Strains of *Lactobacillus plantarum* in Grape Must are also Present in the Intestinal Tract of Vinegar Flies

W.H. Groenewald, C.A. van Reenen and L.M.T. Dicks*

Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa

Submitted for publication: November 2005

Accepted for publication: April 2006

Key words: *Lactobacillus plantarum*, vinegar flies

Twenty-one lactic acid bacteria isolated from the intestinal tract of *Drosophila simulans* Stuvervant and nine from Merlot noir 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. Concluded from these results, *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). A few strains of *L. plantarum* with strong malolactic fermentation have been used as starter cultures (Davis *et al.*, 1985).

Drosophila is a common agricultural pest. The flies lay their eggs on fruit, which nourishes the developing larvae (Demerec, 1950). A number of yeasts have been isolated from the intestinal tract of *Drosophila* (Mortimer and Polsinelli, 1999; Ganter, 2006). *Wolbachia*, a rickettsia-like organism (Rousset *et al*., 1992) and *Vibrio cholerae* (Park *et al*., 2005) have also been isolated from *Drosophila*. However, little is known about the presence of lactic acid bacteria in the gut of vinegar flies. Kvasnikov *et al*. (1971) reported the presence of *L. plantarum* and enterococci in *Drosophila* 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 that are often not reliable (Van Reenen & Dicks, 1996; Kullen *et al*., 1998). In a recent study (Groenewald, 2005), *L. plantarum* (70%), *Lactobacillus paracasei* (3.3%), *Lactobacillus sanfranciscensis* (6.7%), *Leuconostoc mesenteroides* subsp. *mesenteroides* (3.3%), *Lactococcus lactis* subsp. *lactis* (3.3%), *Enterococcus faecalis* (6.7%) and *Pediococcus pentosaceus* (6.7%) have been isolated from vinegar flies.

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 a vector.

MATERIALS AND METHODS

Collection of samples

Five kilograms of Merlot noir 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

*Corresponding author: E-mail address: lmtd@sun.ac.za

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

remaining two sets of plates were incubated aerobically at 30°C. All plates were examined for growth after 24 and 48 h.

Preliminary identification of the isolates

Colonies were randomly selected from plates between 50 and 300 colonies and re-streaked on corresponding media to obtain pure cultures. All cultures were stored at -80°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 and McCance (1976). Production of CO_2 from glucose and gluconate was monitored according to the method described by Dicks and 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

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).

API strips were incubated at 30°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 region in the 16S rDNA molecule. The methods of Felske *et al*. (1997) and Garbers *et al*. (2004) were used, but with the Taq Takara polymerase.

The amplified fragments 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.

RAPD-PCR analysis

Genomic DNA from the isolates was amplified with eight decamer primers, (OPL-01, OPL-02, OPL-03, OPL-04, OPL-05, OPL-08, OPL-12, OPL-20), as described by Van Reenen and Dicks (1996). The primers were of arbitrary sequence from the OPL set (Operon Technologies Inc., Alameda, CA, USA). Taq Supertherm polymerase (Hoffmann-La Roche, Nutley, NJ) was used.

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, 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, β-gentiobiose, gluconate, glycerol, 2-keto-gluconate, mannitol, melezitose, rhamnose, salicin, sorbitol, D-turanose and D-xylose were recorded (see Table 1). Similar results have been reported for other strains of *L. plantarum* (Zanoni, 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, 1987; Van Reenen & Dicks, 1996). Based on carbohydrate fermentation reactions, the isolates were preliminary classified as strains of *L. plantarum.* Sugar fermentation reactions did not show correlation with RAPD groupings. Strains of *L. plantarum* have been isolated from *Drosophila* (Kvasnikov *et al*., 1971). However, the genotypic relatedness of these strains has not been studied. Comparisons with the latter strains are not possible, since they have not been deposited into a culture collection.

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 that were 97.1% (832 of 857 bp sequenced) to 99.0% (849 of 858 bp sequenced) homologous to the 16S rDNA of *L. plantarum* WCFS1 (NCBI nucleotide sequence database, accession number AL935258.1), confirming their classification as *L. plantarum*.

Identical RAPD-PCR profiles were recorded with primers OPL-01, OPL-02 and OPL-12. Primer OPL-20 produced an incomplete profile, primer OPL-03 two different profiles, and primer OPL-08 three different profiles (results not shown). Similar results were recorded in a previous study with 27 different strains of *L. plantarum* (Van Reenen & Dicks, 1996). Amplification with primers OPL-04 and OPL-05 grouped the 30 isolates into four genotypically well-separated clusters (see Fig. 1). Identical DNA profiles were recorded with duplicate RAPD-PCR runs. 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 (see Fig. 1). This also suggests that at least one strain of *L. plantarum* developed the ability to colonise the intestinal tract of *D. 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.

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%

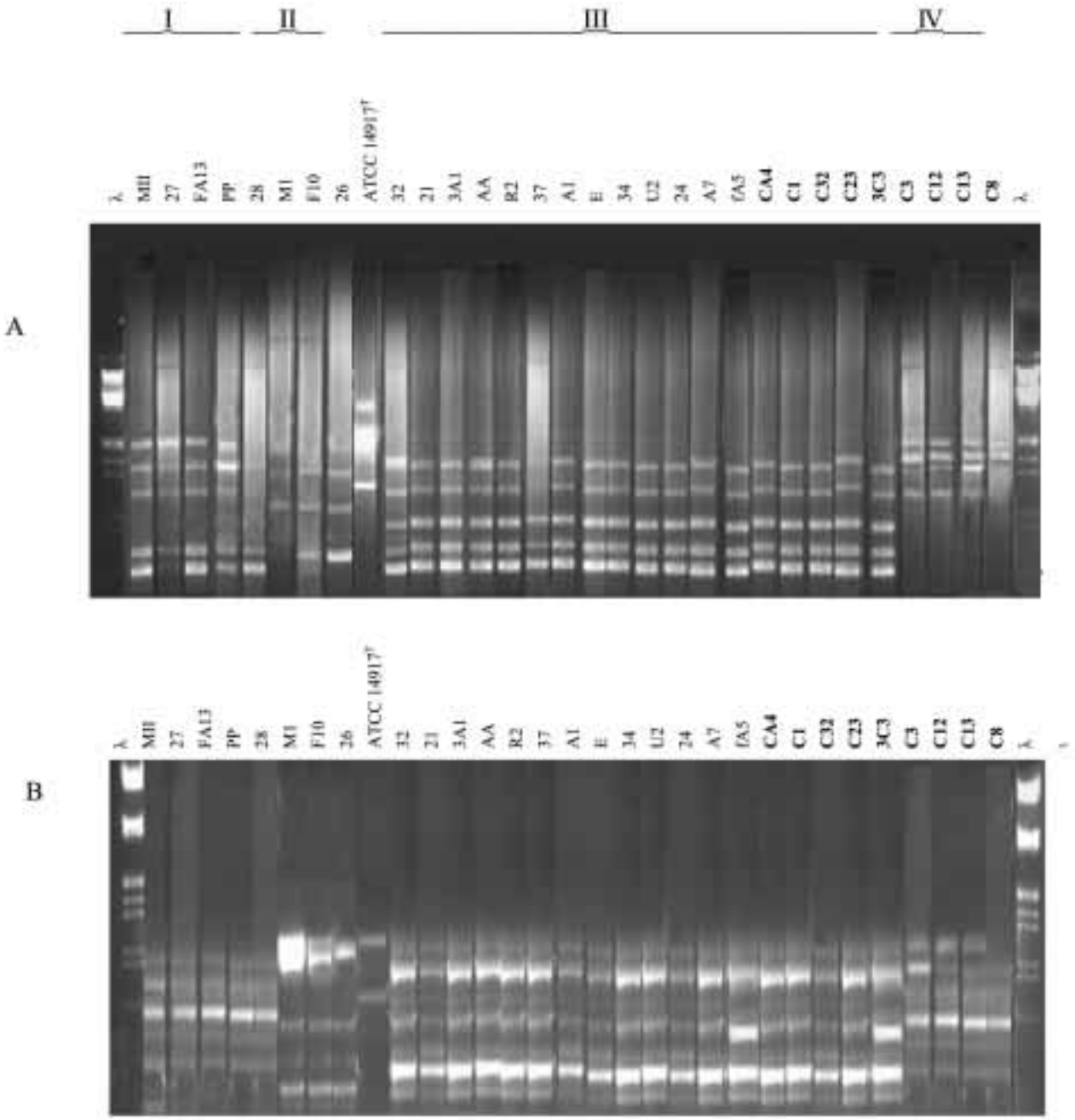

FIGURE 1

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

LITERATURE CITED

Berg, R.D., 1996. The indigenous gastrointestinal microflora. Trends Microbiol. 4, 430-435.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H. 1985. Practical implications of malolactic fermentation. A review. Am. J. Enol. Vitic. 36, 290-301.

Dellaglio, F., Bottazzi, V. & Trovatelli, L.D., 1973. Deoxyribonucleic acid homology and base composition in some thermophilic lactobacilli. J. Gen. Microbiol. 74, 289-297.

Demerec, M. , 1950 (1st ed). Biology of *Drosophila*. John Wiley & Sons, New York.

Dicks, L.M.T. & Van Vuuren, H.J.J., 1987. A modification of the hot tube method for the detection of carbon dioxide produced by heterofermentative *Lactobacillus* strains. J. Microbiol. Meth. 6, 273-275.

Dillon, R.J. & Dillon, V.M., 2004. Gut bacteria of insects. Nonpathogenic interactions. Ann. Rev. Entomol. 49, 71-92.

Du Plessis, H.W., Dicks, L.M.T., Pretorius, I.S., Lambrechts, M.G. & du Toit, M., 2004. Identification of lactic acid bacteria isolated from South African brandy base wines. Int. J. Food Microbiol. 91, 19-29.

Du Toit, M.G. & Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: Using weapons from nature's own arsenal - A review. S. Afr. J. Enol. Vitic. 21, 74-96.

Felske, A., Rheims, H., Wolterink, A., Stackebrant, E. & Akkermans, A.D.L., 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Acinetobacteria in grasslands soil. Microbiol. 143, 2983-2989.

Ganter, P.F., 2006. Yeast and invertebrate associations. In: Rosa, C. & Péter, G. (eds). Biodiversity and ecophysiology of yeasts. Springer. pp. 303-370.

Garbers, I-M., Britz, T.J. & Witthuhn, R.C., 2004. PCR-based denaturing gradient gel electrophoretic typification and identification of microbial consortiums present in Kefir grains. World J. Microbiol. Biotechnol. 20, 687-693.

Groenewald, W.H., 2005. A taxonomic study on lactic acid bacteria isolated from fruit flies. MSc. thesis, University of Stellenbosch.

Harrigan, W.F. & McCance, M.E., 1976 (1st ed). Laboratory Methods in Food and Dairy Microbiology. Academic Press, New York.

Kandler, O. & Weiss, N., 1986. Regular, nonsporing Gram-positive rods. In: Sneath, P.H.A., Mair, N., Sharpe, M.E., & Holt, J.G. (eds). Bergey's manual of systematic bacteriology, vol II. Williams & Wilkins, Baltimore. pp. 1208-1234.

Kullen, M.J., Khil, J., Busta, F.F., Gallaher, D.D. & Brady, L.J., 1998. Carbohydrate source and bifidobacteria influence the growth of *Clostridium perfringens in vivo* and *in vitro*. Nutr. Res. 18, 1889-1897.

Kvasnikov, E.I., Kovalenko, N.K. & Nesterenko, O.A., 1971. Lactic acid bacteria in insects. Mikrobiologia 40, 144-151.

Lilley, A.K., Hails, R.S., Cory, J.S. & Bailey, M.J., 1997. The dispersal and establishment of pseudomonad populations in the phyllosphere of sugar beet by phytophagous caterpillars. FEMS Microbiol. Ecol. 24, 151-57.

Mortimer, R. & Polsinelli, M., 1999. On the origins of wine yeast. Res. Microbiol. 150, 199-204.

Park, S.Y., Heo, Y.J, Kim, K.S. & Cho, Y.H., 2005. *Drosophila melanogaster* is susceptible to *Vibrio cholerae* infection. Mol Cells 20, 409-415.

Rousset, F., Vautrin, D. & Solignac, M., 1992. Molecular identification of *Wolbachia*, the agent of cytoplasmic incompatibility in *Drosophila simulans*, and variability in relation with host mitochondrial types. Proc. Biol. Sci. 247, 163-168.

Torriani, S., Felis, G.E. & Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactobacillusd paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. Appl. Environ. Microbiol. 67, 3450-3454.

Van Reenen, C.A. & Dicks, L.M.T., 1996. Evaluation of numerical analysis of random amplified polymorphic DNA (RAPD)-PCR as a method to differentiate *Lactobacillus plantarum* and *Lactobacillus pentosus*. Curr. Microbiol. 32, 183-187.

Zanoni, P., Farrow, J.A.E., Phillips, B.A. & Collins, M.D., 1987. *Lactobacillus pentosus* (Fred, Peterson and Anderson) sp. nov., nom. rev. Int. J. Syst. Bacteriol. 37, 339-341.