between the two primers is formed. The number and location of these random primer sites vary for different strains of a bacterial species. The separation of the amplification products by agarose gel electrophoresis results in a pattern of bands which is characteristic of the particular bacterial strain (Welsh and McClelland, 1990; Van Reenen and Dicks, 1996).

In most cases the sequences of the RAPD primers which generate the best DNA pattern for differentiation must be determined empirically by fingerprinting assays. This allows for some standardization of the procedure (Vila et al., 1996).

RAPDs assays have been used to distinguish among *L. pentosus*, *L. acidophilus*, *L. plantarum L. reuteri*, *L. fermentum*, *L. brevis* and *L. buchneri* (Du Plessis and Dicks, 1995; Van Reenen and Dicks, 1996). The genetic diversity of strains of *L. plantarum* and *O. oeni* has been assessed using RAPD-PCR (Van Reenen and Dicks, 1996; Reguant and Bordons, 2003). Vila et al. (1996) found that the RAPD assay was more discriminating than restriction fragment length polymorphism (RFLP) analysis of either the 16S rDNA genes or the 16S-23S rDNA spacer region, but less discriminating than repetitive extragenic palindromic PCR (Rep-PCR).

Disadvantages of RAPD are a lack of reproducibility, standardisation and difficulty when interpretating profiles. Many of the priming events are the result of imperfect hybridisation between the primer and the target site since the primers are not directed against any particular genetic locus. Therefore the amplification process is extremely sensitive to slight changes in the annealing temperature which can lead to variability in the banding patterns. The use of empirically designed primers, each with its own optimal reaction conditions and reagents, also makes standardisation of the technique difficult (Reguent and Bordons, 2003).

DNA-DNA hybridization

The percentage DNA binding (De Ley, 1970), the DNA-DNA hybridization value, or the relative binding ratio (Brenner et al., 1969; Grimont et al., 1980) are all indirect parameters of the sequence similarity between two genomes. The most common methods are the hydroxyapatite method (Brenner et al., 1969), the optical renaturation method (De Ley, 1970) and the S1 nuclease method (Grimont et al., 1980).

This technique is used to determine close relationships (at species and subspecies level) between species and has been used in the description of new species, in some cases having been the only way to resolve identification problems (Wayne et al., 1987; Dicks et al., 1995; Van Reenen and Dicks, 1996).

Contention remains as to whether data obtained with short oligonucleotides and experimentally induced mispairings can be extrapolated to entire genomes, making it impossible to convert a percentage DNA-binding or DNA-DNA hybridisation value into a percentage of whole genome similarity (Vandamme et al., 1996; Fournier et al., 2003).

DNA-DNA hybridization studies have several disadvantages. As the technique depends on physiochemical parameters, the results are not cumulative. It is labour intensive and requires the use of large quantities of DNA (Stackebrandt and Goebel, 1994).

16S/23S rDNA sequencing analysis

A new standard for identifying bacteria developed in the 1980's. It was shown that phylogenetic relationships of bacteria and all other life-forms could be determined by comparing a stable part of the genetic code (Woese et al., 1985; Woese, 1987).

Candidates for this genetic area in bacteria included the genes that code for the 5S, the 16S (also called the small subunit), the 23S rDNA and the spaces between these genes. The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rDNA gene (Bottger, 1989; Garrity and Holt 2001; Tortoli, 2003; Harmsen and Karch 2004). The 16S rDNA gene can be compared not only among all bacteria but also with the 16S rDNA gene of archeobacteria and the 18S rDNA gene of eukaryotes.

Woese (1987) defined the important properties of this gene. Foremost is the fact that it behaves as a molecular chronometer. The degree of conservation is assumed to result from the importance of the 16S rDNA as a critical component of cell function. This is in contrast to the genes needed to make enzymes. Mutations in the latter genes can usually

be tolerated more frequently than in the former as their products are not as unique and essential as rDNA. If a bacterium does not have the gene to make the enzymes needed to utilize lactose, it can use an alternative sugar or protein as an energy source. Thus, few other genes are as highly conserved as the 16S rDNA gene. Although the absolute rate of change in the 16S rDNA gene sequence is not known, it does mark evolutionary distance and relatedness of organisms (Kimura, 1980; Thorne et al., 1998; Harmsen and Karch, 2004).

Problems in assigning a numerical value to this rate of change include the possibility that (a) the rate of change of 16S rDNA gene may not be identical for all organisms (different taxonomic groups could have different rates of change), (b) the rates could vary at times during evolution, (c) the rates could be different at different sites throughout the 16S rDNA gene. There are so-called "hot spots" which show larger numbers of mutations (Tortoli, 2003). These areas are not the same for all species. 16S rDNA is also the target for several antimicrobial agents. Mutations in the 16S rDNA gene can affect the susceptibility of the organism to these agents. Therefore the 16S rDNA gene sequence can distinguish phenotypic resistance to antimicrobial agents. However, these characteristics do not prevent the use of 16S rDNA gene sequence for bacterial identification or assignment of close relationships at the genus and species level. They can, however, have a greater impact on the assignment of relationships of the deeper (more distantly related) branches. It has however, been observed that the trees based on whole-genomic analysis and the 16S rDNA gene trees are similar (Harmsen and Karch, 2004). The new edition of *Bergey's Manual of Systematic Bacteriology*, the most widely used and authoritative reference on bacterial taxonomy, is organized using 16S rDNA gene sequence analysis as the backbone.

The 16S rDNA gene sequence is about 1,550 bp long and is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecific polymorphisms, to provide distinguishing and statistically valid measurements. Universal primers are usually chosen to be complementary to the conserved regions at the beginning of the gene and at either the 540-bp region or at the end of the whole sequence

(at about the 1,550-bp region). The sequence of the variable region in between is used for comparative taxonomy (Chen et al., 1989; Relman, 1999). Although 500 and 1,500 bp are common lengths to sequence and compare, sequences in databases can be of various lengths.

The 16S rDNA gene sequence has been determined for a large number of strains. GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rDNA gene. There, are therefore, many previously deposited sequences against which to compare the sequence of an unknown strain. The 16S rDNA gene is universal in bacteria so relationships can be measured among all bacteria (Woese et al., 1985; Woese, 1987). In general, the comparison of the 16S rDNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria. In addition, it allows the classifying of strains at multiple levels, including what we now call the species and subspecies level. The occasional exceptions to the usefulness of 16S rDNA gene sequencing usually relate to more than one well-known species having the same or very similar sequences. Results of rDNA sequencing led to the taxonomic reassignment of many *Lactobacillus* species to other taxa. Collins and Wallbanks (1992) transferred *Lactobacillus minutus*, *Lactobacillus rimae* and *Lactobacillus uli* to the genus *Atopobium*.

Sequencing of the entire 1,500-bp fragment is desirable and usually required when describing a new species. Sometimes sequencing the entire 1,500-bp region is necessary to distinguish between particular taxa or strains (Sacchi et al. 2002, a and b). For most isolates the initial 500-bp sequence provides adequate differentiation for identification and in fact can provide a bigger percent difference between strains because the region shows slightly more diversity per kilobase sequenced (Sacchi et al., 2002, a and b). Other researchers have made identifications using sequences of about 400 bp (Bosshard et al., 2003) or even less than 200 bp (Wilck et al., 2001).

There is no consensus on the exact degree of genetic difference in the 16S rDNA that defines a species or on the mathematical algorithm to be used to generate the data.

Stackebrandt and Ludwig (1994) suggested organisms that generally share more than 97% rDNA sequence similarity may belong to a single species as this value corresponds to two organisms having 70% or greater DNA similarity which is the value used for the delineation of bacterial species. Bosshard et al. (2003) used 99% similarity to define a species and 95% to 99% to define a genus. Fox et al. (1992) proposed a difference of at least 5 to 15 bp in the whole 16S rDNA gene sequence to define a species. Turenne et al. (2001) designated the reportable range for a species as 0.8 to 2.0% and suggested that a sequence could obviously be called unique, i.e., representing an organism whose sequence has not yet been deposited and thus might be a novel species, if there were at least 20 to 38 bp difference in sequence. Tang et al. (1998) suggested a 0.5% difference as the limit for species designation. A strain with a small genotypic difference (less than 0.5%) has often been considered a subspecies (Chen et al., 2002). When there is a clear phenotypic uniqueness, genogroups with less than 1.0% differences in sequence have in fact been named as new species (Roth et al., 2003; Tortoli, 2003).

The total amount of intraspecies variability to be allowed is also not clear. For example, using the guideline that an unknown strain should be less than 1% different from the type strain, might mean that hypothetical strain 1 and strain 2 of the same species are 2% different from each other. In general, there is agreement that all sequences of strains within the same species should be close, no more than 1 to 1.5% differences in base pair sequence. A 5 to 7% divergence in the 16S rDNA sequence is considered enough to distinguish between genogroups.

In conclusion it is not possible to give a definite value to define genus and species. This is in part because different values are generated by analyzing separate databases and using different methods. The percent difference can vary if it is calculated using only the first 500 bp or all 1,500 bp and can also vary with the program used for the calculations. It also is probable that a single value for the definition of a genus or species on the basis of the 16S rDNA gene sequence is not appropriate for all genera (Tortoli, 2003; Harmsen, and Karch, 2004).

Whole genome sequencing

Full-genome sequencing is the most detailed typing system available to researchers for the classification of microorganisms. The first complete genome of the LAB group was published on *Lactococcus lactis* subsp. *lactis* IL1403 by Bolotin et al. (2001) and currently around 29 other LAB genomes have either been sequenced or are in the process of being sequenced (Kleerebezem et al., 2003). Among the total genome projects ongoing, there are several cases where genome sequences will become available for multiple strains of the same species, notably *L. lactis* (three strains), *L. casei* (two strains), *L. delbrueckii* (three strains), *Streptococcus thermophilus* (three strains), *O. oeni* (two strains) and *B. longum* (two strains).

Once full-genome sequences are available for multiple isolates of a single bacterial species, all genetic variables can be catalogued. The nature of the mutations thus identified can be helpful in clarifying the relatedness between these isolates. Alternatively, if multiple isolates from multiple species have been sequenced in full, the data collection will also define the relatedness or lack thereof between microbial species and genera (Kleerebezem et al., 2003). The differences between *L. plantarum* and *L. johnsonii*, both in genome organization and in gene content, were found to be exceptionally large for two bacteria of the same genus (Suyama and Bork, 2001). This low degree of symmetry between *L. plantarum* and *L. johnsonii* suggests that they are only marginally more related to each other than to other Gram-positive bacteria. These findings emphasize the difficulty in taxonomic classification of LAB

The ability to discriminate between genomes is not only essential to taxonomy but also to studies of evolutionary mechanisms, phylogenetic relationships, population genetics of microorganisms, and microbial epidemiology. Comparison of the genome sequences of multiple LAB species and strains is expected to provide a critical view of microbial adaptation and genetic events leading to their adaptation to specialized environments.

Comparative genomics among the microbes sequenced thus far has already illustrated that essential housekeeping gene functions are widely conserved among microbes and horizontal gene transfer commonly occurs. An expected outcome of comparative genomics of LAB will be the definition of conserved and unique genetic functions in LAB that enable core functions, e.g., production of lactic acid, proteolytic and peptidase activities, survival at low pH, stress tolerance, production of antimicrobials, transport systems, cell signaling, and attachment/retention in dynamically mobile environments (Bolotin et al., 2001; Kleerebezem et al., 2003).

It is anticipated that IS-elements, bacteriophages, and mobile genetic elements provide the major routes through which horizontal gene transfer occurs. They will indicate the most interesting and practically significant genetic regions that underscore the unique and beneficial properties of the LAB. It is well documented that the LAB undergo conjugation, exist in phage contaminated environments where gene transfer may occur by transduction and harbour sets or remnants of competence genes for transformation (Bolotin et al., 2001).

Understanding gene transfer, particularly in environments where LAB coexists will provide an important view of their evolution, adaptation and potential for unique applications. Conjugation has played a key role in the evolution and adaptation of *Lactococcus lactis* to a milk environment ensuring the attributes for growth in milk, including lactose and casein utilization and bacteriophage resistance. The explosion of available genome sequences for LAB will accelerate their exploitation in both traditional and non-traditional arenas.

Conclusion

Taxonomy is increasingly being based on more detailed phenotypic and genotypic data. Ribosomal DNA sequence information has allowed many taxonomic revisions during the last two decades. Traditional phenotypic analyses still plays an important role in the identification of strains. The re-evaluation of phenotypic classification systems for lactic

acid bacteria has been possible through the use of phylogenetic methods. An example of this is the effective phenotype description of new genera such as *Oenococcus* and *Weisella*, which form well-defined phylogenetic and phenotypic entities (Vandamme et al., 1996). DNA-DNA hybridisation studies still play a role in determining the inter- and intraspecific relationship among strains, which is not revealed by rDNA sequencing analysis.

A polyphasic approach, i.e. using a combination of all phenotypic and genotypic techniques, is the most effective way to identify unknown isolates. The unprecedented detail in which microbial isolates can now be typed will strengthen taxonomic coherence and at the same time bridge the gap between taxonomy on the one hand and evolutionary genetics and microbial epidemiology on the other.

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3. Identification of lactic acid bacteria from vinegar flies based on phenotypic and genotypic characteristics

Submitted for publication in the Journal of Systematic and Applied Microbiology

Identification of lactic acid bacteria from vinegar flies based on phenotypic and genotypic characteristics

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Keywords: Lactic acid bacteria, vinegar flies

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Abstract

Thirty lactic acid bacteria were isolated from the intestinal tract of *Drosophila simulans* Stuvervant collected from a winery in the Stellenbosch region, South Africa. The isolates were grouped according to morphological, biochemical and physiological characteristics. Strains selected from each group were identified to species level by PCR with species-specific primers, PCR-based DGGE and 16S rDNA sequencing. The majority of isolates belonged to the species *Lactobacillus plantarum,* but *Lactobacillus paracasei, Lactobacillus sanfranciscensis, Leuconostoc mesenteroides* subsp. *mesenteroides, Lactococcus lactis* subsp. *lactis, Enterococcus faecalis* and *Pediococcus pentosaceus* have also been identified. As far as we could determine, this is the first report on the isolation of *L. paracasei, L. sanfranciscensis, L. mesenteroides* subsp. *mesenteroides, L. lactis* subsp. *lactis, E. faecalis* and *P. pentosaceus* from vinegar flies. The genotypic relatedness between strains of *L. plantarum* was determined by RAPD-PCR.

Introduction

The intestinal tract of insects is a rich source of nutrients and supports the growth of a number of microorganisms [5]. However, only a few studies have been published on the presence of lactic acid bacteria (LAB) in insect gut. *Enterococcus faecalis* and *Lactococcus lactis* subsp. *lactis* have been isolated from the hindgut of termites [4,41], *Streptococcus* spp. from the gut of desert locusts [22] and crickets [44], *Lactococcus* spp. from the gut of mosquitoes [33], and *Lactococcus* spp., *Lactobacillus* spp. and *Leuconostoc* spp. from the intestinal tract of wasps [36]. The digestive tract of adult honeybees contains lactobacilli, bacilli, bifidobacteria [35] and enterococci [24]. *Melissococcus pluton* is closely associated with the brood of honey bees [2].

Little research has been done on the microbiota of vinegar flies, and early reports are contradictory. According to Gukasyan [19], the intestinal tract of vinegar flies is mostly free of bacteria. Kvasnikov et al. [28], on the other hand, reported the presence of *Lactobacillus plantarum* and enterococci in vinegar flies, but based their identification on physiological and biochemical characteristics, including sugar fermentation profiles which are often not reliable [27,45].

Our interest in the presence of LAB in vinegar flies (genus *Drosophila*) was fueled by the hypothesis that they may serve as a vehicle in the distribution of lactic acid bacteria in the vineyard, similar to what has been observed for vinegar flies in breweries [28]. *Drosophila* lives primarily on plant material and lay their eggs on unripened or slightly ripened fruit which is used by the developing larvae as primary source of nutrition [10,13].

The role these organisms play in the insect digestive tract is not always known. In a more recent paper [24], the role of LAB in the gut of bees was proposed to be the decomposition and detoxification of non-digested food. Lactic acid bacteria may also serve to protect insects from the invasion of intestinal pathogens (similar to probiotic strains in humans and animals), produce vitamins or form complex interactions with the immune system of the host [3,12].

In this study, strains isolated from vinegar flies collected from vineyards were identified to species level based on PCR with species-specific primers, PCR-based DGGE, 16S rDNA sequencing and RAPD-PCR.

Materials and methods

Isolation of lactic acid bacteria from vinegar flies

Vinegar flies were collected from Merlot vineyards using specially designed traps with grape must. The flies were sterilised with 2.5% (v/v) sodium hypochlorite, followed by rinsing with sterile distilled water. Water from the last washing was tested for the presence of LAB by plating onto MRS agar (Biolab, Biolab Diagnostics, Midrand, SA). The plates were incubated at 30 °C and examined after 48 h.

Approximately 100 surface-sterilised flies were placed in a sterile 50 ml centrifuge tube with 2 ml peptone water and vortexed with 20 glass beads (approximately 2 mm diameter) for 3 min at 25 °C. The fly homogenate was serially diluted in sterile distilled water and plated out, in triplicate, onto MRS agar, MRS agar supplemented with 20% (v/v) apple juice and adjusted to pH 5.5 with 1 N NaOH, *Melissococcus* agar [2] and Zuniga agar [51], respectively. All media contained 100 μg ml^{-1} Delvocid (GistBrocades, Delf, Netherlands) to inhibit the growth of yeast and fungi. One of three plates was incubated in an anaerobic flask (Oxoid, Basingstoke, Hampshire, England) in the presence of an Anaerocult gas generating kit (Oxoid). All plates were incubated at 30 ^{o}C for 24-48 h.

Preliminary identification of lactic acid bacteria

Colonies of different morphology were selected from each plate and streaked onto corresponding media to obtain pure cultures. All cultures were stored at -80 ^{o}C in MRS broth supplemented with sterile glycerol (30%, v/v, final concentration). Gram reaction and catalase activity were determined according to the methods described in

Harrigan and McCance [20]. Gram-positive and catalase-negative isolates were divided into rods and cocci and tested for the production of D (-) and L (+)-lactic acid using an enzymatic kit (Roche Molecular Chemicals GmbH, Mannheim, Germany). Production of CO_2 from glucose and gluconate was determined as described by Dicks and Van Vuuren [11]. Isolates with coccoid morphology were tested for growth at 45 ^{o}C and in medium supplemented with 6.5% (w/v) NaCl.

Carbohydrate fermentations

Carbohydrate fermentation reactions were recorded using the API 50 CHL system (BioMerieux, Marcy L'Etoile, France) according to the manufacturer's instructions. For two of the isolates provisionally identified as *Enterococcus* spp., the API 20 STREP system (BioMerieux) was used. Incubation of all API strips was at 30 ^{o}C. Results were recorded after 24, 48 and 72 h, respectively.

PCR with species-specific primers

The primers and reference strains used in this study are listed in Table 1. Primers were selected based on species previously identified from insects and closely related species. DNA was isolated according to the method of Dellaglio et al. [9]. Isolates were subjected to PCR amplification using the primer sets described in Table 1.

Denaturing gradient gel electrophoresis (DGGE)

Approximately 200 bp of the 5' end of the V3 variable region of the 16S rDNA gene was amplified using the eubacteria specific primers F341 (5'-<u>CGC CCG CCG CGC GCG GCG GGC GGG</u> GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and R534 (5'-ATT ACC GCG GCT GCT GG-3'), published by Muyzer et al. [31]. The GC clamp sequence is underlined. PCR reactions were performed according to the method of Garbers et al. [15]. Separation by DGGE was performed in a BioRad DCode Universal Mutation Detection System (Biorad Laboratories, Hemel Hempstead,

Hertfordshire, England), according to the method of Garbers et al. [15]. Controls used were the reference strains listed in Table 1.

Sequencing of DNA

Strains with different DGGE profiles were selected and their DNA amplified with primers F8 (5'-CAG GCA TCC AGA CTT TGA TYM TGG CTC AG-3') and R1512 (5'-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3'), as described by Felske et al. [14]. PCR was carried out according to the method described by Garbers et al. [15]. The amplified fragments (approximately1.5 kb long) were purified using the High Pure PCR Purification Kit (Roche Diagnostics GmbH, Manheim, Germany), according to the manufacturer's instructions. The fragments were sequenced and compared with sequences in Genbank using BLAST [1].

RAPD-PCR analysis

DNA of 22 isolates identified as *L. plantarum* were amplified with primers OPL-04 (GACTGCACAC) and OPL-05 (ACGCAGGCAG) obtained from Operon Technologies (Alameda, California, United States). RAPD-PCR was performed according to the method described by Van Reenen and Dicks [45]. In this case the Taq Supertherm polymerase (Hoffmann-La Roche, Nutley, NJ) was used. *L. plantarum* ATCC 14917^T was used as a control. PCR reactions were performed in the Eppendorf Mastercycler Personal (Westbury, New York, United States).

Polymerase chain reactions (PCR) were performed in duplicate. To avoid problems of reproducibility all isolates were typed at the same time.) Lambda DNA, digested with *Eco*R1 and *Hin*dIII (Boehringer Mannheim), was used as molecular weight marker.

Results and discussion

No microbial growth was detected in water collected from the last washing of the vinegar flies, indicating that all surface-bound microorganisms had been removed. Plates incubated anaerobically displayed less growth than those incubated aerobically. This is not surprising, since the intestinal tract of insects is aerobic due to its relative small size and large surface area [41]. From a total of 136 isolates, 30 were selected based on gram reaction, morphology and catalase activity. All isolates were Gram-positive and catalase negative. Twenty-two rod-shaped isolates produced DL- or L (+)-lactic acid from D-glucose and CO_2 from D-gluconate, but not from D-glucose and were tentatively classified as members of Group II (facultatively heterofermentative) *Lactobacillus* spp.

Carbohydrate fermentation reactions recorded for 21 of the 22 facultatively heterofermentative isolates corresponded to that of the type strain of *L. plantarum,* ATCC 14917^T (Table 2). Variations in the fermentation of glycerol, L- arabinose, D-xylose, galactose, rhamnose, sorbitol, esculin, salicin, cellobiose, melezitose, β-gentiobiose, D-turanose, gluconate and 2-keto-gluconate have been recorded (Table 2). Similar results have been reported for other strains of *L. plantarum* [45, 49]. None of the facultative heterofermentative strains fermented α-methyl-D-glucoside, which is characteristic for the type strain of *L. plantarum,* ATCC 14917^T [45, 49].

DNA amplification with species-specific primers on strains selected from the *L. plantarum*-group produced a 318 bp fragment (not shown), which is identical in size to that reported for *L. plantarum* ATCC 14917^T [43]. Amplification of the DNA with eubacterial specific primers F341 and R534, and separation of the 16S rDNA by DGGE, confirmed the classification of these strains as *L. plantarum* (Fig. 1). Sequencing of the 16S rDNA amplicons for 8 strains of *L. plantarum* and comparison with nucleotide sequences in Genbank, revealed DNA homology between 97.1% and 99.0% with *L. plantarum* WCFS1 (Table 3). This suggested that the strains of *L. plantarum* isolated from vinegar flies do not represent a homogeneous collection. The latter was confirmed by RAPD-PCR, which grouped 13 strains into cluster I, five strains into cluster II and three strains into cluster III, respectively (Fig. 2). Randomly amplified polymorphic DNA-PCR was repeated for different amplification assays. The profiles obtained were

slightly different for different PCR amplifications of the same DNA (results not shown) even though the PCR reaction conditions had been normalized, and the reactants and thermocycler were the same. Therefore, the reproducibility of different amplification reactions for the same sample was not good enough to make an exhaustive analysis of the strains. We attempted primer hybridisation at 36, 40 and 45 ^{o}C and at different primer and DNA concentrations. Results were best at 5 picamoles OPL-04 and OPL-05, 40ng of genomic DNA and slightly better at 40 ^{o}C than at other temperatures. Higher primer concentrations gave lower discriminating power. Reproducibility was good and the patterns produced by the various reactions showed no differences.

RAPD-PCR grouped the 30 isolates into four genotypically distinct clusters (Fig. 1). The use of primers OPL-04 and OPL-05 under the optimised conditions developed here was highly discriminating, rapid and reproducible, all necessary characteristics if a method is to be used for typing l plantarum strains. However Van Reenen and Dicks [45] used slightly different reaction conditions and primers for the typing of different *Lactobacillus plantarum* strains suggesting that there is not a universal reaction conditions for the typing of all plantarum strains.

In all the profiles there was a serious of coincident bands which demonstrates the relative genetic intraspecific homogeneity of the strains which agree with the strong similarity among the 16s rDNA sequence results (Table6). It is interesting to note ATCC14917^{T} did not have any coincident bands with the *lactobacillus plantarum* strains isolated from vinegar flies and grape must. The validity of the method was confirmed by the clear separation of the profiles corresponding to three strains of species other than *Lactobacillus plantarum*: *Pediococcus pentosaceus, Lactobacillus casei, Leuconostoc mesenteriodes* subsp. *mesenteriodes* and *Enterococcus faecium* (results not shown) However, some common bands were found on all 4 species which suggest that OPL-04 and OPL-05 used below its T_m probably hybridizes with different points of the genome with a minimum of sequence similarity and caution should be exercised in using this technique to differentiate among different species of LAB as previously [34]. It can be concluded from these findings that at least three genotypic groups of *L. plantarum* had been isolated from vinegar flies. Furthermore, the RAPD-PCR profiles of these strains differed from that of the type strain of *L. plantarum*, ATCC 14917^{T} (Fig. 2).

Strain FA7 fermented D-tagatose (Table 2) and produced exclusively L(+)-lactic acid from D-glucose, which corresponds to the description of *Lactobacillus paracasei* [6]. DNA amplification of strain FA7 with species-specific primers produced a 290 bp-fragment (not shown). This corresponded in size to the fragment reported for *L. paracasei* NCFB 2743^T [47]. Classification of strain FA7 as *L. paracasei* was confirmed by DGGE (Fig. 1) and results obtained by sequencing revealed 98.4% homology with a reference strain of *L. paracasei* subsp. *paracasei* (Table 3).

Two isolates (FM2 and FM3) are rod shaped, produce DL- lactic acid from D-glucose, and CO_2 from the fermentation of D-glucose and D-gluconate. Based on these results, they belong to Group III (obligately heterofermentative) *Lactobacillus* spp. The carbohydrate fermentation profiles of both these strains were different from those of other species (Table 2) and they did not generate a PCR-fragment with any of the species-specific primers listed in Table 1. The DGGE profiles of isolates FM3 and FM2 were similar, but different to that of any of the other strains included in this study (not shown). The 16S rDNA amplicon of strains FM3 and FM2 revealed 98% homology to an "uncultured" *Lactobacillus* sp., clone KL-11-1-6, and shared 97.4 % and 97.1% DNA homology, respectively, with the type strain of *Lactobacillus sanfranciscensis,* ATCC 27651^T (Table 3). The threshold for species delineation based on the 16S rRNA gene remains a controversial area. However is generally accepted to be in the 98-99% region [37]. The ability of strains FM2 andFM3 to ferment many more carbohydrates than *Lactobacillus sanfranciscensis,* ATCC 27651^T and their low 16S rDNA sequence homology with *Lactobacillus sanfranciscensis,* ATCC 27651^T suggest they may constitute unknown species. Further phenotypic and genotypic methods, including DNA-DNA hybridisation need to be carried out on these isolates to determine their identity. Isolates FM3 and FM2 thus can not be regarded as strains of *L. sanfranciscensis*.

Isolate F2 is ovoid in shape, produces D(-)-lactic acid from D-glucose, and CO_2 from the fermentation of D-glucose and D-gluconate. No growth was recorded at 45^{o}C or in the presence of 6.5% (w/v) NaCl. According to carbohydrate fermentation reactions (Table 2), strain F2 is a member of *L. plantarum*. However, PCR with primers specific for *Leuconostoc mesenteroides* generated a fragment of 1150 bp (not shown), characteristic for *L. mesenteroides* [29]. The 16S rDNA amplicons generated from strain

F2 migrated to the same position in the DGGE gel as that recorded for the type strains of *Leuconostoc mesenteroides* subsp. *mesenteroides,* NCDO 523^T (Fig. 1). Sequencing of the 16S rDNA amplicon of strain F2 revealed 97.8% homology with the 16S rDNA of the type strain of *L. mesenteroides* subsp. *mesenteroides,* NCFB 523^T (Table 3). Strain F2 is thus classified as *L. mesenteroides* subsp. *mesenteroides*.

Isolate RR is coccoid with cells arranged in tetrad formation and does not produce CO_2 from the fermentation of either D-glucose or D-gluconate. Growth was recorded at 45 ^oC and in the presence of 6.5% (w/v) NaCl. DL- lactic acid was produced from D-glucose. The carbohydrate fermentation pattern resembled that recorded for the type strain of *Pediococcus pentosaceus*, NCDO 813^T (Table 2). PCR with species-specific primers generated a fragment of 872 bp (not shown), characteristic for *P. pentosaceus* [30]. DGGE yielded a DNA band similar in migration to that recorded for *P. pentosaceus* NCDO 813^T (Fig. 1). Sequence analysis revealed 98.8% similarity to the rDNA of *P. pentosaceus* LM2 632 (Table 3). Isolate RR is thus regarded as a strain of the latter species.

Isolates U4 and MI are both cocci, produce L(+)-lactic acid and are homofermentative (no CO_2 production from D-glucose). Both isolates grew at 45 ^oC and in the presence of 6.5% (m/v) NaCl. Strains U4 and MI were classified as *Enterococcus faecalis* based on sugar fermentation reactions recorded by API 20 STREP (not shown). PCR with genus-specific primers yielded a DNA fragment of 112 bp (not shown), characteristic for *Enterococcus* spp. [26]. Furthermore, the migration position of the DNA band obtained from PCR-DGGE was almost identical to that of *E. faecalis* ATCC 19433 ^{T} (Fig. 1) and sequence analysis of isolate U4 revealed 98.8% homology to the 16S rDNA of *E. faecalis* SFL (Table.3). Isolates U4 and MI are thus regarded as members of *E. faecalis*.

Isolates 153 and EE are ovoid in morphology, with cells in chains. No CO_2 production was recorded from the fermentation of D-glucose and D-gluconate. No growth was recorded at 45^oC or in the presence of 6.5% (w/v) NaCl. L(+)-lactic acid was produced from the fermentation of D-glucose. Based on carbohydrate fermentation profiles, the two strains are members of *Lactococcus lactis* (Table 2). DNA primers specific for *L. lactis* generated a fragment of 564 bp (not shown), characteristic of the

latter species [32]. Sequence analyses of the PCR-DGGE fragments obtained for isolates EE and 153 revealed 97.1% and 99.0% homology with the 16S rDNA of *L. lactis* subsp. *lactis* MRS 1 and *L. lactis* subsp. *lactis* SL3, respectively (Table 3). Isolates EE and 153 are thus regarded members of *L. lactis* subsp. *lactis*.

The majority of species isolated belonged to the species *L. plantarum,* but *L. paracasei,, L. mesenteroides* subsp. *mesenteroides, L. lactis* subsp. *lactis, E. faecalis* and *P. pentosaceus* were also identified.

No species-specific PCR products were generated with primers for *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus salivarius*, *Lactobacillus casei*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*, *Lactobacillus rhamnosus*, *Lactobacillus sakei, Lactobacillus zeae*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus hilgardii*, *Lactobacillus reuteri*, *Lactobacillus lindneri*, *Oenococcus oeni*, *Weissella* spp. and *Pediococcus acidilactici* (Table 1), suggesting that these species are not present in the intestinal tract of vinegar flies.

Acknowledgments

This research was funded by the National Research Foundation (NRF), South Africa.

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Table 1. Species-specific primers used in this study

Target organism		Primer pair	Reference
***Lactobacillus* spp:**			
Group I (Homofermentative)			
L. acidophilus	AciF	TCT AAG GAA GCG AAG GAT	
	AciR	CTC TTC TCG GTC GCT CTA	[39]
L. delbrueckii subsp. *delbrueckii*	DelF	ACG GAT GGA TGG AGA GCA G	
	DelR	GCA AGT TTG TTC TTT CGA ACT C	[39]
L. salivarius	SalI	ATT CAC TCG TAA GAA GT	[39]
	lowlac	CGA CGA CCA TGA ACC ACC TGT	
Group II (Facultative heterofermentative)			
L. casei	Y2	CCC ACT GCT GCC TCC CGT AGG AGT	[44]
	casei	TGC ACT GAG ATT CGA CTT AA	
L. paracasei	Y2	CCC ACT GCT GCC TCC CGT AGG AGT	[44]
	para	CAC CGA GAT TCA ACA TGG	
L. paraplantarum	ParaF	GTC ACA GGC ATT ACG AAA AC	[40]
	REV	TCG GGA TTA CCA AAC ATC AC	
L. pentosus	PentF	CAG TGG CGC GGT TGA TAT C	[40]
	REV	TCG GGA TTA CCA AAC ATC AC	
L. plantarum	planF	CCG TTT ATG CGG AAC ACC TA	[40]
	REV	TCG GGA TTA CCA AAC ATC AC	
L. rhamnosus	Y2	CCC ACT GCT GCC TCC CGT AGG AGT	[44]
	RhamF	TGC ATC TTG ATT TAA TTT TG	
L. sakei	Lbs	TTA ATG ATA ATA CTC GAT T	[20]
	302	CGG AAC TTA CCC GAC	
L. zeae	LCZ	TTG GTC GAT GAA C	[35]
	LBL R1	CCA TGC ACC ACC TGT C	
Group III (Obligate heterofermentative)			
L. brevis	Br1	CTT GCA CTG ATT TTA ACA	[17]
	Br2	GGG CGGTGTGTACAAGGC	
L. fermentum	FermI	GTT GTT CGC ATG AAC AAC GCT TAA	[5]
	lowlac	CGA CGA CCA TGA ACC ACC TGT	
L. hilgardii	H2	AAC TGA TTT GAC ATT AAG A	[36]
	8623	CTG GTT CAC TAT CGG TCT C	
L. reuteri	Reut1	TGA ATT GAC GAT GGA TCA CCA GTG	[5]
	Lowlac	CGA CGA CCA TGA ACC ACC TGT	
L. lindneri	DA-40	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	[44]
	907r	ATT ACC GCG GCT GCT GG	
***Leuconostoc* sp.:**			
L. mesenteroides sp.	Lmes-f	AAC TTA GTG TCG CAT GAC	[28]
	Lmes-r	AGT CGA GTT ACA GAC TAC AA	
***Oenococcus* sp.:**			
O. oeni	On1	TAA TGT GGT TCT TGA GGA GAA AAT	[47]
	On2	ATC ATC GTC AAA CAA GAG GCC TT	
***Weissella* sp.:**	WeiF	CGT GGG AAA CCT ACC TCT TA	[22]
	WeiR	CCC TCA AAC ATC TAG CAC	
***Pediococcus* spp.:**			
P. acidilactici	PacF	CGA ACT TCC GTT AAT TGA TTA T	[29]
	PuR	ACC TTG CGG TCG TAC TCC	
P. pentosaceus	PpeF	CGA ACT TCC GTT AAT TGA TCA G	[29]
	PuR	ACC TTG CGG TCG TAC TCC	
***Enterococcus* sp.**	EntF	TAC TGA CAA ACC ATT CAT GAT G	[25]
	EntR	AAC TTC GTC ACC AAC GCG AAC	
***Lactococcus* sp.**			
L. lactis	GADb21	CGT TAT GGA TTT GAT GGA TAT AAA GC	[31]
	GAD7	ACT CTT CTT AAG AAC AAG TTT AAC AGC	

Table 2. Differential carbohydrate fermentation reactions of *Lactobacillus*, *Leuconostoc* and *Pediococcus* isolates, collected from vinegar flies

Isolate	Glycerol	L- arabinose	Ribose	D-xylose	Galactose	D-manose	Rhamnose	Mannitol	Sorbitol	α-Methyl-D-glucoside	Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Lactose	Melibiose	Saccharose	Melezitose	Raffinose	Starch	β-gentiobiose	D-Turanose	D-Tagatose	Gluconate	2-keto-gluconate
L. plantarum ATCC 14917[Ta]	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-
32	d	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-
26	d	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	d	-	+	-
3A1	-	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-
PP	-	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	d	-	d	d
R2	-	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	d	-	+	-
37	-	-	+	-	d	+	-	+	-	-	+	+	d	d	d	+	+	+	d	+	-	+	-	-	d	-
28	-	-	+	-	d	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-
E	d	+	+	d	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	d	-	-	d	-
AA	-	-	+	-	d	+	-	+	-	-	+	+	+	d	d	+	+	+	d	+	-	+	-	-	d	-
U2	d	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-
24	-	-	+	-	d	+	-	+	-	-	+	+	+	+	d	+	+	+	d	+	-	+	-	-	d	-
A7	d	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-
FA5	d	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	d	-	d	d
A1	-	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-
FA13	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	d	-
27	d	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	d	-	+	-
MII	-	-	+	-	d	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-
M1	-	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-
F10	d	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	d	-	d	d
21	-	+	+	-	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	d	-	+	-
34	d	+	+	d	+	+	d	+	+	-	+	+	+	+	+	+	+	+	+	+	-	d	-	-	d	-
L. paracasei NCFB 2743[Tb]	-	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	-	+	+	-	-	+	-	+	-	-
FA7	d	-	+	d	d	+	-	d	+	-	+	+	+	+	+	d	d	+	+	-	-	+	-	+	-	-
L.sanfrancincensis ATCC27651[Td]	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	+	-
FM3	d	-	+	d	+	+	d	+	d	-	+	+	-	+	+	+	+	+	+	-	-	d	-	+	d	-
FM2	d	-	+	d	+	+	d	+	d	-	+	+	-	+	+	+	+	+	+	d	-	d	-	+	d	-
Leuconostoc mesenteriodes subsp. *mesenteriodes* NCDO 523[Tf]	-	+	d	d	+	+	-	-	-	+	+	+	+	+	+	d	+	+	-	-	-	+	+	-	-	-
F2	-	d	d	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	-
P. pentosaceus NCDO 813[Te]	-	+	+	d	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	-	+	-	-
RR	d	-	+	+	+	+	d	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	-	+	-	d
L. lactis ATCC 19435 [Tc]	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-
153	-	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-	+	-	-	d	+	-	-	d	-
EE	-	+	d	+	d	+	-	d	-	-	d	+	+	+	+	d	-	+	-	-	d	+	-	-	d	-

+, positive reaction: -, negative reaction: d, variable reaction. All strains fermented N-acetyl-glucosamine, D-fructose, D-glucose, maltose, saccharose, sorbitol and trehalose. None of the strains fermented adonitol, D-arabinose, D-arabitol, dulcitol, L-arabitol, erythritol, D-fucose, L-fucose, glycogen, inositol, inulin, 5-keto-gluconate, D-lyxose, α-methyl-D-mannoside, β-methyl-xylosidase, L-sorbose, D-turanose, xylitol and L-xylose.

[a] Data obtained from Kandler and Weiss [23]

[b] Data obtained from Collins et al. [6]

[c] Data obtained from Stolz et al. [36], Vogel et al. [42] and the API 50 CHL databank

[d] Data obtained from Collins et al. [6] and Vogel et al. [42]

[e] Data obtained from Garvie [15] and the API 50 CHL databank

[f] Data obtained from Garvie [14] and the API 50 CHL databank

Table 3. Percentage similarity of isolates from vinegar flies to species in the NCBI nucleotide sequence database, based on partial 16S rRNA sequence analysis

Isolate	Phylogenetic affiliation and accession number	% similarity
AA	*Lactobacillus plantarum* WCFS 1 (AL935258)	99.0%
A1	"	99.0%
FA13	"	98.8%
21	"	98.8%
26	"	98.6%
E	"	98.4%
R2	"	98.2%
MII	"	97.1%
FA7	*Lactobacillus paracasei* subsp. *paracasei* (AY773951)	98.4%
153	*Lactococcus lactis* subsp. *lactis* SL3 (AY675242)	99.0%
EE	*Lactococcus lactis* subsp. *lactis* MRS1 (AJ488173)	97.1%
U4	*Enterococcus faecalis* SFL (AY850358)	98.8%
FM3	*Lactobacillus sanfranciscensis* ATCC 27651^T (X76327)	97.4%
FM2	*Lactobacillus sanfranciscensis* ATCC 27651^T (X76327)	97.1%
RR	*Pediococcus pentosaceus* LM2 632 (AY675245)	98.8%
F2	*Leuconostoc mesenteriodes* subsp. *mesenteriodes* NCFB 523^T (AB023242)	97.8%

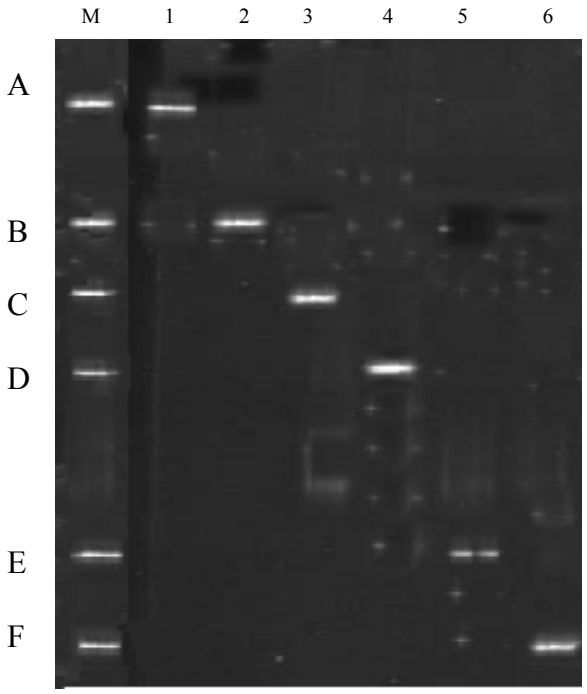

Fig. 1. DGGE of the 16S rDNA PCR amplicons of lactic acid bacteria.

M=ladder. A, *Enterococcus faecalis* ATCC 19433^{T}; B, *Lactobacillus plantarum* ATCC 14917^{T}; C, *Leuconostoc mesenteriodes* subsp. *mesenteriodes* NCDO 523^{T}; D, *Pediococcus pentosaceus* NCDO 813^{T}; E, *Lactococcus lactis* subsp. *lactis* ATCC 19435^{T}; F, *Lactobacillus paracasei* NCFB 2743^{T}.

Lane 1: Strains U4 and MI; Lane 2: Strains 32, 26, 3A1, PP, R2, 37, 28, E , AA, U2, 24, A7, FA5, A1, FA13, 27, MII, M1, F10, 21 and 34; Lane 3: Strain: F2; Lane 4: Strain RR; Lane 5: Strains EE and 153; Lane 6: Strain FA7

ATCC= American Type Culture Collection; NCDO= National Collection of Dairy Organisms; NCFB= National Collection of Food Bacteria.

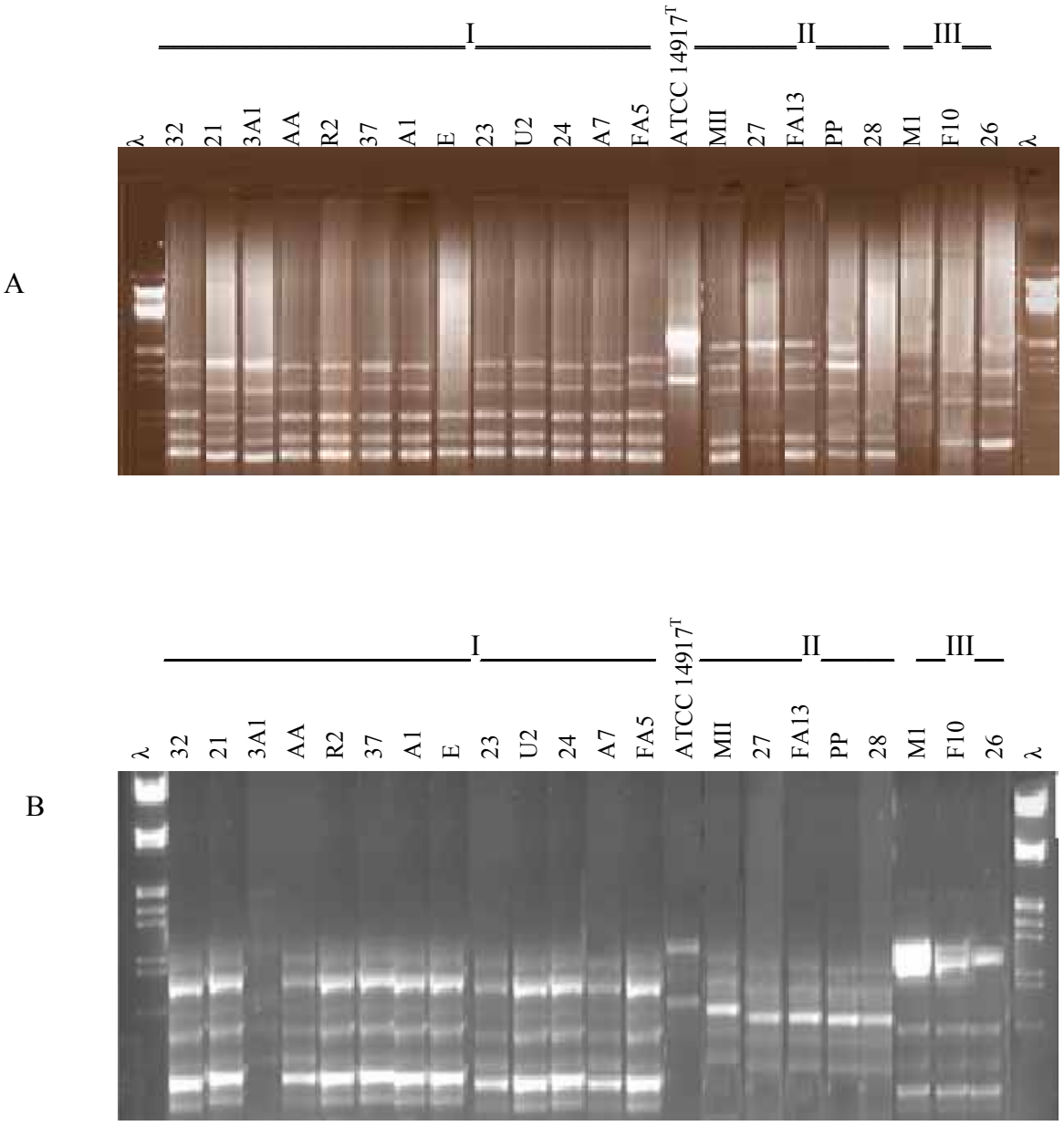

Fig. 2. DNA fingerprints obtained after RAPD-PCR of the genomic DNA of isolates identified as *Lactobacillus plantarum*. A: Primer OPL-4 (GACTGCACAC), B: Primer OPL-5 (ACGCAGGCAC).

4. Strains of *Lactobacillus plantarum* in grape must are also present in the intestinal tract of vinegar flies

Submitted for publication in The South African Journal of Enology and Viticulture

Strains of *Lactobacillus plantarum* in grape must are also present in the intestinal tract of vinegar flies

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Acknowledgements: This research was funded by the National Research Foundation (NRF), South Africa

Submitted for publication:

Accepted for publication:

Key words: *Lactobacillus plantarum*, vinegar flies

Condensed title: *L. plantarum* from vinegar flies

ABSTRACT

Twenty-one lactic acid bacteria isolated from the intestinal tract of *Drosophila simulans* Stuvervant and nine from merlot grapes were identified as *L. plantarum* by PCR with species-specific primers and 16S rDNA sequencing. The 30 isolates grouped into four clusters based on RAPD-PCR banding patterns, suggesting that they belong to at least four genotypic groups. Thirteen isolates from grape must and five from the flies yielded identical RAPD-PCR banding patterns and grouped into one cluster, suggesting that they are descendants from the same strain. It may be concluded from these results that *L. plantarum* (or at least descendants from a specific strain) has the ability to use vinegar flies as a host and vector to infect grape must. Further research is needed to determine the role of this specific strain in wine fermentations.

INTRODUCTION

Lactobacillus plantarum is one of the most widely distributed lactic acid bacteria, probably due to its ability to adapt to various niches. The species is commonly isolated from grape must (Davis *et al*., 1985; Du Plessis *et al.,* 2004) and is present in fairly high cell numbers during the first few days of fermentation. Although the cell numbers of *L. plantarum* usually decreases during secondary fermentation, some strains withstand the high SO_2 and ethanol concentrations and may cause spoilage of bottled wine (Davis *et al*., 1985; Du Toit & Pretorius, 2000).

Drosophila is a common agricultural pest. The flies lay their eggs on fruit, which nourishes the developing larvae (Demerec, 1950). Little research has been done on the microflora of vinegar flies and early reports are contradictory. According to Gukasyan (1966), the intestinal tract of vinegar flies is mostly free of bacteria. However, Kvasnikov *et al*. (1971) reported the presence of *L. plantarum* and enterococci in vinegar flies and suggested that they may contaminate fermentation processes. The latter strains were identified based on physiological and biochemical characteristics (Kvasnikov *et al*., 1971), including sugar fermentation profiles which are not reliable (Van Reenen & Dicks, 1996; Kullen *et al*., 1998).

The role of lactic acid bacteria in the insect gut is not known. They may be involved in the detoxification of plant allelochemicals such as flavonoids, tannins, and alkaloids (Dillon & Dillon, 2004), or prevent the colonisation of non-indigenous pathogenic micro-organisms by competitive exclusion (Berg, 1996).

This study was conducted to confirm the presence of *L. plantarum* in vinegar flies and to determine if the species can use the insect as vector.

MATERIALS AND METHODS

Collection of samples

Merlot grapes collected from a vineyard in Stellenbosch were crushed in sterile plastic bags and the must removed aseptically. Vinegar flies were captured from the same vineyard using specially designed traps. The flies were sterilised with 2.5% (vol/vol) sodium hypochlorite and rinsed several times with sterile distilled water. Water from

the last washing was inoculated onto MRS agar (Biolab, Biolab Diagnostics, Midrand, SA) to evaluate the efficiency of the washing process. The plates were incubated at 30°C and examined for microbial growth after 48 h.

Approximately 100 flies were placed in a sterile 50 mL centrifuge tube with 2 mL sterile peptone water and glass beads (approximately 2 mm in diameter). The flies were homogenised for 3 min at 25°C on a vortex. The homogenate was serially diluted in sterile distilled water and plated out, in triplicate, onto MRS agar (Biolab), supplemented with 20% (vol/vol) apple juice and adjusted to pH 5.5 with 1 N NaOH. The medium was supplemented with 100 μg/mL Delvocid (GistBrocades, Delft, Netherlands) to inhibit the growth of yeast and fungi. One set of plates was incubated in an anaerobic flask (Oxoid, Basingstoke, Hampshire, England) in the presence of an Anaerocult gas generating kit (Oxoid). The remaining two sets of plates were incubated aerobically at 30^{o}C. All plates were examined for growth after 24 and 48 h.

Preliminary identification of the isolates

Colonies were randomly selected from plates with between 50 and 300 colonies and re-streaked on corresponding media to obtain pure cultures. All cultures were stored at -80^{o}C in MRS broth supplemented with sterile glycerol (30%, vol/vol, final concentration). Gram reaction and catalase activity were determined according to the methods described by Harrigan & McCance (1976). Production of CO_2 from glucose and gluconate was monitored according to the method described by Dicks & Van Vuuren (1987). Facultative heterofermentative, Gram-positive and catalase negative rods were selected and the configuration of lactic acid produced determined by using an enzymatic kit (Roche Diagnostics GmbH, Mannheim, Germany).

Carbohydrate fermentations

Carbohydrate fermentation reactions were recorded by using the API 50 CHL system (BioMerieux, Marcy L'Etoile, France). All API strips were incubated at 30^{o}C and readings were taken after 24 and 48 h, respectively.

PCR with species-specific primers

Isolates with carbohydrate fermentation reactions corresponding to that of the type strain of *L. plantarum* (ATCC 14917) were selected and their genomic DNA isolated according to the method described by Dellaglio *et al*. (1973). The DNA was amplified with primers planF (CCG TTT ATG CGG AAC ACC TA) and REV (TCG GGA TTA CCA AAC ATC AC), and Taq Takara polymerase (Otsu, Shiga, Japan), according to the method used by Torriani *et al*. (2001). *L. plantarum* ATCC 14917^T was used as reference strain.

Sequencing of 16S rDNA

Isolates with DNA fragments identical in size to that of *L. plantarum* ATCC 14917^T were selected and their genomic DNA annealed to primers 8f (5'-CAG GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and 1512r (5'-GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT-3') to amplify a conserved region in the 16S rRNA molecule. The methods of Felske *et al*. (1997) and Garbers *et al*. (2004) were used, but with the Taq Takara polymerase.

The amplified fragments, 1500 bases in size, were purified using the High Pure PCR product Purification Kit (Roche), according to the manufacturer's instructions. The fragments were sequenced and compared with sequences in Genbank by using the BLAST programme (Altschul *et al*., 1997).

RAPD-PCR analysis

Genomic DNA from the isolates were amplified with primers OPL-04 (GACTGCACAC) and OPL-05 (ACGCAGGCAG) from Operon Technologies (Alameda, California, United States), as described by Van Reenen & Dicks (1996). In this case the Taq Supertherm polymerase (Hoffmann-La Roche, Nutley, NJ) was used. *L. plantarum* ATCC 14917^T was used as a control. PCR reactions were performed in the Eppendorf Mastercycler Personal Polymerase chain reactions (PCR) were performed in duplicate. To avoid problems of reproducibility all isolates were

typed at the same time. Lambda DNA, digested with *Eco* R1 and *Hind*III (Boehringer Mannheim), was used as molecular weight marker.

RESULTS AND DISCUSSION

No viable micro-organisms were isolated from the water washings, indicating that all surface-bound cells had been washed off. Plates incubated anaerobically displayed less growth than those incubated aerobically.

From a total of 158 isolates, 30 were Gram-positive rods, catalase negative and produced CO_2 from D-gluconate, but not from D-glucose. Based on these characteristics, the isolates were classified as members of Group II (facultatively heterofermentative) *Lactobacillus* spp. All strains produced DL-lactate from D-glucose. Carbohydrate fermentation reactions corresponded to that recorded for the type strain of *L. plantarum* (ATCC 14917^T, Table 1). Variations in the fermentation of L- arabinose, cellobiose, esculin, galactose, gentiobiose, gluconate, glycerol, 2-keto-gluconate, mannitol, melezitose, rhamnose, salicin, sorbitol, D-turanose and D-xylose were recorded (Table 1). Similar results have been reported for other strains of *L. plantarum* (Zanoni et al., 1987; Van Reenen & Dicks, 1996). None of the strains fermented α-methyl-D-mannoside, which is characteristic for the type strain of *L. plantarum* ATCC 14917^T (Zanoni et al., 1987; Van Reenen & Dicks, 1996). Based on carbohydrate fermentation reactions, the isolates were preliminarily classified as strains of *L. plantarum.*

Amplification of genomic DNA with species-specific primers yielded a 318-bp fragment, which is identical in size to that reported for *L. plantarum* ATCC 14917^T (Torriani *et al*., 2001). Furthermore, amplification of the genomic DNA of the isolates with primers 8f and 1512r yielded 16S rDNA amplicons which were 97.1% to 99.0% homologous to the 16S rDNA of *L. plantarum* WCFS1 (Table 2), confirming their classification as *L. plantarum*. However, the variations recorded in 16S rDNA sequencing suggested that the isolates belonged to more than one genotypic group. Randomly amplified polymorphic DNA-PCR was repeated for different amplification assays. The profiles obtained were slightly different for different PCR amplifications of the same DNA (results not shown) even though the PCR reaction conditions had been normalized, and the reactants and thermocycler were the same. Therefore, the reproducibility of different amplification reactions for the same sample was not good enough to make an exhaustive analysis of the strains. In order to increase reproducibility and discriminatory power we attempted primer hybridisation at 36, 40

and 45 ^{o}C and at different primer and DNA concentrations. Results were best at 5 picamoles OPL-04 and OPL-05, 40ng of genomic DNA and slightly better at 40 ^{o}C than at other temperatures. Higher primer concentrations gave lower discriminating power. Reproducibility was good and the patterns produced by the various reactions showed no differences.

RAPD-PCR grouped the 30 isolates into four genotypically distinct clusters (Fig. 1). The use of primers OPL-04 and OPL-05 under the optimised conditions developed here was highly discriminating, rapid and reproducible, all necessary characteristics if a method is to be used for typing l plantarum strains. However Van Reenen and Dicks [] used slightly different reaction conditions and primers for the typing of different *Lactobacillus plantarum* strains suggesting that there is not a universal reaction conditions for the typing of all plantarum strains.

In all the profiles there were a serious of coincident bands which demonstrates the relative genetic intraspecific homogeneity of the strains which agree with the strong similarity among the 16s rDNA sequence results (Table6). It is interesting to note ATCC14917^{T} did not have any coincident bands with the *lactobacillus plantarum* strains isolated from vinegar flies and grape must. The validity of the method was confirmed by the clear separation of the profiles corresponding to three strains of species other than *Lactobacillus plantarum*: Pediococcus pentosaceus, *Lactobacillus casei, Leuconostoc mesenteriodes subsp. mesenteriodes* and *Enterococcus faecium*(results not shown) However, some common bands were found on all 4 species which suggest that OPL-04 and OPL-05 used below its T_m probably hybridizes with different points of the genome with a minimum of sequence similarity and caution should be exercised in using this technique to differentiate among different species of LAB as previously (Olive and Bean, 1996).

Isolates in clusters I and II were from vinegar flies and isolates in cluster IV from grape must. Cluster III contained five isolates (C4, C1 C32, C23 & 3C3) from vinegar flies and 13 from grape must. The identical DNA banding patterns obtained for all the isolates in cluster III suggests that they are descendants from the same strain, but genotypically different from the type strain of *L. plantarum* (ATCC 14917^{T}) which did not group in any of the four RAPD-PCR clusters (Fig. 1). This also suggests that at least one strain of *L. plantarum* developed the ability to colonise

the intestinal tract of *Drosophila simulans* Stuvervant and use the insect as a vector. Insects are known to serve as vectors for bacteria (Lilley *et al*., 1997). As far as we could determine, this is the first evidence of vinegar flies acting as a vector for *L. plantarum.* Further research is needed to determine the role of this specific strain (isolates from cluster III, Fig. 1) in wine fermentations.

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TABLE 1

Differential carbohydrate fermentation reactions of *Lactobacillus plantarum* isolates collected from vinegar flies and grape must. Isolates from grape must are printed in bold.

Isolate	**Glycerol**	**L-arabinose**	**D-xylose**	**Galactose**	**Rhamnose**	**Mannitol**	**Sorbitol**	**Esculin**	**Salicin**	**Cellobiose**	**Melezitose**	**β-gentiobiose**	**D-turanose**	**Gluconate**	**2-keto-gluconate**
L. plantarum ATCC 14917[Ta]	-	+	-	+	-	+	+	+	+	+	+	+	-	+	-
32	d	+	-	+	d	+	+	+	+	+	+	+	-	+	-
26	d	+	-	+	d	+	+	+	+	+	+	+	d	+	-
3A1	-	+	-	+	d	+	+	+	+	+	+	+	+	+	-
PP	-	+	-	+	d	+	+	+	+	+	+	+	d	d	D
R2	-	+	-	+	d	+	+	+	+	+	+	+	d	+	-
37	-	-	-	d	-	+	-	d	d	d	d	+	-	d	-
28	-	-	-	d	d	+	+	+	+	+	+	+	-	+	-
E	d	+	d	+	d	+	+	+	+	+	+	d	-	d	-
AA	-	-	-	d	-	+	-	+	d	d	d	+	-	d	-
U2	d	+	-	+	d	+	+	+	+	+	+	+	+	+	-
24	-	-	-	d	-	+	-	+	+	d	d	+	-	d	-
A7	d	+	-	+	d	+	+	+	+	+	+	+	+	+	-
FA5	d	+	-	+	d	+	+	+	+	+	+	+	d	d	d
A1	-	+	-	+	d	+	+	+	+	+	+	+	-	+	-
FA13	-	+	-	+	-	+	+	+	+	+	+	+	-	d	-
27	d	+	-	+	d	+	+	+	+	+	+	+	d	+	-
MII	-	-	-	d	d	+	+	+	+	+	+	+	-	+	-
M1	-	+	-	+	d	+	+	+	+	+	+	+	-	+	-
F10	d	+	-	+	d	+	+	+	+	+	+	+	d	d	D
21	-	+	-	+	d	+	+	+	+	+	+	+	d	+	-
34	d	+	d	+	d	+	+	+	+	+	+	d	-	d	-
C8	-	+	-	+	d	+	+	+	+	+	+	+	-	d	-
C1	-	+	-	+	d	+	+	+	+	+	+	+	d	+	D
C32	-	+	-	+	d	+	+	+	+	+	+	+	-	d	-
CA4	d	+	-	+	d	+	+	+	+	+	+	+	-	d	D
C23	-	+	-	+	d	+	+	+	+	+	+	+	d	+	-
3C3	d	+	d	+	d	+	+	+	+	+	+	+	-	+	-
C3	-	-	-	+	-	+	-	+	d	d	d	+	-	d	-
C12	d	+	-	+	d	-	+	+	+	+	+	+	+	+	-
C13	-	-	-	d	-	+	-	+	+	+	d	+	-	d	-

+, positive reaction; -, negative reaction; d, variable reaction. All strains fermented: *N*-acetyl-glucosamine, amygdalin, arbutin, D-fructose, D-glucose, lactose, maltose, D-mannose, melibiose, raffinose, ribose, saccharose and trehalose. None of the strains fermented adonitol, D-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, glycogen, inositol, inulin, 5-keto-gluconate, D-lyxose, α-methyl-D-glucoside , α-methyl-D-mannoside, β-methyl-xyloside, L-sorbose, starch, D-tagatose, xylitol and L-xylose.

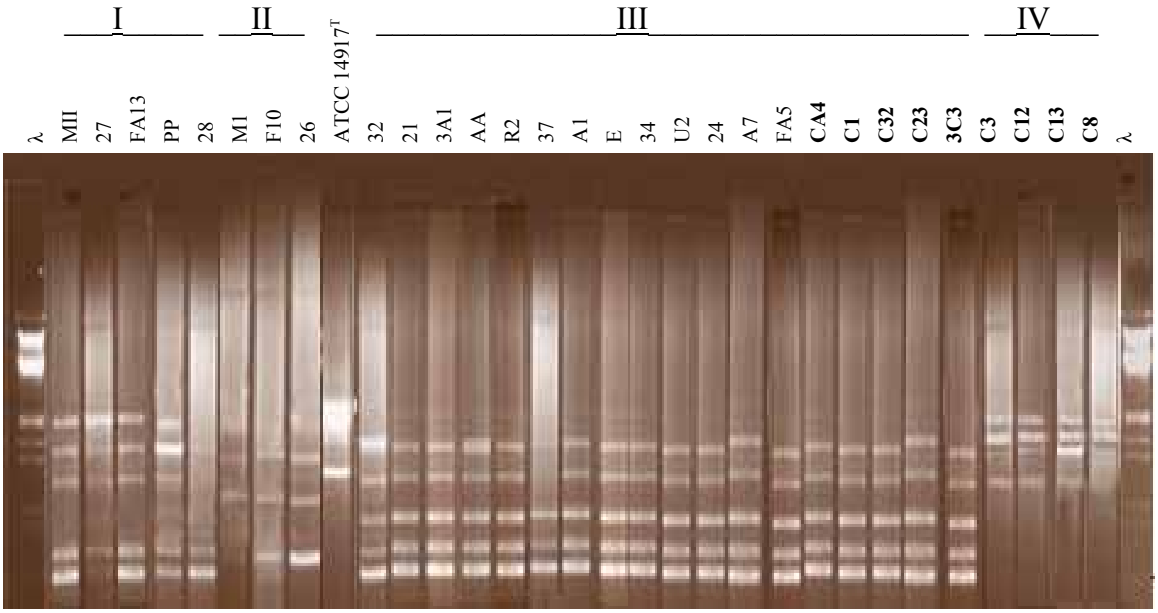
[a] Data from Kandler and Weiss (1986).

TABLE 2

Percentage similarity of isolates to species in the NCBI nucleotide sequence database, based on partial 16S rDNA sequence analysis. Isolates from grape must are printed in bold.

Isolate	**Phylogenetic affiliation and accession number**	**% similarity**
AA	*Lactobacillus plantarum* (AL935258.1)	99.0%
A1	*Lactobacillus plantarum* (AL935258.1)	99.0%
21	*Lactobacillus plantarum* (AL935258.1)	98.8%
CA4	*Lactobacillus plantarum* (AL935258.1)	98.8.%
FA13	*Lactobacillus plantarum* (AL935258.1)	98.8%
C1	*Lactobacillus plantarum* (AL935258.1)	98.7%
26	*Lactobacillus plantarum* (AL935258.1)	98.6%
E	*Lactobacillus plantarum* (AL935258.1)	98.4%
C8	*Lactobacillus plantarum* (AL935258.1)	98.2%
R2	*Lactobacillus plantarum* (AL935258.1)	98.2%
MII	*Lactobacillus plantarum* (AL935258.1)	97.1%

A



B

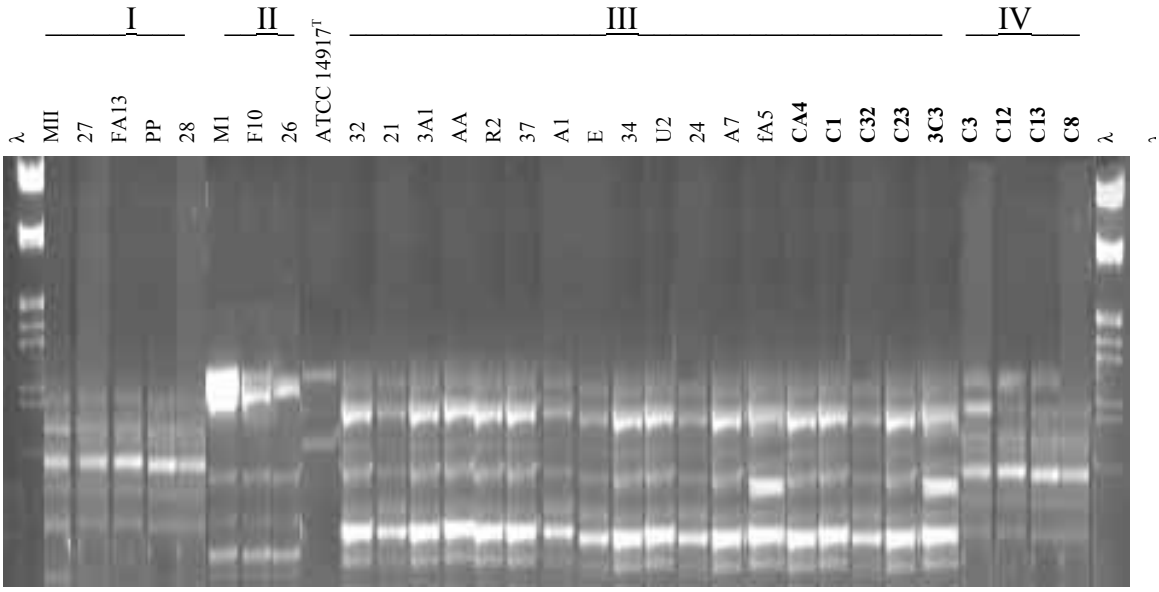


FIGURE 1

DNA fragments obtained after RAPD-PCR amplification of the genomic DNA of isolates identified as *Lactobacillus plantarum*. A: Primer OPL-4 (GACTGCACAC), B: Primer OPL-5 (ACGCAGGCAC).

5. **General Discussion and Conclusion**

The insect gut is a nutrient-rich environment that supports the growth of a number of microorganisms (Bignell, 1984; Dillon and Dillon, 2004). Only a limited number of studies have been published on the presence of LAB in the insect gut. Insect species derive their microbiota from the surrounding environment such as plants or the skin of the animal host, and can act as a vector for certain bacteria (Demerec, 1950; Bignell, 1984). The role of these microorganisms is most probably similar to probiotic bacteria in the intestinal tract of mammals, i.e., they contribute to food digestion, produce essential vitamins and inhibit the development of intestinal pathogens (Dillon and Dillon, 2004).

The microbiota of the vinegar fly was studied by Kvasnikov et al. in 1971. The authors isolated *Lactobacillus plantarum*, enterococci and "hetero-enzymatic" cocci from vinegar flies. Identification was based on physiological and biochemical characteristics, including sugar fermentation profiles. The enterococci and "hetero-enzymatic" cocci could not be identified due to their overlapping phenotypic characteristics. Physiological and biochemical characteristics are often not a reliable means of identification due to overlapping phenotypic characteristics (Van Reenen and Dicks, 1996). Since the seventies, classification of LAB has become dependent on genotypic methods.

In this study LAB were isolated from vinegar flies and Merlot grapes collected at the same vineyard in Stellenbosch, South Africa. Isolates were identified using a combination of phenotypic and genotypic methods. The phenotypic methods included morphology and analysis of carbohydrate fermentation patterns using API 50 CHL and API 20 STREP carbohydrate fermentation kits. Genotypic methods included 16S sequence analysis, denaturing gradient gel electrophoresis (DGGE) and PCR with species-specific primers. The genotypic relatedness between strains were analysed using RAPD-PCR.

Strains isolated from the vinegar flies in this study belonged to the species *L. plantarum, L. paracase, L. mesenteroides* subsp. *mesenteroides, L. lactis* subsp. *lactis, E. faecalis*

and *P. pentosaceus*. As far as we could determine, this is the first report on the isolation of *L. paracasei, L. mesenteroides* subsp. *mesenteroides, L. lactis* subsp. *lactis, E. faecalis* and *P. pentosaceus* from vinegar flies.

Seventy percent of strains isolated from the fly intestine were identified as *Lactobacillus plantarum*. The only LAB identified from Merlot grapes was *L. plantarum. Lactobacillus plantarum* is one of the most abundant and widely distributed lactic acid bacteria. The ecological flexibility of *L. plantarum* is reflected by its relatively large genome size, large number of proteins involved in regulation and transport functions, and its high metabolic potential (Kleerebezem et al., 2003). Variations in the fermentation of carbohydrates were recorded, which is typical for *L. plantarum* (Zanoni et al., 1987; Van Reenen and Dicks, 1996). None of the strains fermented α-methyl-D-mannoside, which is characteristic for the type strain of *L. plantarum* ATCC 14917^T (Zanoni et al., 1987; Van Reenen and Dicks, 1996).

The heterofermentative rods isolated from the vinegar flies, strains FM3 and FM2, could not be identified by carbohydrate fermentation patterns. This confirmed previous reports (Du Toit et al., 2003) that carbohydrate fermentations cannot be used to differentiate obligately heterofermentative *Lactobacillus* spp. The 16S rDNA amplicon of strains FM3 and FM2 revealed 97.4 % and 97.1% DNA homology, respectively, with the type strain of *Lactobacillus sanfranciscensis,* ATCC 27651^T. The threshold for species delineation based on the 16S rRNA gene is generally accepted to be in the 98-99% region (Roth et al., 2003). The ability of strains FM2 andFM3 to ferment many more carbohydrates than *Lactobacillus sanfranciscensis,* ATCC 27651^T and their low 16S rDNA sequence homology with *Lactobacillus sanfranciscensis,* ATCC 27651^T suggest they may constitute unknown species. Further phenotypic and genotypic methods, including DNA-DNA hybridisation need to be carried out on these isolates to determine their identity. Isolates FM3 and FM2 thus can not be regarded as strains of *L. sanfranciscensis*.

The API 50 CHL system identified isolate F2 as a member of *L. plantarum* group despite its ovoid shape, ability to produce CO_2 from the fermentation of D-glucose and D-

gluconate and exclusive production of D(-)-lactic acid from D-glucose. However genotypic tests performed identified strain F2 as *L. mesenteroides* subsp. *mesenteroides*. "Hetero-enzymatic" cocci isolated from the vinegar fly by Kvasnikov et al. (1971) could also not be identified to species level using carbohydrate fermentation patterns due to overlapping characteristics.

RAPD-PCR grouped isolates identified as *L. plantarum* into four genotypically well-separated clusters, suggesting that the *L. plantarum* strains do not form a homogenous collection. Thirteen isolates from grape must and five from the flies yielded identical RAPD-PCR banding patterns and grouped into one cluster (III), suggesting that they are descendants from the same strain. This suggests that *L. plantarum* has the ability to use vinegar flies as a vector. Insects are known to serve as vectors for bacteria (Lilley et al., 1997). As far as we could determine this is the first evidence of vinegar flies acting as a vector for *L. plantarum.* Further research is needed to determine the role of this specific strain in wine fermentations.

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