

# IDENTIFICATION OF LACTIC ACID BACTERIA ISOLATED FROM THE UROGENITAL TRACT OF WOMEN

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By

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## 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

Lactic acid bacteria are present in the oral cavity, gastrointestinal tract, vagina, plants, fermented foods, manure and sewage (Kandler & Weiss, 1986). These organisms are acidophilic and thrive at a low pH which renders the environment unsuitable for most other micro-organisms. A number of lactic acid bacteria (LAB) have probiotic properties, e.g. some strains in the gastrointestinal tract inhibit the growth of *Helicobacter* spp. which causes stomach ulcers (Midolo *et al.*, 1995). Some probiotic *Lactobacillus* spp. may also play a role in cholesterol reduction (Mital & Garg, 1995). A few reports have been published on the role of LAB in the prevention of preterm delivery and the protection against bacterial vaginosis (Hillier *et al.*, 1992; Martius *et al.*, 1988; Shalev *et al.*, 1995). A number of species produce antimicrobial peptides that inhibit the growth of other microbes, including potential pathogens.

Strains thus far isolated from the human vagina are *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus casei* (Rogosa & Sharpe, 1960; Giorgi *et al.*, 1987). In most of these studies simple biochemical and phenotypical tests were used. The purpose of this study was to isolate and identify lactic acid bacteria isolated from the posterior fornix of the vagina of non-pregnant and pregnant patients. Advanced taxonomic methods were used, e.g. sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), random amplified polymorphic DNA (RAPD)-PCR and 16S rRNA sequencing.

Numerical analysis of total soluble cell protein patterns grouped 13 of the facultative heterofermentative lactobacilli isolated from the vagina into one cluster with *Lactobacillus pentosus* at  $r \geq 0.70$ . Five other strains clustered at  $r \geq 0.73$  with strains of *L. plantarum*. Similar groupings were obtained by numerical analysis of the RAPD-PCR profiles of the strains, suggesting that they belong to *L. pentosus* and *L. plantarum*, respectively.

Thirty-one of the obligate homofermentative lactobacilli clustered with nine reference strains of the “*L. acidophilus*”-group at  $r \geq 0.67$ . The type strains of *L. gasseri* (DSM 20077<sup>T</sup>), *Lactobacillus crispatus* (NCFB 2752<sup>T</sup>), *Lactobacillus gallinarum* (NCFB 2235<sup>T</sup>), *Lactobacillus johnsonii* (NCFB 2241<sup>T</sup>) and *L. acidophilus* (ATCC 4356<sup>T</sup>, NCIMB 8690<sup>T</sup>) grouped into five different clusters, confirming their separate taxonomic status. Ten strains clustered with the type strains of *L. gasseri* (DSM 20077<sup>T</sup>) and *L. jensenii* (DSM 20557<sup>T</sup>) in one cluster at  $r \geq 0.81$ , suggesting that they are phenotypically closely related. No clear phenotypic distinction was recorded among strains of *L. gasseri* and *L. jensenii*. Four strains grouped with *L.*

*crispatus* NCFB 4, NCFB 2752<sup>T</sup> and NCFB 5 in one cluster at  $r \geq 0.83$ , suggesting that they are phenotypically closely related. Only one strain (TV 1006) grouped at  $r \geq 0.94$  with the type strain of *L. gallinarum* (NCFB 2235<sup>T</sup>) in a separate cluster. Nine strains grouped with the type strain of *L. johnsonii* (NCFB 2241<sup>T</sup>) at  $r \geq 0.79$  and seven strains formed a cluster with *L. acidophilus* ATCC 4356<sup>T</sup> and NCIMB 8690<sup>T</sup> at  $r \geq 0.80$ . Strains within each cluster displayed unique RAPD-PCR banding patterns, confirming the results obtained by SDS-PAGE.

Four of the obligate heterofermentative strains clustered with *Lactobacillus buchneri* ATCC 9460 and ATCC 12935 and *Lactobacillus fermentans* ATCC 14932 at  $r \geq 0.73$ . Seven strains grouped with *L. fermentum* ATCC 11739<sup>T</sup> and ATCC 23271 at  $r \geq 0.74$  in one cluster and three were related to *Weissella viridescens* ATCC 12076<sup>T</sup> and NCIMB 1615 at  $r \geq 0.76$  in a separate cluster. The strains of *Weissella viridescens* were phenotypically well separated from the strains in the other two clusters, as evident by their linking at  $r \geq 0.61$ . Based on the RAPD-PCR banding patterns obtained for *L. buchneri*, *L. fermentans*, *L. fermentum* and *W. viridescens*, the four species are well separated, each species defined in its own cluster.

Twenty-seven strains clustered with *Enterococcus faecium* NCIMB 583, BFE 1170 and BFE 1027 in one protein profile cluster at  $r \geq 0.78$ . Nine strains grouped with *Enterococcus faecalis* ATCC 13665 in cluster III at  $r \geq 0.77$ , well separated from the type strain of *E. faecalis* (NCIMB 775<sup>T</sup>) and *E. faecalis* NCFB 587. Based on these findings, the majority of enterococci (27 out of 36 strains) are members of *E. faecium*. Numerical analysis of RAPD-PCR grouped the vaginal enterococci into four separate clusters, suggesting a higher diversity among the strains than reflected with the phenotypic groupings obtained by their protein profiles. More strains of *E. faecalis*, *E. faecium* and members of other *Enterococcus* spp. will have to be included to evaluate numerical analysis of RAPD-PCR as a taxonomic technique for enterococci.

A new species, *Lactobacillus fornixalis*, was described. Five strains clustered with *L. jensenii* and *L. gasserii* at  $r \geq 0.83$  in one protein profile cluster. Numerical analysis of selected strains indicated that they belong to two different species. Four strains (TV 1010, TG 1013, TV 1018 and TV 1045) grouped into one protein profile cluster at  $r \geq 0.87$ . Strains from this cluster displayed similar RAPD-PCR profiles and grouped at  $R^2 \geq 0.78$ . A new species of rRNA group 1 *Lactobacillus* was identified based on the 16S rRNA sequences of strains TV 1018 and TG 1013. The name *Lactobacillus fornixalis* sp. nov. for strains TV 1010, TG 1013, TV 1018 and TV 1045 were proposed with TV 1018 as the type strain.

## OPSOMMING

Melksuurbakterieë kom voor in die mond, spysverteringskanaal, vagina, plante, gefermenteerde voedsel en riool (Kandler & Weiss, 1986). Hierdie mikro-organismes is asidofilies en floreer by 'n lae pH wat die omgewing ongeskik maak vir meeste ander mikro-organismes. Sommige melksuurbakterieë het probiotiese eienskappe, byvoorbeeld sommige stamme in die spysverteringskanaal inhibeer die groei van *Helicobacter* spp. wat maagsere veroorsaak (Midolo *et al.*, 1995). Sommige melksuurbakterieë mag dalk 'n rol speel in die verlaging van cholesterolvlakke (Mital & Garg, 1995). Die rol van melksuurbakterieë in die voorkoming van vroeggebore babas en die beskerming teen bakteriese vaginose is reeds beskryf (Hillier *et al.*, 1992; Martius *et al.*, 1988; Shalev *et al.*, 1995). Sommige stamme produseer antimikrobiese peptiede wat die groei van ander mikrobe, insluitend potensiële patogene, inhibeer.

Tot dusver is *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus plantarum* en *Lactobacillus casei* uit die menslike vagina geïsoleer (Rogosa & Sharpe, 1960; Giorgi *et al.*, 1987). In die meeste van hierdie studies is van biochemiese- of fenotipiese metodes gebruik gemaak. Die doel van hierdie studie was om melksuurbakterieë uit die vagina van swanger en nie-swanger pasiënte te isoleer en te identifiseer. Gevorderde taksonomiese metodes is gebruik, naamlik numeriese analiese van SDS-poliakrielamied jelelektroforese (SDS-PAGE), lukraak ge-amplifiseerde polimorfiese DNA (RAPD)-polimerase kettingreaksie (PKR) en 16S rRNA volgordebepaling.

Numeriese analiese van totaal oplosbare selproteïen bandpatrone het 13 van die fakultatief heterofermentatiewe lactobacilli wat uit die vagina geïsoleer is in een groep op 'n vlak van  $r \geq 0.70$  met *Lactobacillus pentosus* geplaas. Vyf ander stamme het op 'n vlak van  $r \geq 0.73$  met stamme van *L. plantarum* gegroepeer. Soortgelyke groeperings is met numeriese analiese van RAPD-PKR profiele verkry. Hierdie resultate dui daarop dat die stamme tot die spesies *L. pentosus* en *L. plantarum* behoort.

Een en dertig van die obligaat homofermentatiewe lactobacilli het met nege van die verwysingsstamme van die "*L. acidophilus*"-groep op 'n vlak van  $r \geq 0.67$  gegroepeer. Die tipe stamme van *L. gasseri* (DSM 20077<sup>T</sup>), *Lactobacillus crispatus* (NCFB 2752<sup>T</sup>), *Lactobacillus gallinarum* (NCFB 2235<sup>T</sup>), *Lactobacillus johnsonii* (NCFB 2241<sup>T</sup>) en *L. acidophilus* (ATCC 4356<sup>T</sup>, NCIMB 8690<sup>T</sup>) het in vyf groepe gesorteer en sodoende hul aparte

taksonomiese status bevestig. Tien stamme het met die tipe stamme van *L. gasseri* (DSM 20077<sup>T</sup>) en *L. jensenii* (DSM 20557<sup>T</sup>) in een groep op 'n vlak van  $r \geq 0.81$  gegroepeer, wat voorstel dat hulle fenotopies naverwant is. Geen duidelike fenotopiese onderskeid is tussen stamme van *L. gasseri* en *L. jensenii* gemaak nie. Vier stamme het met *L. crispatus* NCFB 4, NCFB 2752<sup>T</sup> en NCFB 5 in een groep op 'n vlak van  $r \geq 0.83$  gegroepeer, wat daarop dui dat hulle fenotopies naverwant is. Slegs een stam (TV 1006) het op 'n vlak van  $r \geq 0.94$  met die tipe-stam van *L. gallinarum* (NCFB 2235<sup>T</sup>) in 'n aparte groep gesorteer. Nege stamme het met die tipe-stam van *L. johnsonii* (NCFB 2241<sup>T</sup>) op 'n vlak van  $r \geq 0.79$  gegroepeer en sewe stamme het 'n groep met *L. acidophilus* ATCC 4356<sup>T</sup> en NCIMB 8690<sup>T</sup> by  $r \geq 0.80$  gevorm. Die stamme binne 'n bepaalde proteïenprofiel-groep het unieke RAPD-PKR bandpatrone gelewer en bevestig die resultate wat met SDS-PAGE verkry is.

Vier van die obligaat heterofermentatiewe stamme het met *Lactobacillus buchneri* ATCC 9460 en ATCC 12935, asook *Lactobacillus fermentans* ATCC 14932 op 'n vlak van  $r \geq 0.73$  gegroepeer. Sewe stamme het met *L. fermentum* ATCC 11739<sup>T</sup> en ATCC 23271 op 'n vlak van  $r \geq 0.74$  in een proteïenprofiel-groep gesorteer. Drie stamme was verwant aan *Weissella viridescens* ATCC 12076<sup>T</sup> en NCIMB 1615 en het op 'n vlak van  $r \geq 0.76$  gegroepeer. Die stamme van *Weissella viridescens* is fenotopies nie naverwant aan die stamme in die ander twee groepe nie, soos ook aangedui deur hul koppeling by  $r \geq 0.61$ . Gebaseer op die RAPD-PKR bandpatrone verkry met *L. buchneri*, *L. fermentans*, *L. fermentum* en *W. viridescens*, is die vier spesies onafhanklik.

Sewe en twintig stamme het met *Enterococcus faecium* NCIMB 583, BFE 1170 en BFE 1027 in een proteïenprofiel-groep op 'n vlak van  $r \geq 0.78$  gegroepeer. Nege stamme het met *Enterococcus faecalis* ATCC 13665 in een groep op 'n vlak van  $r \geq 0.77$  gegroepeer, apart van die tipe-stam van *E. faecalis* (NCIMB 775<sup>T</sup>) en *E. faecalis* NCFB 587. Gegronde op hierdie bevindings is die meerderheid enterococci (27 uit 36 stamme) lede van *E. faecium*. Numeriese analiese van RAPD-PKR het die vaginale enterococci in vier aparte groepe geplaas, wat 'n groter diversiteit tussen die stamme uitwys as wat met proteïenprofiel verkry is. Meer stamme van *E. faecalis*, *E. faecium* en lede ander *Enterococcus* spp. sal ondersoek moet word ten einde die tegniek numeriese analiese van RAPD-PKR as taksonomiese metode vir enterococci te evalueer.

'n Nuwe spesie, *Lactobacillus fornixialis*, is beskryf. Vyf isolate het met *L. jensenii* en *L. gasseri* by  $r \geq 0.83$  gegroepeer. Vier isolate (TV 1010, TG 1013, TV 1018 en TV 1045) het in een proteïengroep by  $r \geq 0.87$  gegroepeer. Isolate van hierdie groep het dieselfde RAPD-

PKR profiele gehad en het by  $R^2 \geq 0.78$  gegroep. 'n Nuwe spesie van rRNA-groep 1 *Lactobacillus* is geïdentifiseer op grond van die 16S rRNA volgorde van stamme TV 1018 en TG 1013. Die naam *Lactobacillus fornixalis* sp. nov. is vir isolate TV 1010, TG 1013, TV 1018 en TV 1045 voorgestel, met isolaat TV 1018 as die tipe stam.

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## **BIOGRAPHICAL SKETCH**

Marieka Elizma Silvester was born on 19 December 1974 in Heidelberg, Western Cape and matriculated at Gerrit du Plessis Secondary School, Riversdale, in 1992. She enrolled as a B.Sc. student at the University of Stellenbosch in 1993 and obtained the degree in 1995, majoring in Microbiology and Biochemistry. She obtained the Hons. B.Sc. in Microbiology in 1996 at the University of Stellenbosch.

## **PREFACE**

The literature study is discussed in two reviews. In the first part the microbial flora of the urogenital tract is discussed. The taxonomy of lactic acid bacteria, with special emphasis on the species found in the human vagina, is discussed in a second part.

The results of this study are presented in the form of two manuscripts prepared for publication in *International Journal of Systematic Bacteriology*.

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## DEDICATION

This thesis is dedicated to my parents.

***“the sure and definite determination (of species of bacteria) requires so much time, so much acumen of the eye and judgement, so much of perserverance and patience that there is hardly anything else so difficult” - Mueller***

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## 1. INTRODUCTION

At present the lactic acid bacteria (LAB) are divided into nine genera, viz. *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Carnobacterium* and *Vagococcus*. They produce lactic acid from hexoses and are widely used in fermented foods and beverages (Jay, 1992). 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 & Weiss, 1986) and the intestinal tracts and mucous membranes of humans and animals (Schlegel, 1986). Species such as *Streptococcus lactis*, *Streptococcus thermophilus*, *Streptococcus lactis* and *Lactobacillus bulgaricus* are used as starter cultures in milk and other fermentation products. *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus lactis*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Enterococcus faecium*, *Enterococcus faecalis* and *Bifidobacterium* spp. are used in probiotic products (Hammes *et al.*, 1992).

Lactic acid bacteria are found at cell numbers of  $10^4$ .ml<sup>-1</sup> in the stomach,  $10^4$ .ml<sup>-1</sup> in the jejunum,  $10^3$ - $10^7$ .ml<sup>-1</sup> in the ileum and  $10^4$ - $10^8$ .ml<sup>-1</sup> in the colon (Evaldson *et al.*, 1982). Anaerobic cocci, lactobacilli and *Bacteroides* spp. are the predominant bacteria in the lower genital tract (Bartlett *et al.*, 1977). Most of the strains thus far isolated from the human gastro-intestinal and urogenital tracts belong to the genera *Lactobacillus* and *Bifidobacterium* (Evaldson *et al.*, 1982). These bacteria start to colonise the intestinal tract within the first 25 hours after birth, followed by anaerobic cocci (Bertazzoni *et al.*, 1978).

The bacterial population of the intestinal tract is mainly controlled by the gastric acidity and peristaltic movement. The lower the pH, the lower the bacterial count and vice versa (Evaldson *et al.*, 1982). Several lactic acid bacteria isolated from the human intestinal tract have probiotic properties, some of which inhibit the growth of *Helicobacter* spp. which causes stomach ulcers (Midolo *et al.*, 1995). Probiotic strains of *Lactobacillus* spp. have also been shown to reduce serum cholesterol levels (Mital & Garg, 1995). *L. casei*, *Lactobacillus rhamnosus*, *L. acidophilus*, *Lactobacillus fermentum*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *L. salivarius*, *L. plantarum* and *Lactobacillus cellobiosus* have been isolated from the oral cavity (Rogosa *et al.*, 1953). *L. acidophilus* and *L. fermentum* were isolated from the stomach. In the upper duodenum *L. acidophilus*, *L. fermentum* and *L. salivarius* dominate. In the large intestine *L. acidophilus*, *L. fermentum*, *L. salivarius*, *L. lactis*, *L. casei*, *L. plantarum*, *L. brevis*, *L. buchneri*, *Bifidobacterium infantis*, *Bifidobacterium longum* and *Bifidobacterium adolescentis* have been isolated (Rasic & Kurmann, 1983; Sharpe, 1981). In the vagina *L. acidophilus*, *L. fermentum*, *L. rhamnosus* and *L. cellobiosus* have been described (Rogosa & Sharpe, 1960).

The vaginal microflora is part of a complex but finely balanced ecosystem. Lactobacilli are the predominant micro-organisms in the vagina of healthy women (Redondo-Lopez *et al.*, 1990) and helps to maintain a pH of 3.5-4.5 (Bokkenhauser, 1993). The low pH and the production of hydrogen peroxide by some LAB, especially *L. crispatus* and *L. jensenii*, prevents the growth of pathogenic micro-organisms (Eschenbach *et al.*, 1989; Hillier, 1998; Redondo-Lopez *et al.*, 1990). Some claims have been made which links LAB to the prevention of preterm delivery (Hillier *et al.*, 1992; Martius *et al.*, 1988; Shalev *et al.*, 1995). Clearly more research is needed to determine the role of vaginal lactic acid bacteria.

In the past, taxonomic studies on the vaginal flora was performed by using conventional classification techniques such as sugar fermentation reactions and other biochemical assays (Rogosa & Sharpe, 1960; Fagnant *et al.*, 1982). These techniques were not always reliable and many misclassifications were made. One of the best examples is the recent designation of certain strains of *L. acidophilus* as *L. gallinarum*, *L. johnsonii* and *L. crispatus* (Fujisawa *et al.*, 1992; Cato *et al.*, 1993). This reclassification was based on results obtained from using modern taxonomic techniques like DNA-DNA hybridization studies. Giorgi *et al.* (1987) used DNA-DNA homology data to classify lactobacilli isolated from the vagina of healthy women. Although very accurate, the technique is laborious and only provides data on a genetic level.

The phenotypic characteristics of strains are equally important. Numerical analysis of total soluble cell protein patterns proved to be a reliable technique that corresponded well with results obtained by DNA homology studies (Dellaglio *et al.*, 1991; Dicks and Van Vuuren, 1987; Dicks *et al.*, 1990, 1995, 1996; Du Plessis and Dicks, 1994; Torriani *et al.*, 1996; Van Reenen and Dicks, 1996). This is not surprising, since the DNA sequence of the microbial genome is the primary level of information and is expressed at the second level in the structure of protein molecules (Kerstens, 1985).

The present study was undertaken to identify the diverse LAB population present in the human vagina by using the methods numerical analysis of total soluble cell protein patterns, numerical analysis of RAPD-PCR banding patterns and 16S rRNA sequencing. In previous studies, results obtained by numerical analyses of total soluble cell protein patterns and RAPD-PCR banding patterns corresponded well (Van Reenen & Dicks, 1996).

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## **2. MICROFLORA OF THE UROGENITAL TRACT**

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# MICROFLORA OF THE UROGENITAL TRACT

## INTRODUCTION

*Lactobacillus*, *Streptococcus* and *Staphylococcus* spp. are the dominant bacteria in the vagina and cervix (Bartlett *et al.*, 1977). Cell counts as high as  $10^7$ - $10^8$ .ml<sup>-1</sup> *Lactobacillus* spp.,  $10^4$ .ml<sup>-1</sup> *Staphylococcus* spp. and  $10^5$ .ml<sup>-1</sup> *Streptococcus* spp. have been reported from vaginal secretions (Hillier *et al.*, 1993; Redondo-Lopez *et al.*, 1990). The composition of the microflora is influenced by various parameters, e.g. the amount of glycogen present in the vaginal lumen, personal hygiene, age, menarche, hormonal changes, pregnancy and menopause (Hillier, 1998; Redondo-Lopez *et al.*, 1990). Vaginal douching for instance partially eliminates the normal vaginal microflora (Onderdonk *et al.*, 1992), including *Lactobacillus* spp. (Hillier, 1991). This may lead to conditions such as chlamydial infection, pelvic inflammation (Scholes *et al.*, 1993), ectopic pregnancy (Chow *et al.*, 1985) and tubal infertility (Baird *et al.*, 1996).

Priestley *et al.* (1997) studied the composition of the vaginal microflora of healthy women over time in relation to hormonal changes, sexual activity and hygiene habits. Only four of the 26 patients studied had "normal" vaginal microflora throughout (i.e. mainly *Lactobacillus* spp.), while 10 had intermittent bacterial vaginosis and the remaining patients intermittent candidiasis. In another study by Goldacre *et al.* (1979), 21% of the patients suffered from yeast and fungal infections, while 1% had *Trichomonas vaginalis*. These studies raised doubts about what should be regarded as normal flora. It calls into question the significance of finding bacterial vaginosis, *Candida*, *Mobilincus hominis*, *Ureaplasma urealyticum* or  $\beta$ -haemolytic streptococci on a single occasion in asymptomatic women, as well as the significance of finding normal flora on a single occasion in symptomatic women.

Although *Lactobacillus* spp. are the predominant micro-organisms in the vagina ( Hillier *et al.*, 1993; Redondo-Lopez *et al.*, 1990; Stahl & Hill, 1986), they are not transmitted from the mother to the child and, therefore, do not colonise in the digestive tract of newborn infants. This was clearly shown by the variations in plasmid profiles and genetic relatedness recorded among strains isolated from mothers and their infants (Tannock *et al.*, 1990). On the other hand, in nearly 80% of the cases studied, intestinal strains of *Enterobacteriaceae* and bifidobacteria were passed on from the mother to her child.

A study conducted on girls from the age of three months to fifteen years have shown that anaerobic bacteria are the most predominant in the vagina (Hill *et al.*, 1995). *Lactobacillus* spp. were seldom isolated from girls between the age of three months and five years. In these girls *Peptostreptococcus* spp., *Prevotella* spp. and *Actinomyces* spp. were frequently isolated (Hill *et al.*, 1995). The reason for the apparent lack in high cell numbers of lactobacilli might be pH linked. The pH of the vaginal fluid of prepubertal children is between 6.0 to 7.0 (Hill *et al.*, 1995). This might also explain why lactic acid bacteria are more frequently isolated from girls aged eleven years and older. *Lactobacillus* spp. were present in 88% of the latter patients (Hammerschlag *et al.*, 1978). Other organisms isolated from teenage girls were *Corynebacterium vaginale* (13.5%), yeast (28%), genital mycoplasmas (28%), *Neisseria gonorrhoeae* (1%) and *Trichomonas vaginalis* (2%). *Candida tropicalis* was most frequently isolated from children under the age of three years, while *Candida albicans* was more common among older girls.

Shafer *et al.* (1985) studied the prevalence of *Gardnerella vaginalis*, *Lactobacillus* spp., *Mycoplasma* spp., *Ureaplasma urealyticum*, *Staphylococcus aureus*, yeast, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *T. vaginalis* in adolescent girls. Specific attention was given to differences in sexual activity, the use of contraceptive devices and the presence of non-specific vaginitis. Sexually active girls had significantly more *G. vaginalis*, *Lactobacillus* spp., *Mycoplasma* spp. and *U. urealyticum*. *Neisseria gonorrhoea*, *C. trachomatis* and *T. vaginalis* were isolated only from sexually active girls.

According to Hillier and Lau (1997) the vaginal microflora of postmenopausal women who never received estrogen replacement therapy is similar to the vaginal microflora of prepubertal girls. *Lactobacillus* spp. were recovered in 49% of these women at an average cell count of ca.  $10^6$  cfu.g<sup>-1</sup> vaginal fluid. Strains of *G. vaginalis* were isolated from 27% of the women, *U. urealyticum* from 13%, *C. albicans* from 1%, *Prevotella bivia* from 33% and coliforms from 41% of the patients. Coliforms were recovered at a higher frequency from premenopausal women, while the other organisms were recorded at a lower frequency.

In another study involving elderly women who had not received estrogen therapy and with a vaginal pH of 6.5, faecal bacteria dominated. High cell numbers of *Lactobacillus* spp. were present in 19.4% of the women studied. There was a good correlation ( $p < 0.001$ ) between vaginal pH and the absence or presence of lactobacilli. No association could be found between vaginal pH and the absence or presence of faecal bacteria (Milsom *et al.*, 1993). The more alkaline vaginal pH of older women makes them more vulnerable to urogenital

diseases. The more alkaline the pH, the lesser lactobacilli in the vagina. The vaginal pH does not have an effect on faecal bacteria (Milsom *et al.*, 1993).

Information on the normal vaginal flora of elderly females is limited. Farooqui *et al.* (1996) studied the vaginal flora of elderly women to understand the interpretation of vaginal colonization. The most common organism in the vaginal flora of institutionalised elderly females is *Enterococcus* spp. (72.6%), followed by corynebacteria (57.5%), *Escherichia coli* (51.9%), *Proteus mirabilis* (34.9%) and *Klebsiella pneumonia* (28.3%). In community-residing elderly females, coagulase negative staphylococci (37.2%), corynebacteria (34.9%), *Enterococcus* spp. (32.6%) and *E.coli* (23.2%) have been isolated (Farooqui *et al.*, 1996). The researchers concluded that elderly females, whether institutionalized or community residing, remain asymptomatic for urogenital infections despite demonstrated vaginal colonization.

Hormonal levels play an important role in growth stimulation of vaginal lactobacilli, as reflected in studies on the karyopyknotic index (KPI) of a patient. The KPI, a sensitive indicator of hormone stimulation, is higher in women weighing more than 85 kg and with a diastolic blood pressure  $\geq 100$  mmHg. The KPI is also higher in menopausal women, usually older than 53 years. In general cigarette smoking has a negative affect on estrogen metabolism and increases the KPI (Baron *et al.*, 1990). However, Milsom *et al.* (1993) reported a lower vaginal pH among smokers. This, and the increased nicotine levels in the bloodstream, might explain the increased cell numbers of lactobacilli as reported by Milsom *et al.* (1993).

## FACTORS WHICH MAY INFLUENCE THE COMPOSITION OF THE VAGINAL MICROFLORA

### Menstruation

Corbishley (1977) found no significant changes in the total vaginal flora at different stages of the menstrual cycle. However, in a study by Wilks and Tabaqchali (1987) the mean number of vaginal species isolated declined from ca.  $10^5$  cfu.ml<sup>-1</sup> in week 1 of the menstrual cycle to ca.  $10^3$  cfu.ml<sup>-1</sup> in week 4. The decrease was accounted for by a general decrease in the incidence of most types of bacteria. The pH of the vagina decreased from 6.6 in week 1 to

4.3 in week 4, but it was not attributed to the action of lactobacilli, since the cell numbers remained constant during the menstrual cycle.

### Contraception

Significantly more anaerobic bacteria were isolated from the cervix of women using oral contraception or an intrauterine device (IUD). In women using barrier contraception lactobacilli alone dominated the microbial flora. The women who participated in the study were all healthy and had no microbial infections. Barrier contraception with a condom prevented this anaerobic shift and maintained a lactobacilli-dominated flora in the cervix. Semen makes the vagina more alkaline. This may lead to an anaerobic shift and a decrease in the number of lactobacilli in the vagina. By preventing contact of semen with the vagina a lactobacilli-predominant vaginal ecosystem is ensured (Haukkamaa *et al.*, 1986).

### Gynaecological surgery

Microflora from the cervix may cause post-operative pelvic infections (Schwarz, 1976). With knowledge on the composition of the patient's vaginal flora the bacteria causing post-operative infection can be predicted. Anaerobic organisms predominate in patients with invasive cervical cancer. In these patients the cell numbers of lactobacilli, *Staphylococcus epidermidis* and enterococci decreases, but the numbers of *E. coli* and *Bacteroides* increase (Mead, 1978).

### Pregnancy

There are two primary vaginal flora patterns in pregnant women, namely (i) normal microflora which consists of predominantly lactobacilli and (ii) bacterial vaginosis, i.e. no lactobacilli present (Table 1). A third less distinct transitional pattern, namely intermediate flora, refers to a condition of reduced lactobacilli counts, i.e. inbetween a state of normal and bacterial vaginosis (Table 1). Group B streptococci and yeast are associated with normal or intermediate flora, while *N. gonorrhoeae* and *Chlamydia trachomatis* are more frequently isolated from patients with intermediate flora or bacterial vaginosis. *T. vaginalis* is mostly associated with intermediate flora (Hillier *et al.*, 1992).



The microbial flora of the lower genital tract of women in labour were studied by Mason *et al.* (1989). The patients were divided into four groups: (i) patients with a normal vaginal delivery, (ii) meconium stained liquor (test done to indicate possible foetal distress), (iii) a history of prolonged rupture of membranes, and (iv) patients who gave birth by a caesarean. *T. vaginalis* was identified in 19% of the women, but was not associated with any specific group. *Chlamydia* was detected in 13% of the women and *N. gonorrhoeae* isolated from 7% of the patients. Gonococci, group B streptococci and *Bacteroides* spp. were isolated more frequently from patients with a history of prolonged ruptured membranes than from patients who had a normal vaginal delivery. *Lactobacillus* spp. were isolated from 20% of the patients (Mason *et al.*, 1989).

It is generally accepted that pregnant women with a *Lactobacillus*-predominant flora have a reduced risk of preterm delivery (Martius *et al.*, 1988), while women with a disturbed vaginal flora associated with bacterial vaginosis have an increased risk of preterm delivery (Martius *et al.*, 1988; Gravett *et al.*, 1986), amnionitis (Silver *et al.*, 1989) and postpartum endometritis (Watts *et al.*, 1990). Contrarily to this Kotzé *et al.* (1997) concluded that the absence or presence of lactobacilli in the vagina did not have an effect on the prevalence of preterm delivery or the duration of pregnancy. This phenomenon may be because of a few possible factors like the lack of protection by a specific *Lactobacillus* sp., low lactobacilli frequency or persistent bacterial vaginosis (Kotzé *et al.*, 1997).

Researchers found an association between preterm delivery and the presence of fetal fibronectin in vaginal secretions (Leeson & Maresh, 1993; Lockwood *et al.*, 1991; Nageotte *et al.*, 1994). Damage to the fetal membranes releases fetal fibronectin into the cervix and vagina which in turn provides a biochemical marker for preterm delivery. Such damage may be caused by vaginal micro-organisms. Women with complicated pregnancies such as preterm premature rupture of the membranes (PPROM), preterm delivery and bleeding had higher mean concentrations of vaginal fetal fibronectin than patients with no medical complications. Since the fibronectin concentration is high in amniotic fluid, it may not be influenced by bacterial vaginosis. Bacterial vaginosis is more common in women with

**Table 1.** Recovery of microorganisms from pregnant women with various vaginal flora patterns (Hillier *et al.*, 1992)

	Gram smear interpretation		
	Normal	Intermediate	Bacterial vaginosis
<i>G. vaginalis</i>	1533/4867 (31%)	1040/1292 (80%)	1692/1759 (96%)
<i>Lactobacillus</i> spp.	3871/4863 (80%)	822/1294 (64%)	773/1757 (44%)
<i>Bacteroides</i>	355/4851 (7%)	324/1289 (25%)	642/1751 (37%)
Group B streptococci	928/4872 (19%)	244/1292 (19%)	261/1762 (15%)
<i>M. hominis</i>	625/4835 (13%)	610/1282 (48%)	1169/1748 (67%)
<i>U. urealyticum</i>	3294/4816 (68%)	1018/1278 (80%)	1492/1744 (86%)
<i>N. gonorrhoeae</i>	35/4872 (0.7%)	25/1293 (1.9%)	38/1717 (2.2%)
<i>C. trachomatis</i>	308/4828 (6%)	155/1279 (12%)	207/1732 (12%)
<i>T. vaginalis</i>	441/4847 (9%)	275/1292 (21%)	232/1749 (13%)
<i>Candida</i> spp.	406/4398 (9%)	112/1294 (9%)	78/1760 (4%)

PPROM. These patients also have significantly less H<sub>2</sub>O<sub>2</sub>-producing lactobacilli than patients with a medical complication. Hydrogen peroxide-producing lactobacilli and non-H<sub>2</sub>O<sub>2</sub>-producing floras were significantly less common in the PPRM group compared to patients with no medical complications. Women in the bleeding group had a significantly lower frequency of lactobacilli dominated flora than patients with no medical complications, but not a higher frequency of bacterial vaginosis. No association could be detected between vaginal fetal fibronectin values and the vaginal microflora in women with pregnancies complicated by either preterm labour, PPRM or bleeding. The elevation of fetal fibronectin may be caused by factors other than microorganisms, i.e. amniotic fluid, labour or bleeding. The presence of vaginal fibronectin and absence of H<sub>2</sub>O<sub>2</sub>-producing lactobacilli indicates an increased risk for preterm delivery, i.e. before 34 weeks of pregnancy (Goffeng *et al.*, 1997).

## BACTERIAL VAGINOSIS (BV)

### General

Bacterial vaginosis (non-specific vaginitis) is a polymicrobial vaginal infection caused by an increase in obligate anaerobic organisms (Weaver & Mengel, 1988) and is the most prevalent type of vaginitis (Eschenbach *et al.*, 1988). Under normal circumstances, lactobacilli are the predominant flora, but a large variety of aerobic and anaerobic bacteria are also present. The anaerobic organisms are associated with BV when present in high cell numbers (Levison *et al.*, 1979; Masfari *et al.*, 1986). The bacterial cell count in a healthy vaginal ecosystem is 10<sup>5</sup> - 10<sup>6</sup>.g<sup>-1</sup> secretion, but increases to 10<sup>9</sup> - 10<sup>11</sup>.g<sup>-1</sup> secretion in patients with BV (Levison *et al.*, 1979; Hill *et al.*, 1984). The cause of this shift in the vaginal ecosystem is unknown, but the cell numbers of lactobacilli, especially H<sub>2</sub>O<sub>2</sub>-producing strains are much lower in patients suffering of BV (Eschenbach *et al.*, 1989). *Gardnerella* spp., various anaerobes and genital mycoplasmas may play a role in the development of the full-blown syndrome (Thomason *et al.*, 1991). According to Thorson *et al.* (1998) BV in pregnant women is caused by an intermicrobial interaction in which *G. vaginalis*, anaerobic bacteria and *M. hominis* are dominating.

Bacterial vaginosis-associated organisms which have been identified as potential pathogens are *G. vaginalis*, *M. hominis*, *Prevotella disiens* and *Prevotella melaninogenica*. However, *G. vaginalis* has been isolated in more than 50% of women with no BV (Eschenbach *et al.*, 1988). According to Fredericson *et al.* (1984) the presence of *G. vaginalis* indicates a disturbed ecological situation within the vagina, resulting in a decrease in numbers of

lactobacilli. This may lead to other symptoms of BV like a foul-smelling vaginal discharge and a high vaginal pH. In a study to evaluate the *in vitro* adherence of *Lactobacillus* spp. to epithelial cells, it was found that the presence of *G. vaginalis* which adheres strongly to the vaginal epithelial cells interferes with the adherence of lactobacilli (Wood *et al.*, 1985).

*Mobilincus* spp. is highly specific for BV, but is difficult to identify in wet mount preparations of vaginal secretions because of its physical size (Thomason *et al.*, 1991). Virtually all women with *Mobilincus* spp. in their vaginal secretions suffer from BV (Hallen *et al.*, 1987). These small rods have not been detected in the vaginal and cervical flora of healthy premenopausal women or premenarchal vagina (Gorbach *et al.*, 1973; Sautter & Brown, 1980).

Anaerobic bacteria are present in higher frequencies in women with BV and play a causal role. Whether overgrowth is the primary pathogenic event or merely a consequence of a yet unknown factor is still unclear (Weaver & Mengel, 1988).

Patients with BV have a low tissue redox potential and a high pH in the vagina. This increases the potential of infection (Holmes *et al.*, 1985). In patients with gynaecologic problems, BV is associated with proved pelvic disease, urinary tract infections, endometritis and postoperative vaginal cuff infections as shown by laparoscopy (Hooten *et al.*, 1989; Larson *et al.*, 1990; Soper *et al.*, 1990; Stamm *et al.*, 1989; Westrom, 1983). In patients with obstetric disease, BV is related to preterm labour, premature rupture of membranes, choroamnionitis and postcaesarean and postpartum endometritis (Faro *et al.*, 1988; Hillier *et al.*, 1988; Newton *et al.*, 1990; Watts *et al.*, 1990).

### **Risk factors and the role of contraceptives**

There is no direct association between any of the nonbarrier contraceptive methods and the occurrence of various vaginitidies (Roy, 1991). Women using a spermicidal foam or jelly are less likely to develop BV than women using no contraception at all. The nature of this possible protective effect is unknown. Oral contraceptive and condom use show significant protection against BV (Shoubnikova *et al.*, 1997). However, oral contraceptive use vs. other contraceptive methods is associated with a greater or similar frequency of candidiasis, increased numbers of anaerobic microorganisms, an increased or similar frequency of *C. trachomatis* and a reduced frequency of BV and trichomoniasis (Roy, 1991). A major risk

factor identified for BV is the use of an intra-uterine device (IUD) (Amsel *et al.*, 1983; Lefevre *et al.*, 1988; Majoroni, 1991). In contrast to this, Shoubnikova *et al.* (1997) found that the use of IUD showed no association with BV. There may be various reasons for this, as summarised below:

- \* In previous studies, IUD users were compared with oral contraception and condom users. This might lead to the conclusion that IUD users have an increased risk of having BV, since they were compared with groups of women who are at a reduced risk for changing their vaginal flora.
- \* Non-users of contraceptives were not included as control groups in previous studies. The definition of women who should have a vaginal milieu not influenced by any contraceptive use must be women who do not use any contraceptives.
- \* The inability to adjust for age differences between cases and controls in previous studies. Women with BV tend to be a little older than women without BV (Nilsson *et al.*, 1997). The same is true for IUD users. Doctors are reluctant to recommend the use of IUD in young women. IUD use and occurrence of BV show an association only because the age of these women is similar (Amsel *et al.*, 1983).

Multiple sex partners concurrent or prior to *T. vaginalis* infection is also considered a risk factor (Majoroni, 1991).

### **Sexual transmission**

Unequivocal sexual transmission of BV remains to be proved. Nilsson *et al.* (1997) found that BV is associated with sexual behaviour risk factors similar to those associated with genital *Chlamydia trachomatis* infection, except for a higher frequency of experience of casual sex in the latter group. Berger *et al.* (1995) concluded that with respect to BV, lesbians in monogamous relationships usually have concordant vaginal secretions, which demonstrates the sexual transmission of BV. In 72% of the cases the partners of women with BV were also infected while only 10% of the partners of women without BV were infected (Berger *et al.*, 1995).

## Diagnosis

The only consistent symptom observed for patients with BV is a profuse, malodorous vaginal discharge. More than 50% of women suffering from BV have no symptoms (Thomason *et al.*, 1991). Clinical signs of BV infection are the development of clue cells, i.e. epithelial cells surrounded with coccobacilli which obscure the borders of the cells so that the edges appear fuzzy rather than smooth; homogenous vaginal discharge; release of a fishy odour when 10% KOH is added; and an elevated pH (Gardner & Dukes, 1955; Amsel *et al.*, 1983). If three of these symptoms occur a patient is diagnosed positive for BV.

The sensitivity and specificity of the Gram-stain on vaginal bacteria, as interpreted by the Nugent criteria for the identification of BV (Nugent *et al.*, 1991) is calculated at 89%, compared with the Amsel criteria (Amsel *et al.*, 1983) which has a specificity of 83%. The lower specificity obtained with the Amsel criteria may lead to the underdiagnosis of BV (Schewbke *et al.*, 1996). Thomason *et al.* (1992) proposed a new simplified Gram stain interpretative method which combines clue cells with bacterial morphotypes as diagnostic markers. If clue cells are present and the non-lactobacilli morphotypes exceed lactobacilli morphotypes, bacterial vaginosis can be diagnosed without the need for exact bacterial counts.

The use of the Papanicolaou-stained smear (Pap smear) is also an accurate diagnosis of BV. The sensitivity of this test to diagnose BV is 88.7% and the specificity is 98.8%. The positive predictive value is 89.8 and the negative predictive value 98.7 (Giacomini *et al.*, 1998).

## BV and preterm delivery

Women with BV are 40% more likely to give birth to a premature, low-weight infant than women without BV (Hillier *et al.*, 1995). A study conducted on women from a developing country indicated that 29% of asymptomatic patients did not have any microbiological evidence of a lower genital tract infection, 52% had BV and mixed vaginal infections of BV, and trichomoniasis were present in 14% (Govender *et al.*, 1996). A total of 46% of the women suffered poor pregnancy outcome. These results highlight the role of BV in preterm delivery.

Pregnant women do not develop BV after 16 weeks gestation, and if present, remits spontaneously in approximately half of those who reach term. Since BV is associated with increased rates of second trimesters miscarriage and preterm delivery, any treatment aimed at its eradication in pregnancy should be given no later than the beginning of the second trimester of pregnancy (Hay *et al.*, 1994). Diagnosis of BV at any point during pregnancy is associated with an increased risk of prenatal complications in spite of spontaneous recovery in subsequent examinations (Gratacos *et al.*, 1998).

Joesoef *et al.* (1995) found that 85% of pregnant women with BV who used 2% clindamycin vaginal cream were cured two weeks after completion of the treatment. The rate of preterm delivery (< 37 weeks) was 15% for clindamycin patients and 13.5% for placebo patients. The rate of low birth weight was 9% for clindamycin patients and 6.8% for placebo patients. Treatment of BV with intravaginal clindamycin did not reduce either preterm delivery or low birth weight. Intravaginal treatment would not be effective against BV-associated microorganisms present in the upper genital tract and systemic treatment may be required to eradicate upper tract infection to reduce preterm delivery. If pregnant women with BV who have also an increased risk for preterm delivery are treated with metronidazole and erythromycin, the rate of premature delivery is reduced (Hauth *et al.*, 1995). The treatment of BV in women with heavy growth of *G. vaginalis* during mid-pregnancy with metronidazole did not reduce the preterm birth rate, but among women with a previous preterm birth, treatment did reduce the risk of spontaneous preterm birth (McDonald *et al.* 1997).

### **BV and cervical cancer**

Most studies have shown that BV often occurs in patients with cervical precancerous changes or early cancer as opposed to women with normal hormonal changes (Neurer & Menton, 1995; Platz-Christensen *et al.*, 1994). It is not possible to say if the association between anaerobic vaginosis and cervical pre-cancer is causal or incidental (Hudson *et al.*, 1997). The important factor in cervical cancer may be the relative absence of H<sub>2</sub>O<sub>2</sub>-producing lactobacilli that may have a protective role in the development of cervical dysplasia (Blackwell, 1996). An increase in vaginal pH may stop squamous metaplasia in the post-pubertal cervix and prolong the period in which the transformation zone is vulnerable to agents promoting dysplasia such as human papilloma virus (Blackwell & Phillips, 1989). It is also possible that in patients with cervical malignancy the disruption of the normal vaginal environment by necrotic tissue and blood contributes to an alteration in the vaginal flora. Hudson *et al.* (1997) suggested that because of the association between BV and cervical

carcinoma, women with a history of recurrent or persistent vaginal anaerobic infection should have cytology performed more frequently.

### **Mechanisms of protection against BV**

The microflora normally present in the vagina helps to protect the patient from pathogens, including those that cause urinary tract infections and sexually transmitted diseases. Lactobacilli are critical in this ecosystem and are the predominant vaginal organisms in healthy women (Hillier *et al.*, 1993). Circulating estrogens during premenopause induces colonisation of lactobacilli. Urogenital problems in elderly females are common among a third of women from the age of 50 years and older. Symptoms from the lower urinary tract include incontinence, urethritis and recurrent urinary tract infections. Vaginal symptoms are vaginal dryness, dyspareunia and recurrent vaginitis which is diagnosed by a foul smelling odour and discharge. The vaginal pH increases to between 6 and 7 (Samsioe, 1998). Uropathogens are *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Staphylococcus* spp., *Klebsiella pneumonia*, *Proteus mirabilis* and *Providencia stuartii* (Nicole, 1994). Lactobacilli may interfere with colonization of uropathogens such as *Escherichia coli* in the vagina and periurethral epithelium. Factors such as a history of recurrent urinary tract infections, bacterial vaginosis, use of nonoxynol-9 or other selected antibiotics, and menopause increase the risk of urinary tract infections and are associated with a decreased amount of vaginal lactobacilli, an elevated vaginal pH and an increase in colonization with *Enterobacteriaceae* (Stamey & Sexton, 1975; Pfau & Sacks, 1981; Hooton *et al.*, 1991).

In premenopausal women with a normal genitourinary (genital and urinary) tract, but with recurrent urinary tract infections, Gram-negative enteric bacteria, mainly *E. coli*, is predominant in the introitus, vagina and urethra. Infections occur in greater numbers and persist for longer intervals than in women who never suffer from urinary infections and in whom the predominant flora in the introitus, vagina and urethra consist of lactobacilli and staphylococci (Pfau & Sacks, 1977). Most cases of urinary tract infections are preceded by a persistent similar Gram-negative vulvovaginal and urethral microbial flora.

Antibiotics like amoxicillin disturb the normal vaginal flora, reduce its adherence to vaginal epithelial cells *in vivo* and promote a persistent vaginal colonization of *E. coli*. In patients with urinary tract infection (UTI) the natural colonization resistance could not clearly be correlated with the presence of lactobacilli, which were only transiently reduced by amoxicillin.



When a monkey was used as a model, resistance was partly restored by vaginal instillation of lactobacilli (Winberg *et al.*, 1993). When postmenopausal women who had urinary tract infections received estriol treatment, the lactobacilli colonization increased, the vaginal pH decreased and the colonization of *Enterobacteriaceae* decreased. The result was a dramatic decrease in the incidence of recurrent urinary tract infections (Raz & Stamm, 1993). The same positive results were obtained with elderly women who suffered from urinary incontinence and related urogenital symptoms (Molander *et al.*, 1991). Since the urogenital tissue is more sensitive to estrogens than other tissues, very low estrogen doses are recommended for controlling urogenital symptoms (Samsioe, 1998).

Hydrogen peroxide-producing lactobacilli (LB<sup>+</sup>) are present in the vagina of most healthy women, but absent from most women with bacterial vaginosis. High cell numbers of these bacteria inhibited *G. vaginalis*. No inhibition of *G. vaginalis* is found when non-H<sub>2</sub>O<sub>2</sub>-producing lactobacilli (LB<sup>-</sup>) were used. Klebanoff *et al.* (1991) suggested that LB<sup>+</sup> might help to control the microbial flora of the vagina. Hawes *et al.* (1996) supported the hypothesis and speculated that H<sub>2</sub>O<sub>2</sub>-producing vaginal lactobacilli may protect women against the development of bacterial vaginosis, but not against vulvovaginal candidiasis or vaginal trichomoniasis. Pregnant women with LB<sup>+</sup> are less likely to suffer from bacterial vaginosis, symptomatic candidiasis and colonisation of *G. vaginalis*, *Bacteroides*, *Peptostreptococcus*, *M. hominis*, *U. urealyticum*, streptococci and *C. trachomatis*.

Another possible mechanism of protection by lactobacilli is the production of lactic acid and fatty acids. Lactobacilli are acidophilic and thrive at a pH of 3.5 to 4.5. *Gardnerella* spp. and other anaerobic bacteria are inhibited by the acid produced from these bacteria (Skarin and Sylwan, 1986).

Adherence of bacteria to the vaginal walls may lead to long-term bacterial colonisation of the vagina (Smith, 1977). Estrogen level fluctuations and alteration in the ionic charge of the epithelium cells could alter bacterial adherence properties and thus affect the composition of the vaginal microflora. *In vitro* studies with vaginal strains of *Lactobacillus* spp. have shown that they compete with *C. albicans* for adherence to the epithelial cells in the vagina (Sobel *et al.*, 1981). However, only 35% of the strains studied showed strong adherence. The fact that these strains had specific and reproducible hemagglutination patterns might have something to do with their adhesion (Andreu *et al.*, 1995).

Lactobacilli are well known for their production of antimicrobial peptides (bacteriocins) which usually inhibit species genetically closely related to the producer cell (Toba *et al.*, 1991; Gonzáles *et al.*, 1994). It might well be that these peptides play a role in the inhibition of indigenous vaginal pathogens (Redondo-Lopez *et al.*, 1990). According to a hypothesis published by Perdigon *et al.*, (1988) the stimulation of macrophages and lymphocytes in animals which have been fed milk products fermented with lactobacilli raises the possibility that an induced local immune system may help control microbial levels in the vagina.

Production of H<sub>2</sub>O<sub>2</sub> by facultative lactobacilli and the maintenance of an acidic vaginal pH controls the growth of pathogenic microorganisms. Patients with BV have a lower *Lactobacillus* cell count (Fredricsson *et al.*, 1984; Spiegel *et al.*, 1980; Weels & Goes, 1981). A reduction in the number of H<sub>2</sub>O<sub>2</sub>-producing lactobacilli allows the unrestrained growth of vaginal anaerobes that induce clinical disease. In a study undertaken by Fontaine *et al.*, (1996) 88.6% of healthy women had vaginal lactobacilli, while only 29.4% of women with BV had lactobacilli. In both groups of patients the predominant species were *L. acidophilus* and *L. plantarum*. The number of H<sub>2</sub>O<sub>2</sub>-producing strains isolated from healthy women and women with BV did not differ much (61% and 73%, respectively). Bacteria-free supernatant containing H<sub>2</sub>O<sub>2</sub> produced by the growth of several strains of lactobacilli, in addition to micromolar solutions of H<sub>2</sub>O<sub>2</sub> and known concentrations of lactic acid and urea, had no inhibitory effect on *B. ureolyticus*, *P. melaninogea* and *Bifidobacterium* spp. However, *G. vaginalis* and *Mobiluncus* spp. were weakly inhibited by the cell-free supernatants and *G. vaginalis* by solutions of nitrates and catalase. Since these organisms are also resistant to known concentrations of analytical H<sub>2</sub>O<sub>2</sub>, the positive inhibitory reaction from these fluids were probably due to an inhibitory substance in the supernatant other than H<sub>2</sub>O<sub>2</sub>. These observations suggest that H<sub>2</sub>O<sub>2</sub> *per se* may be less bactericidal than previously thought in the control of intra-vaginal proliferation by BV-related bacteria *in vivo* (Fontaine *et al.*, 1996).

Support for the study of Fontaine *et al.* (1996) came from Rosenstein *et al.* (1997) who determined the relationship between lactobacilli and bacterial species associated with BV in pregnant patients and the prevalence of H<sub>2</sub>O<sub>2</sub>-producing and non-producing strains of lactobacilli in women whose vaginal flora had already been analysed. They suggested that BV may develop in some women despite the presence of H<sub>2</sub>O<sub>2</sub>-producing strains of lactobacilli and that other factors, as yet unidentified, may be conducive to the appearance of abnormal bacterial flora with progression to vaginosis. The cell numbers of *Lactobacillus* spp. tend to decrease in women whose vaginal flora is recorded grade III (the situation where women have no or almost no vaginal lactobacilli; grade I is normal vaginal microflora while

grade II is intermediate, i.e. a mixed microflora). The lactobacilli which remain are not necessarily H<sub>2</sub>O<sub>2</sub>-negative and may produce H<sub>2</sub>O<sub>2</sub> in large amounts. In some women, the flora reverts spontaneously to normal, possibly before the lactobacilli completely disappear. There may be certain factors relating to the individual women's susceptibility to BV which causes the flora to revert to normal or progress to a grossly abnormal state.

Another study evaluated the hypothesis that women colonised by lactobacilli have a lesser chance of contracting vaginal infections. Over a 2-year follow-up, 50 out of 182 women contracted BV, 25 contracted vulvovaginal candidiasis (VVC) and seven patients vaginal trichomoniasis. Acquisition of BV was independently associated with a lack of vaginal H<sub>2</sub>O<sub>2</sub>-producing lactobacilli or presence of only non-H<sub>2</sub>O<sub>2</sub>-producing lactobacilli. Development of BV was associated with having a new sex partner and douching. Absence of lactobacilli did not increase acquisition of VVC. Trichomoniasis was associated only with having a new sex partner. These results supported the hypothesis that H<sub>2</sub>O<sub>2</sub>-producing vaginal lactobacilli protect against acquisition of BV, but do not protect against VVC or trichomoniasis (Hawes *et al.*, 1996).

Skarin and Sylwan (1986) tested the inhibition of vaginal lactobacilli against *G. vaginalis*, *Mobilincus* spp. and other bacterial species from the vaginal content of women with BV. Inhibition of BV-associated organisms by lactobacilli was observed. Lactobacilli isolated from the vaginas of healthy women (39 strains) and from the vaginal discharge of women with BV (15 strains) were investigated for their binding to fibronectin. Nine out of the 54 strains bound fibronectin at pH 7.2 and the binding were specific. Incubation of lactobacilli with fibronectin for different periods revealed a time-dependent increase in binding. Lowering the pH to 4.0 increased the binding capacity of all the strains, even strains that failed to bind at pH 7.2. Based on these studies, it would appear that the increased binding of fibronectin by vaginal lactobacilli at these low pH values, which are normal for the vagina of healthy women, plays an important role in the maintenance of the ecological balance of the vagina (Nagy *et al.*, 1992).

Treatment with metronidazole resulted in the regrowth of lactobacilli which inhibits the overgrowth of anaerobes (Weaver & Mengel, 1988). The fact that lactobacilli do not reallocate itself in the vagina following treatment with penicillin and ampicillin (two antibiotics much less effective than metronidazole in the treatment of BV) is further evidence of the role of lactobacilli in the pathogenesis of BV (Amsel *et al.*, 1982).

## Treatment

Erythromycin is ineffective at the acid pH level of the vagina (Durfée *et al.*, 1979). Ampicillin is also ineffective, probably because of poor activity against anaerobes, especially with the production of  $\beta$ -lactamases (Bhattacharyya & Jones, 1980). Twice daily douching with various preparations such as povidone iodine and aluminium acetate does not effectively cure BV. Clindamycin and metronidazole are the most effective antibiotics against BV (Hillier & Holmes, 1990). BV is effectively treated with 500 mg metronidazole applied intravaginally twice daily for 7 days (Bistoletti *et al.*, 1986). The use of metronidazole during the first trimester of pregnancy is discouraged because of suspected mutagenicity (Rossenblatt & Edson, 1987). However, a later study concluded that metronidazole is probably safe throughout the whole pregnancy (Sullivan & Smith, 1993). The following are disadvantages to the use of metronidazole:

- \* It is contraindicated during the first trimester of pregnancy.
- \* Relapses occur.
- \* The remaining lactobacilli are mostly no longer able to produce  $H_2O_2$  after therapy (mutagenesis).
- \* Side effects experienced by patients like alcohol intolerance due to a disulfiram-like mechanism of action, gastrointestinal upset, unpleasant metallic taste, rash, dizziness and headaches (Fischenbach *et al.*, 1993).

The systemic use of clindamycin (300 mg twice daily) is also effective against BV. Higher cure rates may be achieved if a 3x per day regimen is used (Thomason *et al.*, 1991). A 2% clindamycin cream preparation used once daily and a 5 g dose has a cure rate of 94% (Livengood *et al.*, 1990). Superinfections with *Candida* spp. are not uncommon subsequent to treatment with clindamycin and metronidazole (Fischenbach *et al.*, 1993).

Non-antibiotic treatments may be tried because of high recurrence rates after antibiotic treatment. The effectiveness of a lactate gel against BV during pregnancy proved to be effective. The main component of the gel was lactic acid and other growth substrates for lactobacilli. Ten pregnant women with BV were treated vaginally with the gel intermittently during six weeks. Reappearance of a lactobacilli-dominated flora was observed after a few days of treatment. Normal vaginal acidity and recolonization of lactobacilli were restored (Holst & Brandberg, 1990). This treatment may be preferable to oral antimicrobial therapy (metronidazole etc.), especially during pregnancy. When women with BV were treated with

yoghurt containing *L. acidophilus*, 57% had normal vaginal wet smear results. After subsequent menstruation, only 10% were free of BV (Hallen *et al.*, 1992). Neri *et al.* (1993) had much more encouraging results and attributed their higher success rate to differences in the technical application, dose used and duration of treatment. Additional studies with a larger number of patients are necessary before final conclusions on the use and potential of alternative treatments can be drawn (Neri *et al.*, 1994).

Shalev *et al.* (1996) compared and assessed ingestion of yoghurt that contained live *L. acidophilus* cultures with pasteurised yoghurt as prophylaxis for recurrent BV and candidal vaginitis. They concluded that daily ingestion of yoghurt enriched with live cells of *L. acidophilus* was associated with an increased prevalence of colonisation of the rectum and vagina by the bacteria. Ingestion of the yoghurt may have reduced episodes of BV. However, they could not show a difference in the episodes of candidal vaginitis between ingesting yoghurt that contained live *L. acidophilus* compared with the period of ingesting pasteurised yoghurt, although a clear reduction in the number of episodes occurred. *L. acidophilus* was chosen for the study because evidence suggested that the species had an improved survival on passage through the gastrointestinal tract compared to other *Lactobacillus* spp. (Sarra & Dellaglio, 1984).

Gynoflor, a vaginal tablet that contains 50 mg of a lyophilisate of viable, H<sub>2</sub>O<sub>2</sub>-producing *L. acidophilus* (at least 10<sup>7</sup> cfu/tablet) and 0.03 mg estriol, can be used to treat BV. The cure rate for non-menopausal women with BV after 2 weeks was 77%. Four weeks after the start of therapy, the cure rate was 88%. After 6 days the lactobacilli were able to recolonise in the vagina. A significant increase in the number of lactobacilli was observed in the Gram-stained vaginal smears. For more serious cases of BV, combined therapy of metronidazole or clindamycin followed by intravaginal application of suitable lactobacilli to reduce the risk of *Candida* superinfection. This two-phase therapy would likely reduce the incidence of the commonly observed relapses or infection with a new pathogen (Parent *et al.*, 1996).

The advantages of lactobacilli substitution therapy:

- \* Suitable for therapy of mild and intermediate cases, where the use of a potent antibiotic is questionable.
- \* A reduced risk of relapse or superinfection.
- \* No systematic side effects expected.
- \* Safe during pregnancy.
- \* The physiological vaginal flora is quicker restored.

\* Recommended for patients where compliance problems with other anti-infectives are expected (Parent *et al.*, 1996).

## Recurrence

Recurrent BV is a serious problem. One study showed an 80% recurrence rate of BV within 9 months of completion of metronidazole. The reasons for this phenomenon are not understood. Possible explanations are: reinfection by a male partner with BV-associated microorganisms, persistence of BV-associated microorganisms that were inhibited, but not killed during therapy, the failure to re-establish the protective lactobacilli-dominant flora after therapy, and the persistence of an unknown host factor which renders the women susceptible to recurrence (Hillier & Holmes, 1990).

## ASSOCIATION AMONG VAGINAL LACTOBACILLI, BACTERIAL VAGINOSIS AND THE HUMAN IMMUNOINEFFICIENCY VIRUS (HIV) INFECTION

The role of the vaginal microbial ecosystem in determining the efficiency of HIV transmission has been largely ignored. The ability of  $H_2O_2$ -producing lactobacilli to kill HIV *in vitro* was first noted by Klebanoff and Coombs (1991).

Women with HIV have seldom a normal vaginal flora. When diagnosed clinically, BV is associated with HIV seroprevalence. HIV infection may promote abnormal vaginal flora, or BV may increase susceptibility to sexual transmission of HIV. Alternatively, the association may result from intervening variables. BV may be a marker or a cofactor of HIV transmission (Cohen *et al.*, 1995). It is possible that lactobacilli could decrease the risk of heterosexual HIV transmission through the virucidal effect of  $H_2O_2$  on cell-free HIV (Klebanoff & Coombs, 1991). Another protective mechanism is the normal acidic pH of the vagina which may prevent HIV infection, in addition to the effect of  $H_2O_2$ .

A study on women in Zimbabwe showed a strong correlation between the absence of vaginal lactobacilli and HIV seropositivity (Gwanzura *et al.*, 1996). Studies in Malawi showed that the prevalence of HIV increased from 12% in pregnant women with normal vaginal flora to 30% among those with BV (Gray *et al.*, 1997). Prostitutes in Kenya who have been vaginally colonized by *Lactobacillus* spp. had a trend towards a decreased incidence of both gonorrhoea and HIV (Martin *et al.*, 1996).

Hillier (1998) proposed that one of the reasons for reduced transmission of HIV could be the presence of microbial products such as  $H_2O_2$  and lactic acid (produced by lactobacilli) that are antagonistic towards pathogens or the presence of microbial products that are permissive to invasive infection such as succinate (produced by anaerobic Gram-negative rods), trimethylamine (produced by *G. vaginalis* and *Mobilincus*) and sialidases (produced by *P. bivia*, *B. fragilis* and *G. vaginalis*).

It is essential that potential microbicides are examined for activity against normal vaginal flora. Rosenstein *et al.* (1998) studied the effect of three intravaginal microbicides potentially active against HIV type 1 on the vaginal flora. Dextrin sulphate did not effect the vaginal flora. Nonoxynol-9 (N-9) caused a decrease of lactobacilli by  $\geq 10^2$  cfu/ml in 53% of the women. Docusate sodium caused the same effect in 63% of the women using the agent. Women using N-9 or docusate sodium were also significantly more likely to become colonized with aerobic Gram-negative rods, than those using placebo, as were women using docusate sodium. Women with reduced lactobacilli were less likely to regain normal flora than those with unaffected lactobacilli. Coliform colonization occurred whether lactobacilli produced  $H_2O_2$  or not. It seems if the continuous use of N-9 could induce susceptibility to urinary and gynecological infection.

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### 3. TAXONOMY OF LACTIC ACID BACTERIA, WITH SPECIAL EMPHASIS ON SPECIES FOUND IN THE HUMAN VAGINA

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## TAXONOMY OF LACTIC ACID BACTERIA, WITH SPECIAL EMPHASIS ON SPECIES FOUND IN THE HUMAN VAGINA

### The genus *Lactobacillus*

#### General characteristics

The genus *Lactobacillus* is part of the *Lactobacillus- Leuconostoc- Pediococcus- Streptococcus* supercluster of the clostridia sub-branch of the Gram-positive bacteria with the type species being *Lactobacillus delbrueckii* (Kandler & Weiss, 1986). Lactobacilli are Gram-positive, non-sporeforming, long slender rods or short coryneform coccobacilli. Gelatine is not liquefied, casein is not digested and indole and H<sub>2</sub>S are not produced (Kandler & Weiss, 1986). All species are catalase and cytochrome negative. Growth temperatures are between 2°C and 53°C with the optimum between 30°C and 40°C (Kandler & Weiss, 1986).

Lactobacilli are strictly fermentative and have complex nutritional requirements. Substrates include carbohydrates, amino acids, peptides, fatty acid esters, salts and vitamins. The species are found in a number of habitats, including fermented foods, plants, sewage, soil, manure and the oral cavity, intestinal tract and vagina of humans and animals (Sharpe, 1981).

Some *Lactobacillus* spp. are adapted to specific ecological niches, e.g. *Lactobacillus delbrueckii* in sour milk, *Lactobacillus sanfrancisco* in sourdough bread and *Lactobacillus mali* in cider and fruit juices (Carr & Davis, 1970; Davis, 1975; Carr *et al.*, 1977). Lactobacilli are the predominant microorganisms in the stomach and small intestine, but only constitute 0.07 to 1.0 % of the microbial flora of the jejunum and large intestine (Sharpe, 1981). *Lactobacillus ruminis* and *Lactobacillus vitulinus* are only found in the bovine rumen.

The oral cavity of humans and animals harbours a variety of LAB, which includes *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus casei*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus salivarius*, *Lactobacillus plantarum* and *Lactobacillus cellobiosus* (Rogosa *et al.*, 1953). The species in the intestinal tract are *L. acidophilus*, *L. fermentum*, *L. salivarius*, *Lactobacillus lactis*, (Sharpe, 1981), *Lactobacillus reuteri* (Kandler & Weiss, 1986), *L. buchneri* and *L. brevis* (Reuter, 1965). The *Lactobacillus* spp. isolated thus far from the human vagina include *L. acidophilus*, *L.*

*fermentum*, *L. rhamnosus*, *L. cellobiosus* (Rogosa & Sharpe, 1960), *L. casei*, *L. plantarum*, *L. brevis*, *Lactobacillus delbrueckii*, *L. lactis*, *Lactobacillus bulgaricus*, *Lactobacillus leichmannii*, *L. salivarius* (Lenzner, 1966; Wylie & Henderson, 1969), *Lactobacillus jensenii* (Sharpe, 1981), *Lactobacillus vaginalis* (Embley *et al.*, 1989), *Lactobacillus gasseri* and *Lactobacillus crispatus* (Kandler & Weiss, 1986). Only small numbers of lactobacilli are present on intact plant material. *L. plantarum*, *L. fermentum*, *L. casei*, *L. brevis*, *Lactobacillus viridescens*, *L. cellobiosus* and *L. salivarius* were isolated from plant material (Hammes *et al.*, 1992). Lactobacilli are more frequently present in soil than in water because of the content of fermentable substrates. *L. fermentum*, *L. reuteri*, *L. brevis*, *Lactobacillus confusus*, *L. plantarum* and *Lactobacillus ruminus* were isolated from aquatic sources. Lactobacilli are not present in fresh or marine water, but have been isolated from manure, e.g. *Lactobacillus curvatus*, *Lactobacillus coryniformis* and *Lactobacillus vaccinoferus* (Hammes *et al.*, 1992).

### **Taxonomy of the genus *Lactobacillus* in the past**

The genus *Lactobacillus* was divided into three subgenera according to their fermentation patterns by Orla Jensen in 1919. The subgenera were *Thermobacterium*, *Streptobacterium* and *Betabacterium*. Currently the subgenera are referred to as Groups I, II and III, respectively (Kandler & Weiss, 1986). All the obligately homofermentative species are represented in Group I. These bacteria lack the enzymes glucose 6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase. Group II consists of the facultatively heterofermentative lactobacilli that utilise glucose mainly via the EMP pathway. The obligate heterofermentative species are allocated to Group III and lack the FDP-aldolase enzyme.

The homofermentative species were divided into groups IA and IB and the obligate heterofermentative species (*Betabacterium* subgroup) into groups II and III by Rogosa (1974). Group II and III were renamed to IIA and B by Sharpe (1979). Until 1996, 67 species were recognised as part of the genus *Lactobacillus* (Curk *et al.*, 1996; Wiese *et al.*, 1996). The genomic G+C content ranges from 32 to 54 mol %.

### **Current taxonomic status of the genus *Lactobacillus***

Homofermentative lactobacilli (Group I) are subdivided into two groups on the basis of DNA-DNA homology. Subgroup 1 consists of *L. delbrueckii* and its subspecies (*delbrueckii*,

*leichmannii*, *bulgaricus* and *lactis*). The DNA homology is 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 by DNA-DNA hybridization studies (Gasser & Janvier, 1980; Johnson *et al.*, 1980; Lauer *et al.*, 1980; Sarra *et al.*, 1980). The six DNA homology groups (Johnson *et al.*, 1980) are currently viewed as valid species, viz. *L. acidophilus* (group A1), *L. crispatus* (group A2) (Cato *et al.*, 1983), *L. amylovorus* (group A3) (Fujisawa *et al.*, 1992), *L. gallinarum* (group A4) (Fujisawa *et al.*, 1992), *L. gasseri* (group B1) (Lauer & Kandler, 1980) and *L. johnsonii* (group B2) (Fujisawa *et al.*, 1992). The DNA homology among these species is 25%. *L. acidophilus*, *L. gasseri*, *L. crispatus* and *Lactobacillus helveticus* forms a tight genotypic group. *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus aviarius*, *Lactobacillus farciminis*, *Lactobacillus hamsteri*, *L. jensenii*, *Lactobacillus kefiranoferens*, *Lactobacillus mali*, *L. ruminis*, *L. salivarius*, *Lactobacillus sharpeae*, *Lactobacillus vitulinus*, *L. acetotolerans* and *Lactobacillus intestinalis* are not related to any of the two complexes (Entani *et al.*, 1986; Fujisawa *et al.*, 1984, 1988, 1990; Kandler & Weiss, 1986; Kaneuchi *et al.*, 1988).

The facultatively heterofermentative lactobacilli consist of 3 genotypic complexes of species, subspecies and species that have no relatedness to each other (Kandler & Weiss, 1986). Subgroup 1 is *L. plantarum*, *L. pentosus* or *Lactobacillus paraplantarum* (Curk *et al.*, 1996). The DNA homology is 80-100%. Subgroup 2 consists of *Lactobacillus zeae*, *L. casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. The complex consists of 3 genotypic clusters, viz.

- \* *L. casei* type strain ATCC 393<sup>T</sup> and *L. zeae* with a DNA homology between 80 and 100%.
- \* *L. casei* subsp. *casei*, *L. casei* subsp. *pseudoplantarum* and *L. casei* subsp. *tolerans* with a DNA homology of 80-100%. This cluster shares 40% DNA homology with *L. casei* ATCC 393<sup>T</sup>
- \* Strains of *L. rhamnosus* (Collins *et al.*, 1989). The DNA homology of this cluster with the other 2 clusters is 30 to 50%.

The final decision on the status of *L. casei* and *L. paracasei* is pending and so are the decision on the neotype strain (Collins *et al.*, 1989; Dellaglio *et al.*, 1991, Dicks *et al.*, 1996). Subgroup 3 contains *Lactobacillus sake*, *Lactobacillus curvatus* and *Lactobacillus bavaricus* (Kagermeier-Callaway & Lauer, 1995; Torriani *et al.*, 1996; Klein *et al.*, 1996). Emended descriptions and subspecies status have been proposed for these three species. DNA



homology between *L. sake* and *L. curvatus* is 50%. Some strains of *L. bavaricus* are 100% homologous with *L. sake*, while others are homologous with *L. curvatus*. Other species which are members of Group 3 are *L. acetotolerans*, *Lactobacillus agilis*, *Lactobacillus alimentarius*, *Lactobacillus coryneformis* subsp. *coryneformis*, *Lactobacillus coryneformis* subsp. *torquens*, *Lactobacillus murinus* (Kandler & Weiss, 1986) and *Lactobacillus graminis* (Beck *et al.*, 1988). These species do not cluster with *L. sake*, *L. curvatus* or *L. bavaricus*.

The obligately heterofermentative species include *Lactobacillus bif fermentans*, *L. buchneri*, *L. brevis*, *Lactobacillus collinoides*, *L. fermentum*, *Lactobacillus fructovorans*, *Lactobacillus hilgardii*, *Lactobacillus kefir*, *Lactobacillus malefermentans*, *Lactobacillus oris*, *Lactobacillus panis* (Wiese *et al.*, 1996), *Lactobacillus parabuchneri*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Lactobacillus sanfrancisco*, *Lactobacillus suebicus*, *Lactobacillus vaccino stercus* and *Lactobacillus vaginalis*.

## THE GENUS *ENTEROCOCCUS*

### General characteristics

Enterococci are Gram-positive cocci which occur mostly in pairs or short chains. They are predominantly found in the intestines of humans and animals (Devriese *et al.*, 1992). *Enterococcus faecalis* and *Enterococcus faecium* are most frequently isolated. *Enterococcus avium* was isolated from children (Watanabe *et al.*, 1981), *E. faecalis*, *E. faecium* and *Enterococcus gallinarum* were from animals (Devriese *et al.*, 1987) and *E. faecalis*, *E. faecium*, *Enterococcus mundtii* and *Enterococcus casseliflavus* from plants (Martin & Mundt, 1972).

Enterococci are chemoorganotrophic and have complex nutritional requirements. The main end product of fermentation is L(+)-lactic acid. Growth occurs at 10°C and 45°C, pH 9.6 and in the presence of 6.5 % (m/v) NaCl. Esculin is hydrolysed and most strains are resistant to 40% (m/v) bile salts (Kandler & Weiss, 1986).

Enterococci are used as indicators of faecal contamination of water and food and are believed to be pathogenic to humans and animals. *E. faecalis* are mostly associated with intra-abdominal and pelvic infections in humans. Enterococci, usually *E. faecalis*, are

responsible for 5-15% of the human endocarditis cases reported (Kaye, 1982). The pathogenicity mechanisms of enterococci are unknown (Devriese *et al.*, 1992).

### Taxonomy of the genus *Enterococcus*

In the past the identification of streptococci was done according to the serological groups of Lancefield. All enterococci, except *Enterococcus cecorum* have the group D antigen. *Streptococcus faecalis* and *Streptococcus faecium*, the faecal streptococci of group D, was classified under a new genus, *Enterococcus*, by Schleifer and Kilpper-Bälz (1984). However, the group D antigen is not confined solely to the enterococci. Other streptococci, e.g. *Streptococcus bovis*, *Streptococcus alactolyticus*, *Streptococcus equinus* and *Streptococcus suis*, also have the group D antigen. *Streptococcus cecorum*, which lacks the group D antigen, was reclassified as *E. cecorum* (Williams *et al.*, 1989). DNA-RNA hybridizations and 16S rRNA sequencing showed that enterococci form a phylogenetically coherent group equivalent in rank to *Streptococcus* and *Lactococcus* (Schleifer & Kilpper-Bälz, 1984). However, Collins *et al.* (1989) found that enterococci are phylogenetically more closely related to some motile group N strains and to strains of *Listeria* than to *Streptococcus* or *Lactococcus*. The authors compared full 16S rRNA sequences using reverse transcriptase.

Chemotaxonomic and phylogenetic studies revealed the presence of 16 new species within the genus *Enterococcus*, viz. *Enterococcus avium*, *Enterococcus casseliflavus*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus malodoratus*, *Enterococcus cecorum*, *Enterococcus saccharolyticus*, *Enterococcus columbae*, *Enterococcus dispar*, *Enterococcus flavescens*, *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus pseudoavium*, *Enterococcus raffinosus* and *Enterococcus sulfurens*.

The species are divided into different subgroups. The first subgroup consists of *E. durans*, *E. faecium*, *E. hirae* and *E. mundtii*. The second subgroup consists of *E. avium*, *E. raffinosus*, *E. malodoratus* and *E. pseudoavium*. The third subgroup is *E. casseliflavus* and *E. gallinarum* which is linked to the *E. avium*-group. *E. faecalis*, *E. saccharolyticus*, *E. sulfureus*, *E. cecorum* and *E. columbae* form separate branches of descent in the phylogenetic tree.

## TAXONOMY

### CONVENTIONAL TAXONOMIC METHODS

The most important methods which are used to differentiate lactobacilli are optimum growth temperatures, sugar fermentation patterns, configuration of lactic acid produced and the production of CO<sub>2</sub> from glucose and gluconate (London, 1976). Davis (1955) modified the classification system used by Briggs (1953) by adding a few simple physiological tests, viz. the production of ammonia from arginine, salt tolerance, the Voges-Proskauer test, reaction in yeast glucose litmus milk and the hydrolysis of Na-hippurate and esculin. Rogosa *et al.* (1953) introduced a list of nutritional requirements characteristic of the main species.

Earlier in the century only morphological, physiological and serological characteristics were used in the classification of lactic acid bacteria. Morphology was used to define genera, physiology was used to define species and serology to define subspecies. This approach lacked predictivity and very few assertions could be made about the taxa. Classifications were unstable because important characteristics were overlooked. Different scientists had contrasting views about the composition and defining features of taxa. Continuing revision of taxa with new descriptions and new names was often the result. Numerical taxonomy provided a satisfactory solution to this dilemma (Priest & Austin, 1993).

In the past, *Lactobacillus rhamnosus* was recognised as a subspecies of *L. casei*. DNA hybridisation studies found a low homology among *L. casei* subsp. *rhamnosus* and the other subspecies of *L. casei* (Collins, 1989). Due to these taxonomic studies, *L. rhamnosus* is now regarded as a separate species. *Lactobacillus jugurti* shared 80-100% homology with *Lactobacillus helveticus* (Dellaglio *et al.*, 1973). For this reason *L. jugurti* was later reclassified as *L. helveticus*.

Results obtained from conventional methods such as sugar fermentations are not consistent and reproducible (Briggs, 1953). Results that rely on optimum growth temperature tests depend on too many external factors and the technique used to determine the configuration of lactic acid was too tedious.

## MODERN TAXONOMIC METHODS

Morphology, biochemical characteristics, carbohydrate fermentation patterns, cell wall compositions and the type of lactic acid produced is helpful in the preliminary identification of isolates. However, these characteristics only relate to the genes that are turned on under specific conditions. Molecular techniques reflect the composition of nucleotides in the DNA and RNA and are more reliable in taxonomic studies.

### Development of differential media

Media have been developed to differentiate between genera such as *Lactobacillus*, *Bifidobacteria* and *Enterococcus*. The basic composition of these media are yeast extract, peptone, sugar, manganese, acetate and Tween 80. The pH of the media is usually between 4.5 to 6.5 (McCann *et al.*, 1996). Sodium azide is used as selective agent for the enumeration of enterococci. MRS media is used for the isolation and growth of lactobacilli (de Man *et al.*, 1960). HHD is a differential medium derived from MRS that can be used to differentiate between homofermentative and heterofermentative LAB (McDonald *et al.*, 1987).

### Plasmids

The ability of plasmids to mediate or participate in the horizontal transfer of genes from one host to another is very important in bacterial systematics (Priest & Austin, 1993). Plasmids encode phenotypes like antibiotic resistance and toxin production. Some plasmids of lactobacilli are cryptic and strain-specific. Plasmid profiling can be used to identify isolates from different environments (Tannock *et al.*, 1990; Roussel *et al.*, 1993; Reid *et al.*, 1996). The method has been used successfully to identify strains of *L. plantarum* in silage fermentations (Hill & Hill, 1986). The disadvantage of this method is the fact that plasmids are usually unstable and must be used in combination with other techniques. Plasmid-derived DNA probes have been used to identify *L. fermentum* in the porcine stomach (Tannock *et al.*, 1992).

## DNA-rRNA Hybridisation

The structural homology between genomes can be determined by using this technique. This method is used to identify bacteria on family and genus level, but not on a species or strain level. The technique has been used in the taxonomy of *Enterococcus* and *Lactococcus* spp. (Garvie & Farrow, 1981; Schleifer *et al.*, 1985). This method was for instance used to determine the taxonomic status of the current subspecies of *L. delbrueckii*. Simonds *et al.* (1971) found 86-100% homology between *Lactobacillus lactis* and *Lactobacillus bulgaricus*. Miller *et al.* (1971) found 98-100% homology between *L. lactis* and *Lactobacillus leichmannii*. Based on these studies *L. lactis*, *L. bulgaricus* and *L. leichmannii* were given subspecies status of *L. delbrueckii* (Weiss *et al.*, 1983). Ribosomal (rRNA) sequence analysis replaced this method.

## Restriction enzyme analysis

Chromosomal DNA are digested with rare-cutting restriction enzymes, separated by gel electrophoresis and analysed by computer if a large number of DNA bands are formed. *Sma*I, *Bgl*II, *Apa*I and *Sfi*I are the most commonly used restriction enzymes for lactobacilli (Roussel *et al.*, 1993; Ferrero *et al.*, 1996; Charteris *et al.*, 1997). The *L. acidophilus*-group (Roussel *et al.*, 1993) and *L. casei* (Ferrero *et al.*, 1996) are examples of strains which were characterised by using this method.

## Oligonucleotide probes

Species and strain-specific probes are mostly derived from 16S and 23S rRNA sequences (Betzl *et al.*, 1990; Pot *et al.*, 1993, Charteris *et al.*, 1997). 16S rRNA is highly conserved and stable. It is a simple and rapid method to screen a large number of bacteria. The primers which are often used as probes are listed in Table 1. During this procedure a specific probe binds to the target-DNA which is followed by PCR amplification, *in situ* hybridisation and dot blot hybridisation or reverse dot blot hybridisation. This method has been successfully used in the identification of lactobacilli (Delley *et al.*, 1990; Bringel *et al.*, 1996), *Bacillus subtilis*, *Salmonella typhi*, *Haemophilus influenza* and DNA and RNA viruses (Delley *et al.*, 1990; Petrick *et al.*, 1988). 23S rRNA has an advantage over DNA probes because the biological function and stability of a random cloned DNA fragment is unknown.

The rRNA has high copy numbers, is ubiquitously distributed and DNA-rRNA hybrids are more stable.

### Random amplified polymorphic DNA (RAPD)-polymerase chain reaction (PCR)

Primers used in this technique are usually 10 basepairs long and are derived from either specific DNA or RNA sequences or randomly selected DNA primers. The primers anneal to the target DNA under low stringency conditions to multiply and produce randomly sized DNA fragments. This method has been successfully used in the rapid typing of strains within the *L. acidophilus*-group, *L. plantarum* and *L. pentosus* (Du Plessis & Dicks, 1995; Van Reenen & Dicks, 1996). Computer-assisted analysis is needed for the interpretation of the DNA fingerprints.

### Ribotyping

This method uses species-specific probes which are amplified by PCR. The advantage of using ribotyping is the fact that fewer bands are obtained than with RAPD-PCR. The results are thus easier to interpret. The method was used with success in studying the genetic relatedness and the heterogeneity among strains of *L. plantarum* (Duffner & O'Connell, 1995; Johanssen *et al.*, 1995).

Table 1. Primers used in 16S rRNA sequencing.

Sequence of primer	Position where primer binds
CACGYGTTACKCACCCGTCC	101-127
CTGCTGCSYCCCGTAG	342-357
GWATTACGCGGCKGCTG	519-536
TCTACGRATTTACCCYCTAC	685-704
CCGTCAATTCMTTTRAGTTT	907-926
ACGAGCTGACGACRGCCA	1056-1073
AGGGTTGCGCTCGTTG	1100-1115
CGGTGTGTRCAAGGCC	1385-1401
GGTTACCTTGTTACGACTT	1492-1510

K = G/T; M = A/C; R = A/G; W = A/T; S = G/C (Lane *et al.*, 1985)

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

Whole cell proteins of bacteria are isolated and separated by using SDS polyacrylamide gel electrophoresis. A large number of bacteria can be identified by using this method. Isolates can be distinguished on species and/or subspecies level if standardised SDS PAGE conditions are used (Pot *et al.*, 1994). A good correlation exists between results obtained by SDS-PAGE, DNA-DNA hybridisations and RAPD-PCR (Dicks *et al.*, 1996; Dellaglio *et al.*, 1991). This method has been used successfully in the differentiation of *L. plantarum* and *L. pentosus* (Van Reenen & Dicks, 1996) and *Enterococcus* spp. (Devriese *et al.*, 1995).

### **NUMERICAL ANALYSIS**

Numerical analysis was defined by Sneath and Sokal (1973) as “the grouping by numerical methods of taxonomic units into taxa on the basis of their characteristics”. Current classification of lactic acid bacteria is still largely relying on the work of Orla Jensen in 1919. Today, numerical taxonomy employ the following techniques to determine the identification and classification of organisms: cell wall composition, cell wall antigens, paper chromatography of cellular contents, electrophoretic mobility of enzymes, serology, electrophoresis of proteins, DNA base composition, nucleic acid hybridization, sequencing of 16S rRNA oligonucleotides, plasmid profiling, nucleic acid probes, pulse field gel electrophoresis of restricted genomic DNA, and DNA fingerprinting (Goodfellow & O'Donnell, 1993).

The DNA base sequence is the primary level of information which is expressed at the secondary level in the structure of protein molecules. DNA-DNA and DNA-rRNA hybridization, 16S rRNA studies, electrophoresis of DNA fragments generated by restriction endonucleases and the determination of the DNA base composition are techniques employed to identify, classify and compare bacteria. Amino acid sequencing techniques, gel electrophoresis of proteins, immunodiffusion and immunoelectrophoresis are the techniques used to study the second level of expression of genetic information. At the third and final level information is given by various chemotaxonomic markers revealed by chromatographic procedures such as gas chromatography and mass spectrometry. The amino acid sequence of proteins of the bacterial cell is an indirect copy of the bacterial genome. Molecular weight, net electrical charge and spatial conformation are reflections of the primary structure of

proteins. The electrophoretic separation of these macromolecules is based on one or more of these factors. Gel electrophoresis of cellular proteins of a bacterial strain produces a complex banding pattern called a protein electrophoregram. Bacterial strains grown under identical conditions produce constant electrophoretic protein patterns, which can be used as fingerprints of the strains investigated. In one-dimensional gels, each protein band usually consists of a number of structurally different proteins having identical electrophoretic mobility. The electrophoresis of cellular proteins is a very sensitive technique and provides valuable information on the similarity of strains within the same species or subspecies (Priest & Austin, 1993).

Two different protein electrophoresis techniques are currently used in bacterial taxonomy:

- A mixture of soluble cytoplasmic proteins of a bacterial strain or proteins solubilized by treatment with a denaturing agent is submitted to polyacrylamide gel electrophoresis (PAGE) and stained. The whole banding are compared without any attempt to characterize the individual protein bands.
- The native proteins of bacterial stains are submitted to electrophoresis in a support matrix like starch gel or polyacrylamide gel and are stained for specific enzymes. The electrophoretic mobility variants of enzymes are then compared between strains.

Protein patterns of extracts prepared in non-denaturing conditions reflect the size as well as charge difference of the proteins while patterns of extracts prepared in denaturing conditions reflects only the size of the proteins (Vauterin *et al.*, 1993).

Electrophorograms can be compared visually by gel photographs or by computation. Computer-aided numerical analysis of protein patterns has been used in the delination of new taxa and for train identification. The advantages of using numerical analysis for protein patterns are:

- \* Large numbers of bacterial strains can be compared.
- \* Digitally processed electrophoretic patterns of representative strains can be stored on computer files that can be used to identify other unknown isolates.
- \* Comparison of protein fingerprints gives a reliable measure of taxonomic relatedness.

The disadvantages of the technique are:



- \* The identity of a single colony cannot be established within a few hours after isolation.
- \* Larger amounts of cells are required.
- \* It is slower than other fingerprint techniques like gas chromatography.
- \* A large number of steps are involved, each of which introduce some experimental error.
- \* Extremely standardized and reproducible experimental conditions are required.

There are two ways to identify organisms if given an identification matrix or a minimal data set which describe the taxa. Simultaneous identification involves gathering all the test results for an unknown strain and making some comparison with the information in the identification matrix. Sequential identification schemes (e.g. diagnostic keys) employ test results in order often as a result of a question or prompt from the identification program.

### **Similtaneous identification**

The two methods that are used include numerical codes and computer-assisted identification. Numerical codes that are usually constructed by computers, but do not require a computer for the use of such a method. An example of numerical codes is API kits. Numerical codes require the results of a series of tests and the binary results are converted into a code number. Code numbers appears in an index which give the appropriate identification. This is an effective, popular and extremely convenient system, particularly in the case of well-studied organisms. Computer-assisted identification often use a probabilistic approach. Probabilistic identification uses taxon-radius models and can only be executed with a computer. Taxon-radius models are identification system in which taxa are viewed as hyperspheres in an attribute-space in which the dimensions are characters. The centre of the hypersphere is defined by the centroid and a critical radius,  $r$ , encompassed strains within the cluster. When identifying an unknown strain, the test results for an unknown strain are determined and its position in the a-space calculated. If it lies within a hypersphere of a given radius and position it identifies with that taxon, if it is located at the periphery it is probably an atypical strain of that species, and if it lies between clusters it is unidentified (Priest & Austin, 1993).

A densitometer trace/document scanner image of stained gels provides the quantitative data for the bacterium. The peak heights of the traces are normalised using internal reference proteins and are used as the characters for the organism. Similarities are calculated between each organism using a suitable similarity coefficient like the Pearson product-

moment correlation coefficient or the Dice coefficient. The resultant matrix is clustered using the average linkage algorithm to provide a sorted similarity matrix or dendrogram. Computer programs like GelCompar (Applied Maths, Belgium) and Gel Manager for Windows (BioSystematica, Prague).

Comparative electrophoresis is used in identification and comparison of bacteria on species level, but are of little assistance when comparing distantly related bacteria. Chemical analysis was successful in the classification and identification of organisms in which morphological and physiological characters have been few or have led to confusing classifications through undue importance being placed on them (Priest & Austin, 1993).

### **Numerical analysis of protein patterns**

In the past comparison of resemblance between protein profiles was done visually by comparing electropherograms or photographs side by side. This visual comparison is only reliable when a few strains are compared. When a large group of strains are studied, no reliable comparison can be done with the eye and group them reliably. Estimation of quantitative resemblance is visually impossible. Numerical techniques are therefore employed to quantify resemblance between electrophoretic patterns.

### **Recording**

Optical densities are registered at regular intervals along the traces. Normalisation of the traces is the standardization of the length of the gels and the compensation for inevitable discrepancies within and between gels (Vauterin *et al.*, 1993). Numerical taxonomy of total soluble proteins has proved to be a popular and successful approach to bacterial classification and identification (Dicks & du Plessis, 1995; Dicks & Van Reenen, 1996).

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#### **4. TAXONOMY OF LACTIC ACID BACTERIA ISOLATED FROM THE UROGENITAL TRACT OF WOMEN**

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## Taxonomy of lactic acid bacteria isolated from the urogenital tract of women

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A total of 100 lactic acid bacteria were isolated from the posterior fornix of the vagina of 259 patients. Of these strains, 41 were isolated from non-pregnant women and 59 from pre-natal patients in the first seven months of pregnancy. The strains were identified by using simple physiological and biochemical tests and their phenotypic relatedness determined by numerical analysis of total soluble cell protein patterns. The genetic relatedness of representative strains selected from each of the protein profile clusters was determined by numerical analysis of the DNA banding patterns obtained from RAPD-PCR. The majority of lactobacilli isolated belonged to the species *Lactobacillus fermentum*, *Lactobacillus jensenii*, *Lactobacillus plantarum*, *Lactobacillus johnsonii*, *Lactobacillus gasseri* and *Lactobacillus crispatus*. A few strains of *Lactobacillus buchneri* and *Weissella viridescens* were isolated. The identification of these strains was confirmed by 16S rRNA sequencing. The enterococci were identified as *Enterococcus faecalis* and *Enterococcus faecium*. This is the first report of *L. buchneri*, *W. viridescens*, *E. faecalis* and *E. faecium* associated with the human vagina.

**Keywords:** Taxonomy, *Lactobacillus*, *Enterococcus*, human vagina

## INTRODUCTION

*Lactobacillus* and *Streptococcus* spp. are normally present in the vagina and cervix (Bartlett, 1977). In healthy females as many as  $10^7$ - $10^8$  *Lactobacillus* spp. per ml vaginal fluid has been reported (Redondo-Lopez *et al.*, 1990). However, the microbial composition in the vagina and cervix is influenced by various internal and external parameters, e.g. fluctuation of hormone levels, menstruation (Drasar & Hill, 1974), immunosuppression, antibiotics, gynaecological surgery, cervical cancer (Larsen & Galask, 1980), contraceptive devices, personal hygiene (Hooton *et al.*, 1989) and coitus (Freidrich, 1979). The most drastic changes in the vaginal flora occurs at the onset of puberty, mainly due to an increase in estrogen levels, accompanied by thickening of the vaginal epithelium and an increase in glycogen. All these factors stimulate the growth of lactic acid bacteria, especially *Lactobacillus* and *Streptococcus* spp. (Bartlett, 1977; Fernandes *et al.*, 1987).

Strains of lactobacilli which are usually found in the human vagina are *L. acidophilus*, *L. brevis*, *L. delbrueckii*, *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. fermentum*, *L. plantarum* and *L. casei* (Rogosa & Sharpe, 1960; Wylie & Henderson, 1969; Giorgi *et al.*, 1987). Of these species, *L. acidophilus* and *L. fermentum* were identified as the most dominant species in the vagina (Rogosa & Sharpe, 1960).

In the majority of studies on vaginal lactic acid bacteria simple physiological and biochemical tests, including sugar fermentation profiles, have been used to identify the strains to species level (Rogosa & Sharpe, 1960; Giorgi *et al.*, 1987). With the emergence of more sophisticated taxonomic techniques, e.g. DNA-DNA hybridization, species such as *L. crispatus* and *L. jensenii* were reported as being the most predominant in the vagina (Giorgi *et al.*, 1987).

In this study we have identified the lactic acid bacteria from the posterior fornix in the vagina of non-pregnant and pregnant patients by using numerical analyses of total soluble cell protein patterns and RAPD-PCR profiles, and 16S rRNA sequence analysis.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The lactic acid bacteria included in this study were isolated from swabs obtained from Tygerberg Hospital. Samples were taken from the posterior fornix of the vagina of 259 patients who attended the pre-natal and gynaecology

clinics. All the patients were of a lower income group. The swabs were immersed in 1 ml sterile physiological salt solution (Univar) and immediately spread-plated (100  $\mu$ l) on MRS agar (Biolab). Colonies were selected from the plates after two days of incubation at 37 °C. Reference strains were from National Collection of Food Bacteria (NCFB), the National Collection of Industrial and Marine Bacteria (NCIMB), American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen (DSM) and the culture collection of the Laboratory of Microbiology in Ghent (LMG). All strains were grown in MRS medium (Biolab) as prescribed in the respective culture collection catalogues.

**Numerical analysis of total soluble cell protein patterns.** All strains (Table 1) were grown in MRS broth (Biolab) for 24 h at 37 °C. Protein isolations and gel electrophoresis were done as described by Vauterin *et al.* (1993). The Gelcompar computer program (version 4.0) of Applied Maths (Kortrijk, Belgium) was used to analyze the protein banding patterns. The program recorded the normalized electrophoretic banding patterns of the densitometric traces, grouped the isolates by the Pearson product moment correlation coefficient ( $r$ ) and performed UPGMA cluster analysis of the protein bands.

**Numerical analysis of RAPD-PCR analysis.** The DNA of selected strains (Table 1) were isolated according to the method of Dellaglio *et al.* (1973). Three single primers [TGGGCGTCAA(OPL-02), ACGCAGGCAC (OPL-05) and ACGATGAGCC (OPL-11)] were used. Amplification products were analyzed by electrophoresis in 1.4% (m/v) agarose gels with TAE buffer (Sambrook *et al.*, 1989). Lambda DNA, digested with *Eco*R1 and *Hind* III (Boehringer Mannheim), was used as molecular weight marker. Numerical analysis of RAPD-PCR profiles was done according to the methods described by Du Plessis and Dicks (1995) and Van Reenen and Dicks (1996).

**Sequencing of 16S rRNA.** Conserved primers close to the 3' and 5' ends of a 16S rRNA gene were used to amplify a large fragment of the 16S rRNA gene by PCR. Purification of the PCR products using the Prep-A-Gene kit (Biorad) followed and were directly sequenced by using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (Applied Biosystems). The relatives of the isolates were established by searching the EMBL/Genbank Data Library with the FASTA program. Those sequences were retrieved and aligned with the newly determined sequences by using the program PILEUP.

## RESULTS AND DISCUSSION

Clustering of strains according to their protein profiles and RAPD-PCR banding patterns are shown in Figs. 1-8. Thirteen of the facultative heterofermentative lactobacilli isolated from the vagina clustered at  $r \geq 0.70$  with *Lactobacillus pentosus* NCIMB 8531 in cluster I. Five vaginal strains clustered at  $r \geq 0.73$  with *L. plantarum* ATCC 369, LMG 13556 and ATCC 14719<sup>T</sup> in cluster II (Fig. 1). The two clusters of *L. pentosus* and *L. plantarum* are related at a level of  $r \geq 0.65$  and in this sense similar to previous findings (Van Reenen and Dicks, 1996). Strain TV 1034 linked at  $r \geq 0.52$  with both these clusters, indicating that it is phenotypically not closely related to *L. pentosus* and *L. plantarum*. The taxonomic status of strain TV 1034 could not be determined by its protein profile or RAPD-PCR banding pattern.

In general, the results obtained by numerical analysis of RAPD-PCR profiles (Fig. 2) confirmed the protein profile groupings recorded for *L. pentosus* and *L. plantarum*. However, the RAPD-PCR banding patterns of the type strains of *L. pentosus* (NCFB 363<sup>T</sup>) and *L. plantarum* (ATCC 14719<sup>T</sup>) were very similar, as indicated by their grouping into one cluster at  $R^2 \geq 0.83$  (Fig. 2). Based on these results, the two species are genetically closely related and difficult to distinguish based on RAPD-PCR banding patterns. However, previous findings with more strains included in the study indicated that *L. pentosus* and *L. plantarum* belong to two independent RAPD-PCR clusters (Van Reenen and Dicks, 1996). More strains of both species will have to be included to determine their genetic relatedness as far as RAPD-PCR banding patterns are concerned.

Thirty-one of the obligate homofermentative lactobacilli clustered with nine reference strains of the "*Lactobacillus acidophilus*"-group at  $r \geq 0.67$  (Fig. 3). The type strains of *L. gasseri* (DSM 20077<sup>T</sup>), *L. crispatus* (NCFB 2752<sup>T</sup>), *Lactobacillus gallinarum* (NCFB 2235<sup>T</sup>), *Lactobacillus johnsonii* (NCFB 2241<sup>T</sup>) and *L. acidophilus* (ATCC 4356<sup>T</sup>, NCIMB 8690<sup>T</sup>) grouped into five different clusters (Fig. 3). Ten strains clustered with the type strains of *L. gasseri* (DSM 20077<sup>T</sup>) and *L. jensenii* (DSM 20557<sup>T</sup>) in cluster I at  $r \geq 0.81$ , suggesting that they are phenotypically closely related. No clear phenotypic distinction was recorded among strains of *L. gasseri* and *L. jensenii*. Four strains grouped with *L. crispatus* NCFB 4, NCFB 2752<sup>T</sup> and NCFB 5 in cluster II at  $r \geq 0.83$ , suggesting that they are phenotypically closely related. Only one strain (TV 1006) grouped at  $r \geq 0.94$  with the type strain of *L. gallinarum* (NCFB 2235<sup>T</sup>) in cluster III. Nine strains grouped with the type strain of *L. johnsonii* (NCFB 2241<sup>T</sup>) at  $r \geq 0.79$  in cluster IV and seven strains formed a cluster with *L. acidophilus* ATCC 4356<sup>T</sup> and NCIMB 8690<sup>T</sup> at  $r \geq 0.80$  (cluster V).



The type strains of *L. jensenii*, *L. crispatus*, *L. acidophilus*, *L. johnsonii*, *L. gasseri* and *L. crispatus* produced unique RAPD-PCR banding patterns and grouped into separate clusters (Fig. 4). In general these groupings corresponded well with previous findings (Du Plessis and Dicks, 1995). However, two strains (TV 1005 and TG 1005) identified as *L. johnsonii*, based on their protein profiles (Fig. 3), grouped with the type strain of *L. acidophilus* (NCIMB 8690<sup>T</sup>) in one cluster at  $R2 \geq 0.67$  (Fig. 4). On the other hand, strain TG 1001, identified as *L. acidophilus* (Fig. 3), grouped with the type strain of *L. johnsonii* (NCFB 2241<sup>T</sup>) in cluster IV (Fig. 4). The taxonomic status of strains TV 1005, TG 1005 and TG 1001 could not be clarified with RAPD-PCR.

Four of the obligate heterofermentative strains clustered with *Lactobacillus buchneri* ATCC 9460 and ATCC 12935 and *L. fermentans* ATCC 14932 at  $r \geq 0.73$  (cluster I, Fig. 5). Seven strains grouped with *L. fermentum* ATCC 11739<sup>T</sup> and ATCC 23271 at  $r \geq 0.74$  (cluster II, Fig. 5) and three were related to *Weissella viridescens* ATCC 12076<sup>T</sup> and NCIMB 1615 at  $r \geq 0.76$  (cluster III, Fig. 5). The strains of *Weissella viridescens* were phenotypically well separated from the strains in the other two clusters, as evident by their linking at  $r \geq 0.61$  (Fig. 5). It was, however, interesting to note that clusters I and II linked at  $r = 0.72$ . This suggested that *L. buchneri* and *L. fermentum* are phenotypically closely related.

Based on the RAPD-PCR banding patterns obtained for *L. buchneri*, *L. fermentans*, *L. fermentum* and *W. viridescens*, the four species are well separated, each species defined in its own cluster (Fig. 6).

Twenty-seven strains clustered with *Enterococcus faecium* NCIMB 583, BFE 1170 and BFE 1027 in one protein profile cluster at  $r \geq 0.78$  (cluster I, Fig. 7). Nine strains grouped with *E. faecalis* ATCC 13665 in cluster III at  $r \geq 0.77$ , well separated from the type strain of *E. faecalis* (NCIMB 775<sup>T</sup>) and *E. faecalis* NCFB 587 in cluster II (Fig. 7). Based on these findings, the majority of enterococci (27 out of 36 strains) are members of *E. faecium*.

The vaginal enterococci grouped into four separate RAPD-PCR clusters (Fig. 8), suggesting a higher genetic diversity that reflected with the phenotypic groupings obtained by their protein profiles. The two reference strains of *E. faecalis* and *E. faecium* (LMG 7737 and LMG 2149) grouped into two separate clusters (I and II, Fig. 8). More strains of *E. faecalis*, *E. faecium* and members of other *Enterococcus* spp. will have to be included to evaluate numerical analysis of RAPD-PCR as a taxonomic technique for enterococci.

In general, the results obtained by numerical analysis of total cell protein patterns corresponded well with results obtained by numerical analysis of RAPD-PCR. The strain identifications were confirmed by 16S rRNA sequencing (Table 1).

Our results have shown that *L. plantarum* is the most dominant species in the vagina, followed by *L. fermentum*, *L. crispatus* and *L. jensenii*. Two species, *W. viridescens* (previously *L. viridescens*) and *L. buchneri*, have not previously been reported as being present in the human vagina. We did not isolate any strains of *Lactobacillus casei*, *Lactobacillus rhamnosus* and *Lactobacillus cellobiosus*. The latter three species were, in addition to the species identified in this study, isolated from the human vagina by Rogosa and Sharpe (1960). The species we have isolated are in correlation with that described by Fagnant *et al.* (1982) and Giorgi *et al.* (1987). However, we did not isolate strains of *L. leichmanii*, *L. delbrueckii* and *Lactobacillus salivarius*.

The high frequency of enterococci which we have isolated is of particular interest, since *Enterococcus* spp. are usually dominant in the urogenital tract of older women. In many cases we have not isolated any *Lactobacillus* spp. The plates were usually densely populated with other bacteria and/or yeasts, suggesting that the patients suffered from vaginal infections like bacterial vaginosis or candidal vaginitis. The role which *Enterococcus* spp. play in the urogenital tract is unknown and merits further research.

### Acknowledgements

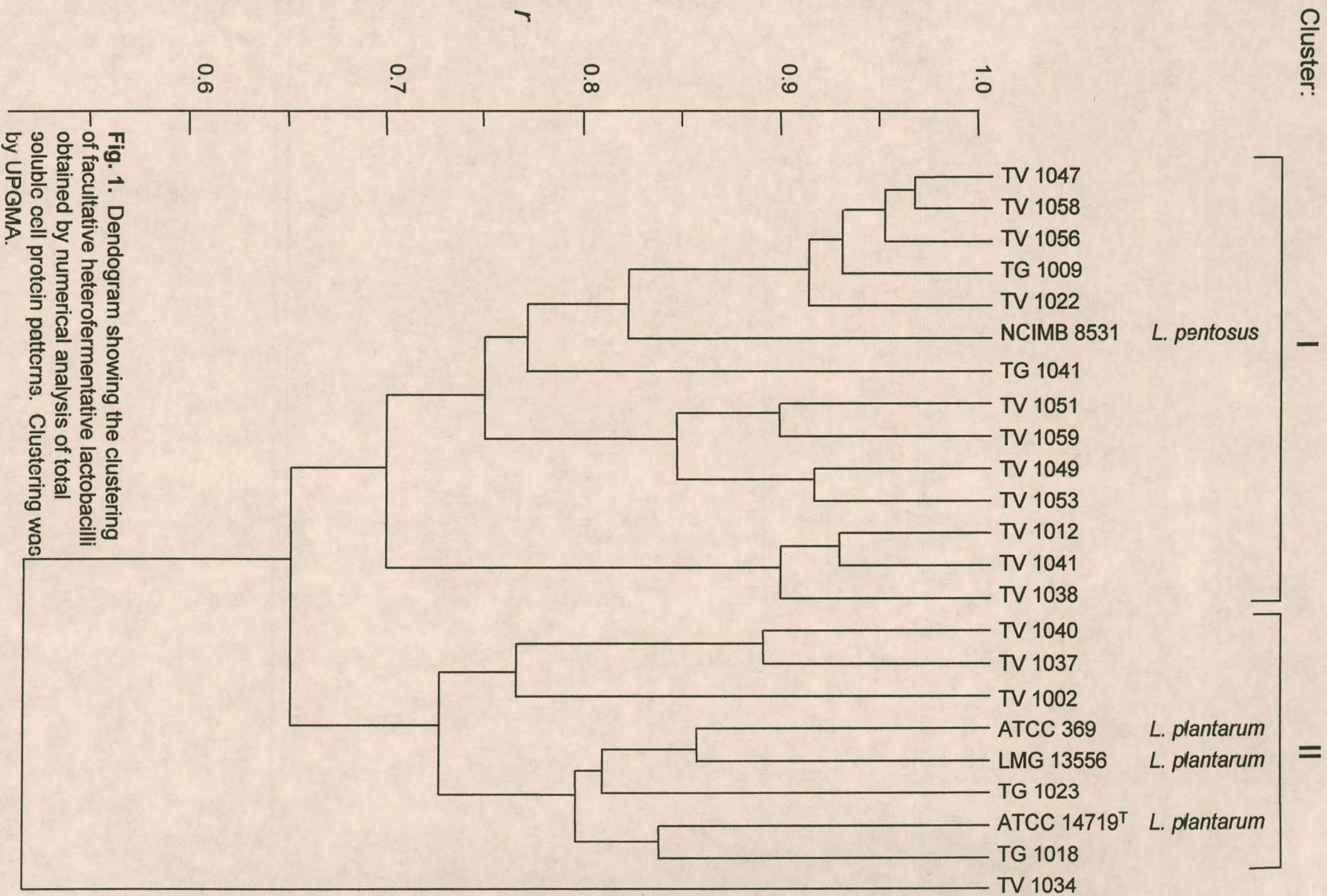
We thank prof. W. Holzapfel from the Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition, Karlsruhe, Germany, for reference strains, prof. H.J. Odendaal, Department of Gynaecology and Obstetrics, University of Stellenbosch, for swabs taken from the patients and prof. J.J. Joubert, Department of Medical Microbiology, University of Stellenbosch for invaluable discussions. We also thank Dr. M. du Toit for assistance with the numerical analysis. This research was funded by the Industrial Development Corporation.

Table 1. Strains and methods used for identification of isolates.

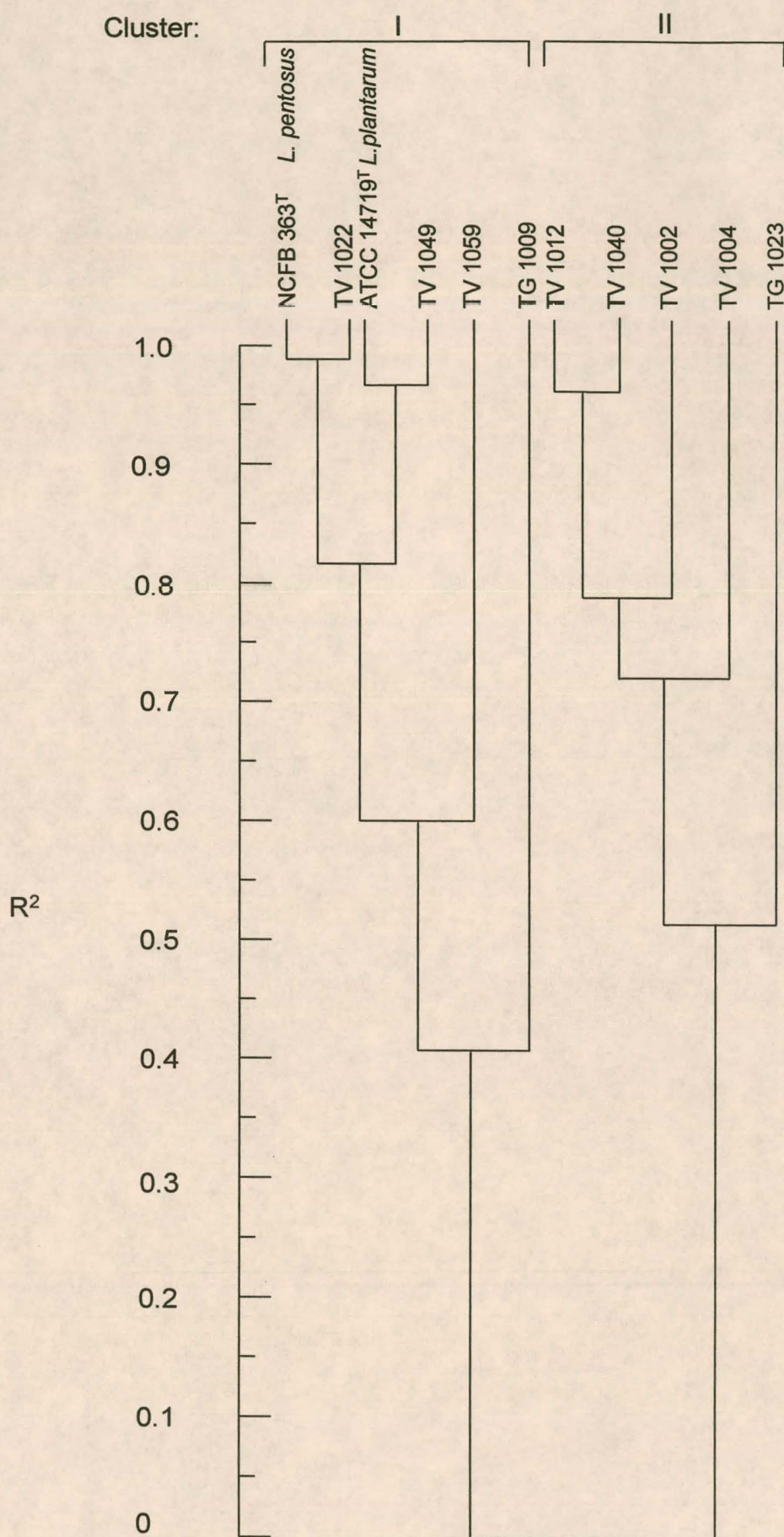
Strain	Identification
ATCC 9460, <b>ATCC 12935</b>	<i>Lactobacillus buchneri</i>
<b>NCFB 2752<sup>T</sup></b> , NCFB 4, NCFB 5, TG 1003, TG 1005, TG 1006, TG 1007, TG 1010, <u>TV 1008</u> , TG 1014, <b>TV 1021</b>	<i>Lactobacillus crispatus</i>
<b>ATCC 11739<sup>T</sup></b> , ATCC 23127, <b>TV 1025</b> , <b>TV 1027</b> , <u>TV 1031</u> , <u>TV 1032</u> , <b>TG 1021</b> , TV 1052, TV 1054, <b>TV 1055</b>	<i>Lactobacillus fermentum</i>
<b>ATCC 14932</b> , <b>TV 1030</b> , TV 1039, <b>TV 1042</b>	<i>Lactobacillus fermentans</i>
<b>DSM 20077<sup>T</sup></b> , <u>TV 1013</u> , TG 1034	<i>Lactobacillus gasseri</i>
<b>DSM 20557<sup>T</sup></b> , <b>TV 1010</b> , TG 1013, <u>TV 1018</u> , TV 1036, TV 1044, TV 1045, <u>TV 1050</u> , TG 1030	<i>Lactobacillus jensenii</i>
<b>NCIMB 8086<sup>T</sup></b> , ATCC 4356 <sup>T</sup> , <u>TV 1001</u> , <b>TV 1005</b> , TV 1048	<i>Lactobacillus acidophilus</i>
<b>NCFB 2235<sup>T</sup></b> , <b>TG 1001</b> , <b>TV 1006</b>	<i>Lactobacillus gallinarum</i>
<b>NCFB 2241<sup>T</sup></b> , TV 1016, <b>TG 1016</b> , TV 1035, <b>TG 1025</b> , TG 1038	<i>Lactobacillus johnsonii</i>
<b>NCFB 363<sup>T</sup></b> , NCIMB 8531, <u>TV 1004</u> , <b>TG 1009</b> , <b>TV 1022</b> , <b>TV 1047</b> , TV 1056, TV 1058, <u>TG 1041</u>	<i>Lactobacillus pentosus</i>
<b>ATCC 14719<sup>T</sup></b> , ATCC 936, LMG 13556, <b>TV 1002</b> , <u>TV 1012</u> , TV 1037, TV 1038, <b>TV 1040</b> , TV 1041, <u>TV 1046</u> , <b>TG 1023</b> , <b>TV 1049</b> , TV 1051, TV 1053, <u>TV 1059</u>	<i>Lactobacillus plantarum</i>
<b>ATCC 12706<sup>T</sup></b> , NCIMB 1615, TG 1008, <b>TV 1011</b> , <b>TV 1023</b>	<i>Lactobacillus viridescens</i>
<b>NCIMB 775<sup>T</sup></b> , NCFB 587, LMG 13665, <b>LMG 7737</b> , TV 1003, <b>TG 1002</b> , <b>TG 1004</b> , TV 1007, <b>TG 1011</b> , TG 1012, <u>TV 1014</u> , <b>TV 1015</b> , <b>TG 1015</b> , TV 1017, <u>TV 1019</u> , TV 1020, TV 1024, TG 1017, TV 1028, <b>TG 1020</b> , TV 1043, TG 1022, <u>TG 1024</u> , <b>TG 1026</b> , TG 1028, <b>TV 1057</b> , <u>TG 1029</u> , <u>TG 1031</u> , TG 1032, TG 1033, <b>TG 1035</b> , <b>TG 1036</b> , TG 1037, <b>TG 1039</b> , <b>TG 1040</b>	<i>Enterococcus faecalis</i>
<b>NCIMB 583</b> , <b>LMG 2149</b> , <u>TV 1009</u> , TV 1026, <b>TV 1029</b> , <b>TV 1033</b> , TG 1019	<i>Enterococcus faecium</i>
<b>TG 1018</b>	<i>Lactobacillus acetotolerans</i>
<b>TV 1034</b> , <b>TG 1027</b>	Unknown

Strains printed in bold were selected for RAPD-PCR analysis.

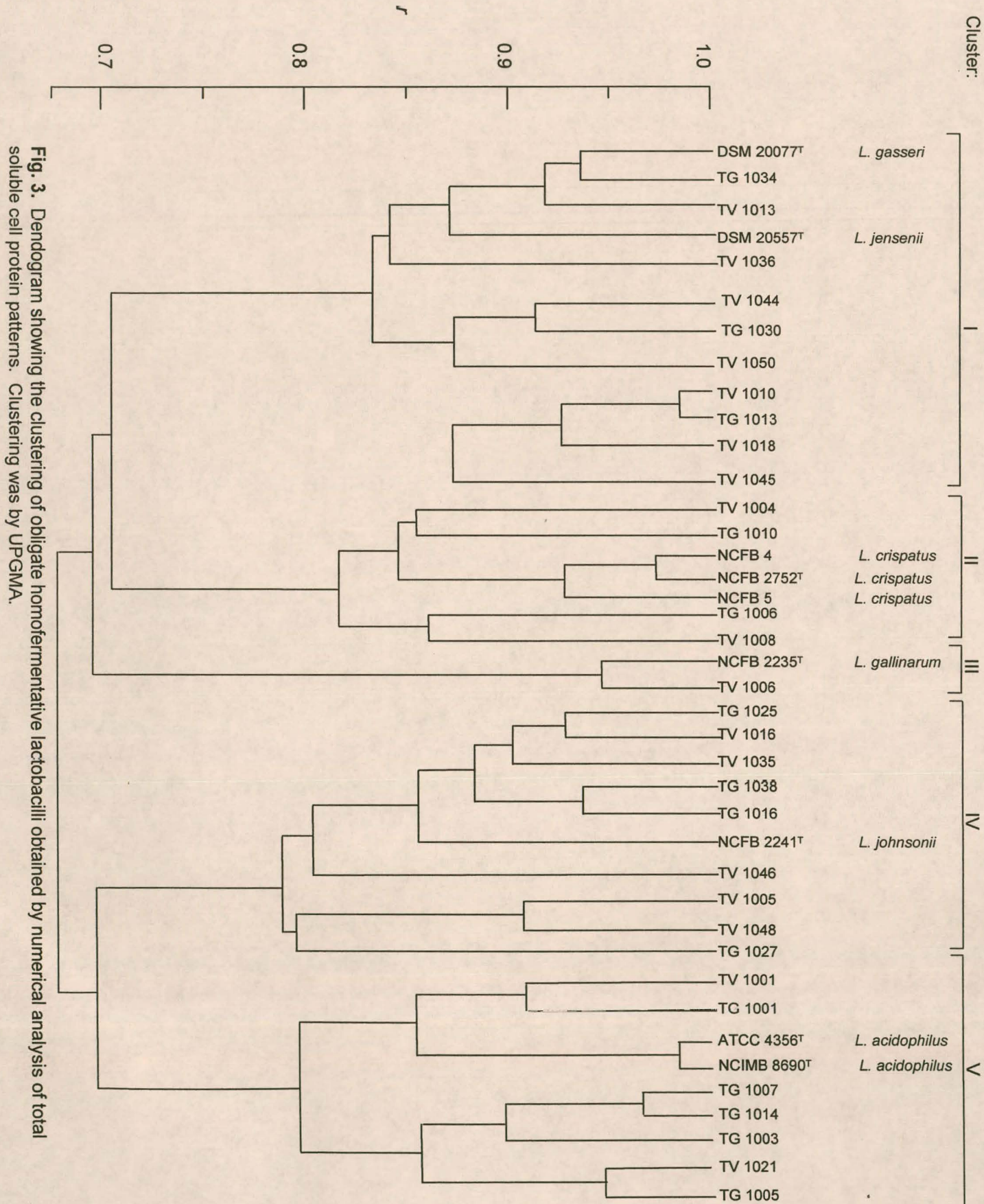
Strains underlined were selected for 16S rRNA sequencing.



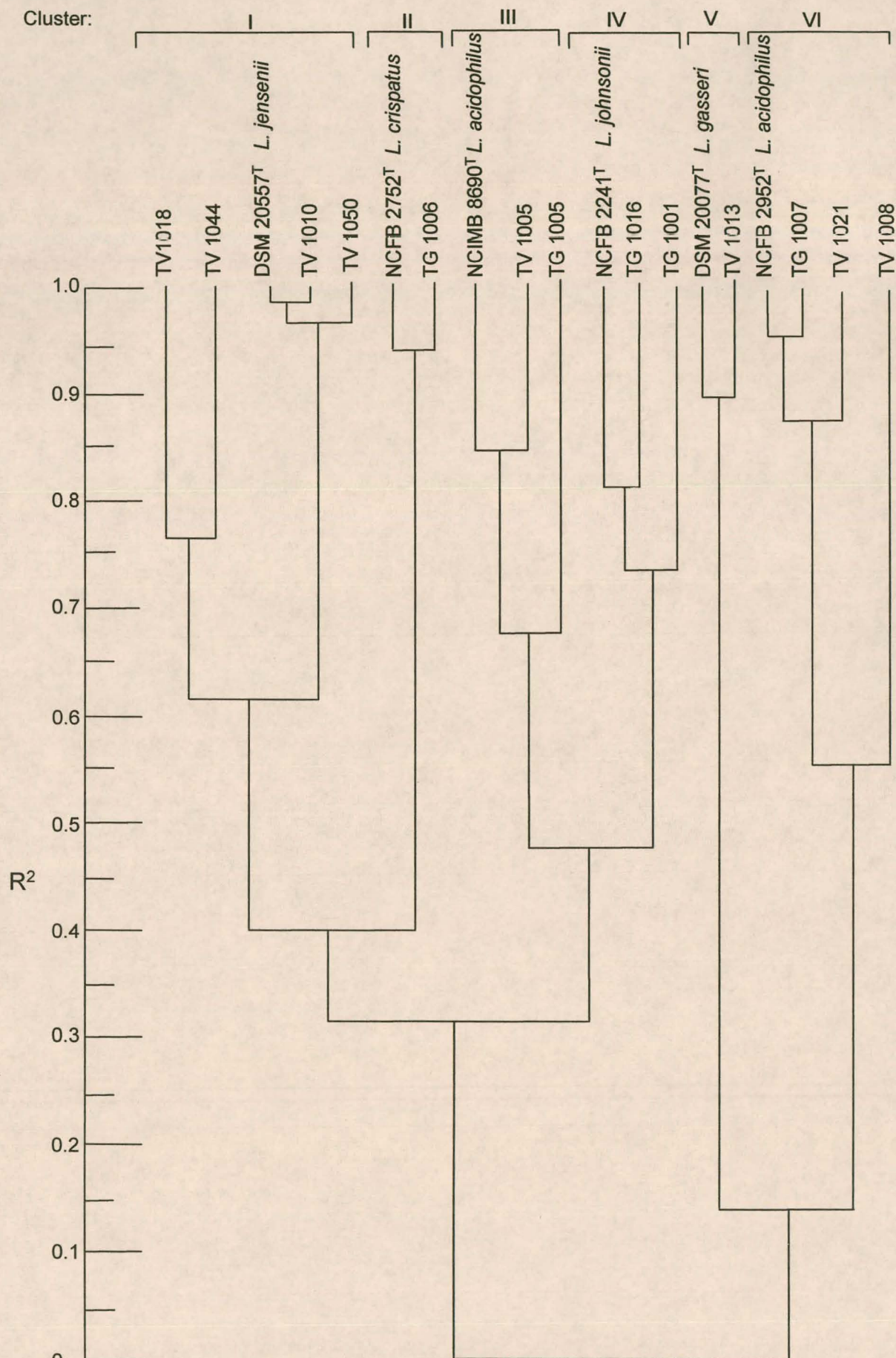
**Fig. 1.** Dendrogram showing the clustering of facultative heterofermentative lactobacilli obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA.



**Fig. 2.** Dendrogram showing the clustering of facultative heterofermentative lactobacilli obtained by numerical analysis of RAPD-PCR profiles. Clustering was by the normalized average linkage analysis. Distances between clusters are expressed in R<sup>2</sup>-values.



**Fig. 3.** Dendrogram showing the clustering of obligate homofermentative lactobacilli obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA.



**Fig. 4.** Dendrogram showing the clustering of obligate homofermentative lactobacilli obtained by numerical analysis of RAPD-PCR profiles. Clustering was by the normalized average linkage analysis. Distances between clusters are expressed in  $R^2$ -values.

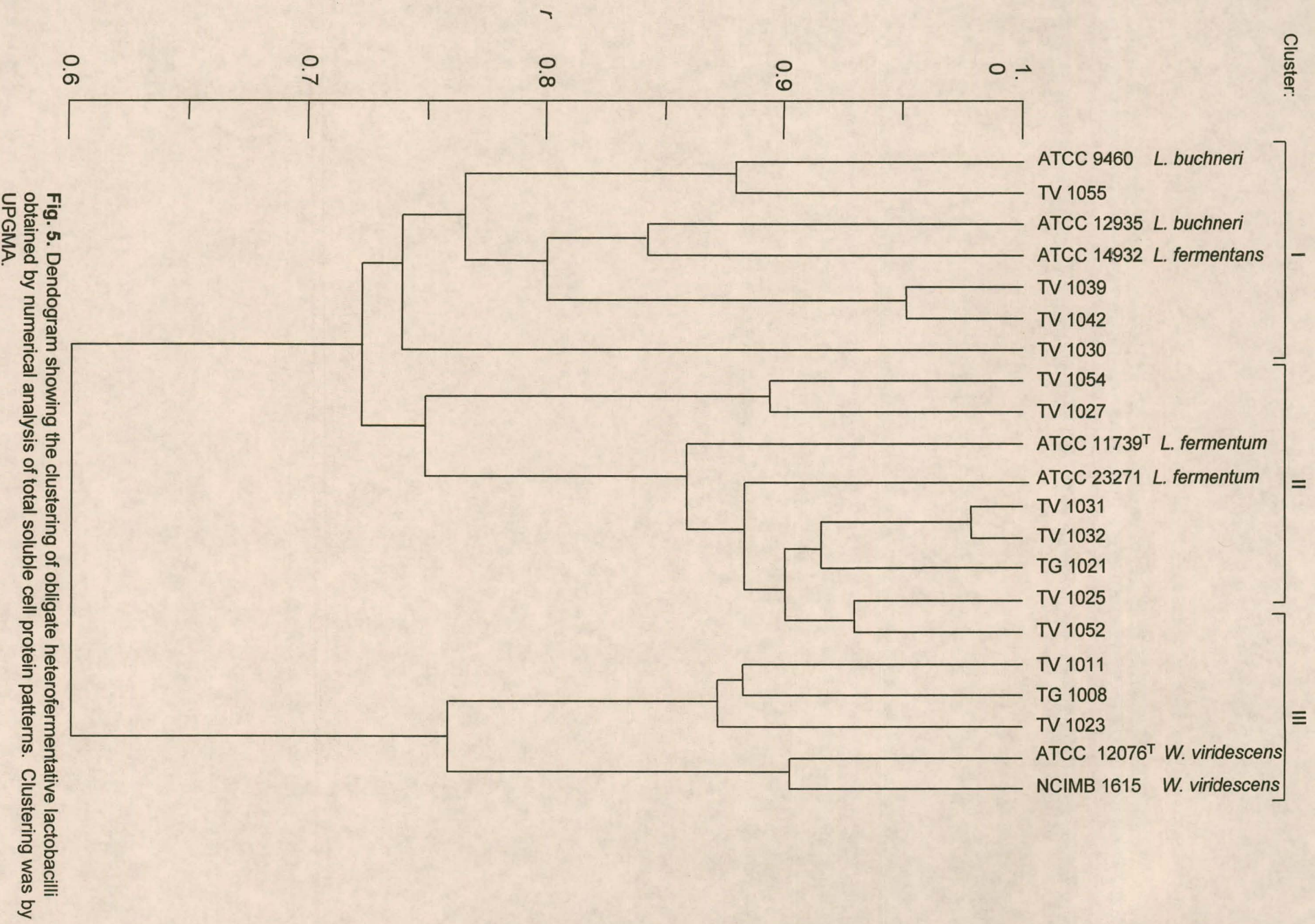
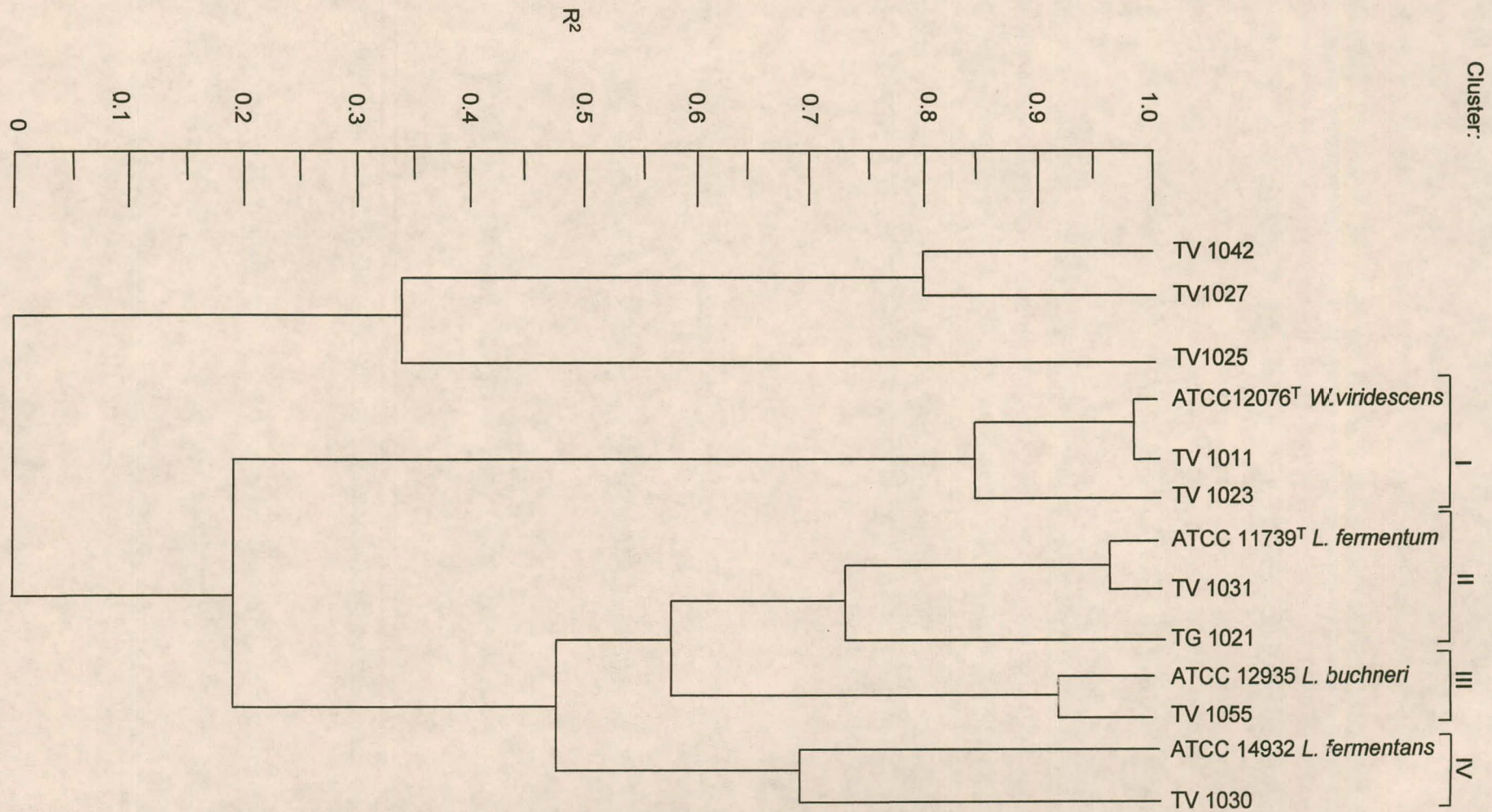
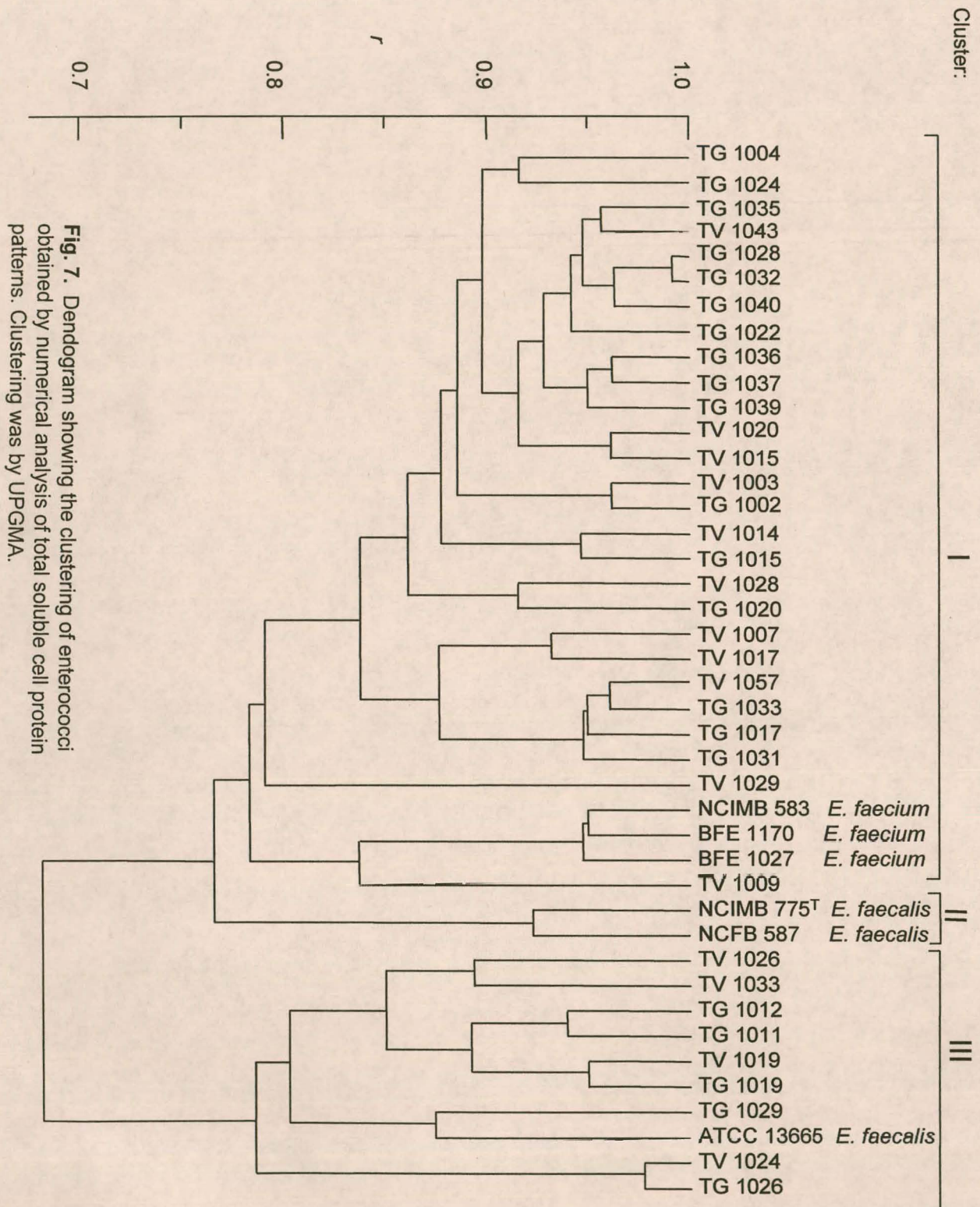


Fig. 5. Dendrogram showing the clustering of obligate heterofermentative lactobacilli obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA.

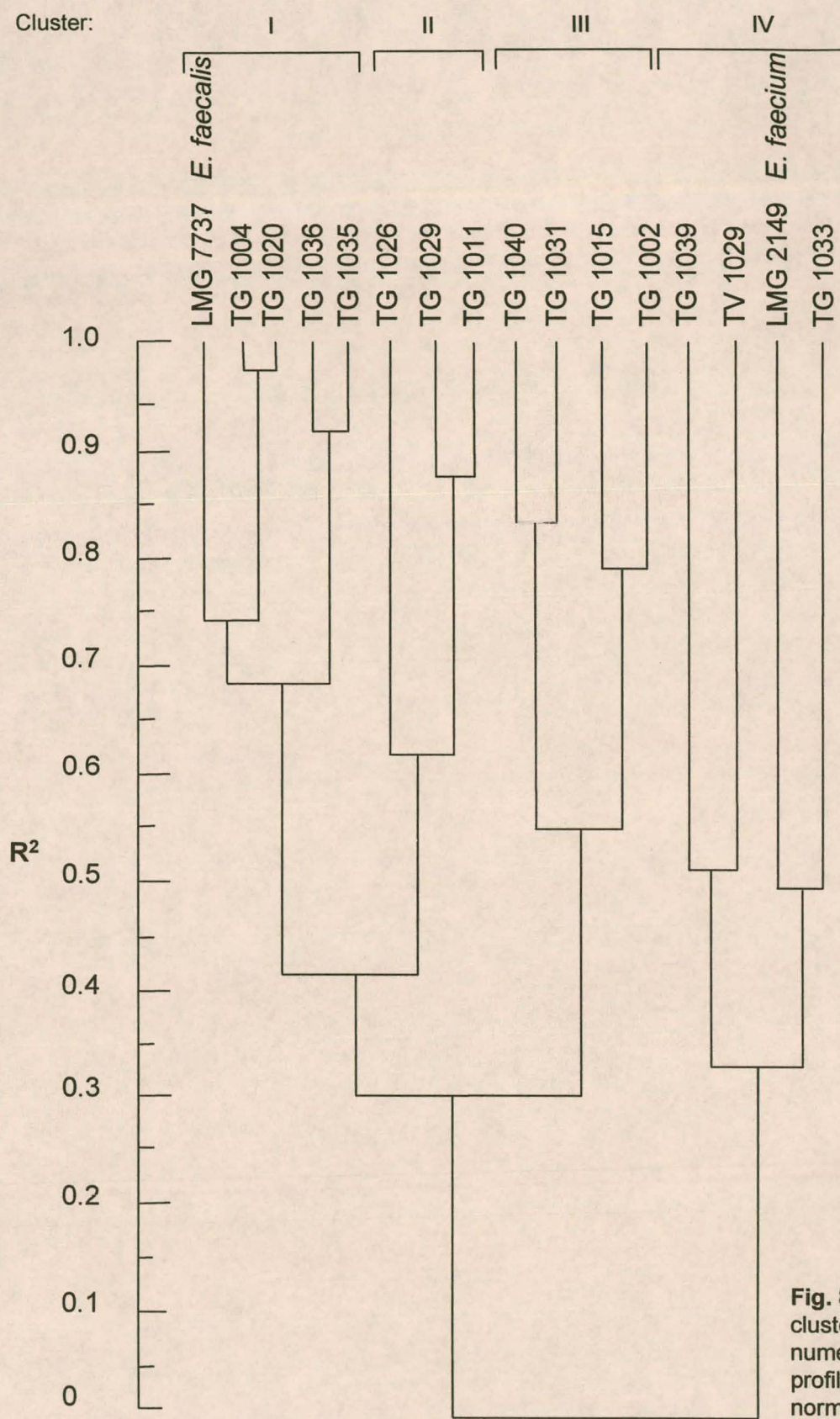




**Fig. 6.** Dendrogram showing the clustering of obligate heterofermentative lactobacilli obtained by numerical analysis of RAPD-PCR profiles. Clustering was by the normalized average linkage analysis. Distances between clusters are expressed in  $R^2$ -values.



**Fig. 7.** Dendrogram showing the clustering of enterococci obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA.



**Fig. 8.** Dendrogram showing the clustering of enterococci obtained by numerical analysis of RAPD-PCR profiles. Clustering was by the normalized average linkage analysis. Distances between clusters are expressed in R<sup>2</sup>-values.

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**5. *LACTOBACILLUS FORNIXALIS* SP. NOV., ISOLATED FROM THE  
POSTERIOR FORNIX OF THE HUMAN VAGINA**

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***Lactobacillus fornixalis* sp. nov., isolated from the posterior fornix of the human vagina**

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**Running title:** *Lactobacillus fornixalis* sp. nov., a new species

**Keywords:** *Lactobacillus fornixalis* sp. nov., human vagina, taxonomy, phylogeny, lactic acid bacteria

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Twelve strains isolated from the posterior fornix fluid of the human vagina were identified as *Lactobacillus johnsonii*, *Lactobacillus acidophilus*, *Lactobacillus gallinarum* and *Lactobacillus crispatus* based on results obtained by numerical analyses of total soluble cell protein profiles and RAPD-PCR banding patterns. Five strains grouped with the type strains of *Lactobacillus gasseri* (DSM 20077<sup>T</sup>) and *Lactobacillus jensenii* (DSM 20557<sup>T</sup>) at  $r \geq 0.83$  in one protein profile cluster, well separated from the other species included in this study. However, numerical analysis of the RAPD-PCR banding patterns of representative strains selected from the *L. gasseri* / *L. jensenii* protein cluster clearly indicated that they belong to two different species. Four strains (TV 1010, TG 1013, TV 1018 and TV 1045) grouped into one protein profile cluster at  $r \geq 0.87$ , well separated from the other species included in this study. Strains selected from this cluster displayed very similar RAPD-PCR banding patterns and clustered at  $R^2 \geq 0.78$ , separate from the other strains examined. Sequencing of the 16S rRNA of two representative strains TV 1018 and TG 1013 of this group indicated that it represents a new member of rRNA group I *Lactobacillus* which includes *Lactobacillus delbrueckii*, the type of the genus, and close relatives *Lactobacillus acetotolerans*, *Lactobacillus kefiranofaciens*, *Lactobacillus iners*, *L. jensenii*, *L. crispatus*, *L. acidophilus*, *Lactobacillus helveticus*, *Lactobacillus amylovorus*, *Lactobacillus hamsteri*, *L. johnsonii*, *L. gasseri* and *Lactobacillus amyolyticus*. We propose the name *Lactobacillus fornixalis* sp. nov. for strains TV 1010, TG 1013, TV 1018 and TV 1045, with strain TV 1018 as the type.



## INTRODUCTION

The genus *Lactobacillus* currently comprises of 64 valid species (Pot *et al.*, 1994), which can be divided into three groups based on the fermentative abilities of the species (Kandler & Weiss, 1986). Group I, the obligately homofermentative species, degrade hexoses almost completely to lactic acid and do not ferment pentoses or gluconate. Species of group II are facultatively heterofermentative and produce acetic acid, ethanol and formic acid under glucose limitation in addition to lactic acid. Pentoses are usually fermented. Group III contains the obligately heterofermentative lactobacilli which ferment hexoses to lactic acid, acetic acid, ethanol and carbon dioxide. Pentoses are fermented to lactic acid and acetic acid by this latter group.

Group I contains 21 species, divided into two subgroups based on DNA:DNA hybridizations (Kandler & Weiss, 1986). Subgroup 1 includes *Lactobacillus delbrueckii* (the type species of the genus) and *Lactobacillus jensenii* (Kandler & Weiss, 1986). Subgroup 2 is represented by *Lactobacillus acidophilus*, *Lactobacillus amylophilus*, *Lactobacillus farciminis*, *Lactobacillus kefiranofaciens*, *Lactobacillus vitulinus*, *Lactobacillus intestinalis*, *Lactobacillus uli* (Pot *et al.*, 1994), and the "*Lactobacillus casei*/Pediococcus group of lactobacilli", viz. *Lactobacillus aviatus*, *Lactobacillus animalis*, *Lactobacillus salivarius*, *Lactobacillus mali*, *Lactobacillus ruminis* and *Lactobacillus sharpae* (Collins *et al.*, 1991). The *L. acidophilus* group was later divided into six genotypic groups based on DNA:DNA hybridizations, with the species *L. acidophilus* in group A1 (Fujisawa *et al.*, 1992). Groups A2, A3 and A4 contains *Lactobacillus crispatus* (Cato *et al.*, 1983), *Lactobacillus amylovorus* and *Lactobacillus gallinarum* (Fujisawa *et al.*, 1992), respectively. Groups B1 and B2 include *Lactobacillus gasseri* (Lauer & Kandler, 1980) and *Lactobacillus johnsonii* (Fujisawa *et al.*, 1992), respectively.

In humans, as many as  $10^7$ - $10^8$  *Lactobacillus* spp. ml<sup>-1</sup> vaginal fluid have been reported (Redondo-Lopez *et al.*, 1990). The obligate homofermentative species thus far isolated include *L. acidophilus*, *L. crispatus*, *L. jensenii*, the facultative heterofermentative species *Lactobacillus plantarum* and *L. casei*, and the obligate heterofermentative species *Lactobacillus brevis* and *Lactobacillus fermentum* (Fernandes *et al.* 1987; Giorgi *et al.*, 1987).

In this study we have investigated the relatedness among 21 strains of obligately homofermentative (group I) *Lactobacillus* spp. isolated from the posterior fornix fluid of the vagina of pre- and post-natal patients and representative strains of *L. acidophilus*, *L. crispatus*, *L. jensenii*, *L. gallinarum*, *L. gasseri* and *L. johnsonii* using both phenotypic and

genotypic methods. Based on the results of this polyphasic taxonomic study we propose a new species *Lactobacillus fornixalis*.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Twenty-one strains of lactic acid bacteria were isolated from the posterior fornix secretions of the vagina of 80 patients who attended the pre- and post-natal clinics at the Tygerberg Hospital in Tygerberg, South Africa (Table 1). The swabs collected from the patients were immersed in 1 ml sterile physiological salt (Univar) and immediately spread-plated (100  $\mu$ l) onto MRS agar (Biolab). Colonies were selected from the plates after two days of incubation at 37 °C. Reference strains were obtained from the National Collection of Dairy Organisms (NCDO), American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen (DSM) and National Collection of Industrial and Marine Bacteria (NCIMB). All strains were cultured in MRS broth (Biolab) at 37 °C.

**Numerical analysis of total soluble cell protein patterns.** Cultures were grown in MRS broth (Biolab) for 24 h at 37 °C. Protein isolation and gel electrophoresis were performed as described by Vauterin *et al.* (1993). The Gelcompar computer program (version 4.0) of Applied Maths (Kortrijk, Belgium) was used to analyse the protein banding patterns. The program recorded the normalised electrophoretic patterns of the densitometric traces, grouped the isolates by the Pearson product moment correlation coefficient ( $r$ ) and performed UPGMA cluster analysis of the protein bands.

**Numerical analysis of RAPD-PCR profiles.** The DNA of selected strains (Table 1) was isolated according to the method of Dellaglio *et al.* (1973). Three single primers [TGGGCGTCAA(OPL-02), ACGCAGGCAC (OPL-05) and ACGATGAGCC (OPL-11)] were used and the DNA amplification performed according to the methods described by Van Reenen and Dicks (1996). Lambda DNA, digested with *EcoR*I and *Hind*III (Boehringer Mannheim), was used as molecular weight marker. Numerical analysis of RAPD-PCR profiles was done using the CLUSTER program of SAS Institute Inc. (1989), according to the methods described by Van Reenen and Dicks (1996).

**Determination of 16S rRNA gene sequences and phylogenetic analyses.** A large fragment of the 16S rRNA gene was amplified by PCR by using universal primers pA (5'-AGAGTTTGATCCTGGCTCAG; positions 8 to 27; *Escherichia coli* numbering) and pH\* (5' –

AAGGAGGTGATCCAGCCGCA; positions 1541 to 1522). The PCR products were purified by using a Prep-A-gene kit (Bio-Rad, Hercules, Ca., USA) according to the manufacturer's instructions and were sequenced by using a *Taq* Dye Deoxy terminator cycle sequencing kit (Applied Biosystems, Inc. Foster City, USA) and a model 373A automatic sequencer (Applied Biosystems, Inc.). The closest known relatives of the new isolates were determined by performing sequence data base searches and the sequences of closely related strains were retrieved from GenBank or Ribosomal Database Project libraries. Sequences were aligned by using the program PILEUP (Devereux *et al.*, 1984) and the alignment was corrected manually. Distance matrices were produced by using the DNADIST program of the PHYLIP package (Felsenstein, 1989) and a phylogenetic tree was constructed according to the neighbor-joining method with the program NEIGHBOR (Felsenstein, 1989). The statistical significance of the groups obtained was assessed by bootstrapping (500 replicates) by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1989).

**Nucleotide sequence accession number.** The determined 16S rRNA gene sequence of strain TV 1018<sup>T</sup> has been deposited in the GenBank Data Library under the accession number Y18654.

**DNA base composition.** DNA was isolated and purified by using the technique described by Dicks *et al.* (1990). The G+C content was calculated by using the method of Marmur & Doty (1962).

**Biochemical and physiological tests.** The biochemical and physiological characteristics were determined by using the methods described by Sharpe (1979). Carbohydrate fermentation characteristics were determined by using the API 50CHL system (La Balme Les Grottes, Montalieu Vercieu, France). Results were recorded after 48 h at 37°C.

## RESULTS AND DISCUSSION

Twenty-one strains isolated from the posterior fornix fluid of the human vagina grouped into six protein profile clusters at  $r \geq 0.82$ , well separated from each other at  $r \geq 0.67$  (Fig. 1). Eight strains of the vaginal isolates grouped with the type strain of *L. johnsonii* (NCDO 2241<sup>T</sup>) in cluster I at  $r \geq 0.82$ , one strain formed a tight cluster with the type strain of *L. acidophilus* (ATCC 4356<sup>T</sup>, NCDO 1748<sup>T</sup>) in cluster II at  $r \geq 0.84$ , one strain linked with the type strain of *L. gallinarum* (NCDO 2235<sup>T</sup>) in cluster III at  $r \geq 0.93$ , and two strains grouped with *L.*

*crispatus* in cluster IV at  $r \geq 0.82$  (Fig. 1). These groupings were confirmed by numerical analysis of RAPD-PCR banding patterns (Fig. 2). Strains within each protein profile cluster displayed similar DNA profiles, whereas strains from different clusters displayed unique banding patterns (Table 2). Based on these results, strains TG 1025, TV 1016, TV 1035, TG 1038, TG 1016, TV 1005, TV 1048 and TG 1027 (cluster I, Fig. 1) are classified as *L. johnsonii*, whereas strains TV 1001 (cluster II, Fig. 1) and TV 1006 (cluster III, Fig. 1) are considered members of *L. acidophilus* and *L. gallinarum*, respectively. Strains TG 1010 and TG 1006 (cluster IV, Fig. 1) are classified as *L. crispatus*.

Five strains (TG 1034, TV 1013, TV 1036, TV 1044 and TG 1030) grouped with the type strains of *L. gasseri* (DSM 20077<sup>T</sup>) and *L. jensenii* (DSM 20557<sup>T</sup>) at  $r \geq 0.83$  in cluster V (Fig. 1), indicating that they belong to the same phenotypic group. However, the RAPD-PCR profile of strain TV 1013, selected as one of the representative strains, was almost identical to that of *L. gasseri* DSM 20077<sup>T</sup>, as shown by the high similarity value ( $R^2 \geq 0.92$ ) recorded between the two strains (Fig. 2). On the other hand, strain TV 1044 shared a high DNA similarity with *L. jensenii* DSM 20557<sup>T</sup>, as revealed by the high correlation ( $R^2 \geq 0.98$ ) recorded in their RAPD-PCR banding patterns (Fig. 2). These findings suggest that numerical analysis of RAPD-PCR banding patterns are more reliable than numerical analysis of total soluble cell protein patterns in differentiating strains of *L. gasseri* and *L. jensenii*. Additional strains will have to be studied to confirm this finding.

Four strains (TV 1010, TG 1013, TV 1018 and TV 1045) were phenotypically different from any of the species included in this study and formed a tight phenotypic group at  $r \geq 0.87$  (cluster VI, Fig. 1). The RAPD-PCR profiles of two representative strains from this cluster (TV 1018 and TV 1010) were found to be very similar, as shown by the high correlation value ( $R^2 \geq 0.78$ ) recorded between the two strains (Fig. 2). To investigate the phylogenetic position of this group, the almost complete 16S rRNA gene sequence (>1400 nucleotides) of strain TV 1018 was determined, and subjected to a comparative analysis. Sequence database searches (data not shown) revealed the bacterium was closely related to the *L. delbrueckii* group of organisms (rRNA group I; see Collins *et al.*, 1991) with other lactobacilli more distantly related. A tree depicting the phylogenetic affinities of strain TV 1018 is shown in Fig. 3. It was evident from both sequence divergence values of >5% with other members of the *L. delbrueckii* rRNA cluster, and the treeing analysis, that the unidentified bacterium represents a hitherto unknown *Lactobacillus* species. The 16S rRNA gene sequence of a second isolate TG 1013 of the novel vaginal bacterium was also sequenced (>1400 bases), and showed 99.9% sequence similarity with strain TV 1018, thereby confirming their

genotypic identity. Thus, based on phenotypic and phylogenetic findings we propose the name *Lactobacillus fornixalis* sp. nov. for strains TV 1010, TG 1013, TV 1018 and TV 1045, with strain TV 1018 as the type. A full description of *L. fornixalis* is given below.

**Description of *Lactobacillus fornixalis* sp. nov. *Lactobacillus fornixalis* (fornixalis. L. gen. n. *fornixalis*, of the posterior fornix).** Gram-positive rods. Nonmotile, non-sporulating, catalase negative and oxidase negative. Colonies on MRS agar are round, smooth, white and approx. 1 mm in diameter. Growth occurs at temperatures between 20 and 40 °C on MRS agar, with the optimum temperature between 35 and 37 °C. Facultatively anaerobic, but grows well on the surface of MRS agar when not incubated under micro-aerophilic conditions. Obligately homofermentative, with no gas production from glucose or gluconate. D(-)- and L- lactic acid are produced from glucose. Esculin is hydrolysed. Voges Proskauer negative. Indole is not formed and nitrates are not reduced. Polysaccharides are not produced from sucrose. Acid is produced from amygdalin, cellobiose, fructose, galactose, glucose, maltose, mannose, mannitol, melezitose, ribose, salicin, sorbitol, sucrose and trehalose. Arabinose, lactose, melibiose, raffinose, rhamnose, starch and xylose are not fermented. The G+C content of the type strain is 37 mol% (as determined by the thermal denaturation method). Isolated from the posterior fornix fluid of the human vagina. The type strain is TV 1018.

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**Table 1.** Genotypic relatedness among *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. fornixalis* sp. nov.

Strain	Protein cluster*	RAPD-PCR cluster†
<i>L. johnsonii</i> <b>TG 1025</b> , TV 1016, TV 1035, TG 1038, <b>TG 1016‡</b> , <b>NCDO 2241<sup>T</sup></b> , <b>TV 1005</b> , TV 1048, TG 1027	I	V
<i>L. acidophilus</i> TV 1001, ATCC 4356 <sup>T</sup> , <b>NCDO 1748<sup>T</sup></b>	II	IV
<i>L. gallinarum</i> <b>NCDO 2235<sup>T</sup></b> , TV 1006	III	III
<i>L. crispatus</i> TG 1010, NCDO 4, <b>NCDO 2752<sup>T</sup></b> , NCDO 5, TG 1006	IV	NC <sup>§</sup>
<i>L. gasseri</i> <b>DSM 20077<sup>T</sup></b> , TG 1034, TV 1013	V	VI
<i>L. jensenii</i> <b>DSM 20557<sup>T</sup></b> , TV 1036, <b>TV 1044</b> , TG 1030	V	II
<i>L. fornixalis</i> sp. nov. TV 1010, TG 1013, <b>TV 1018</b> , TV 1045	VI	I

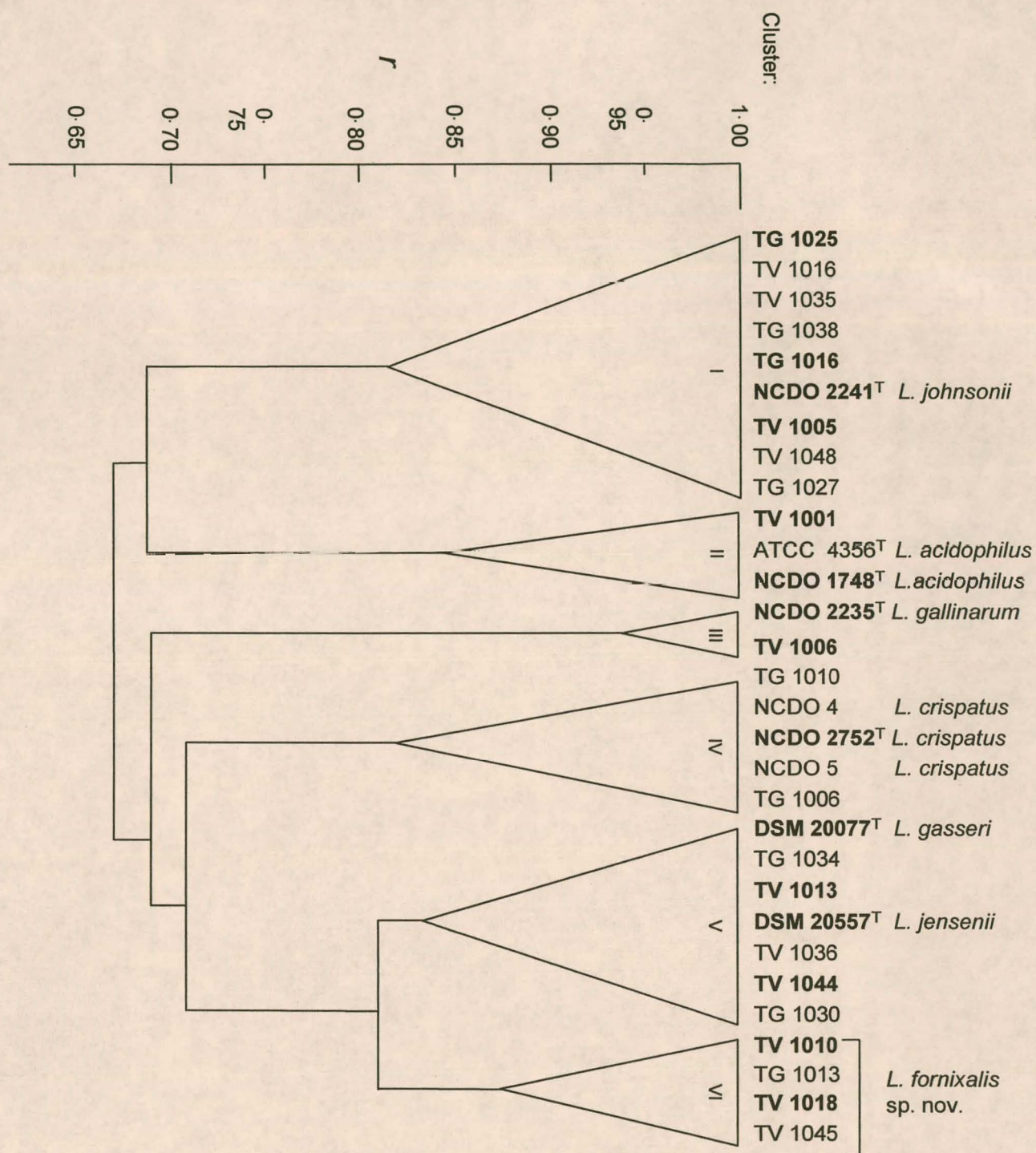
\* From Fig. 1.

† From Fig. 2.

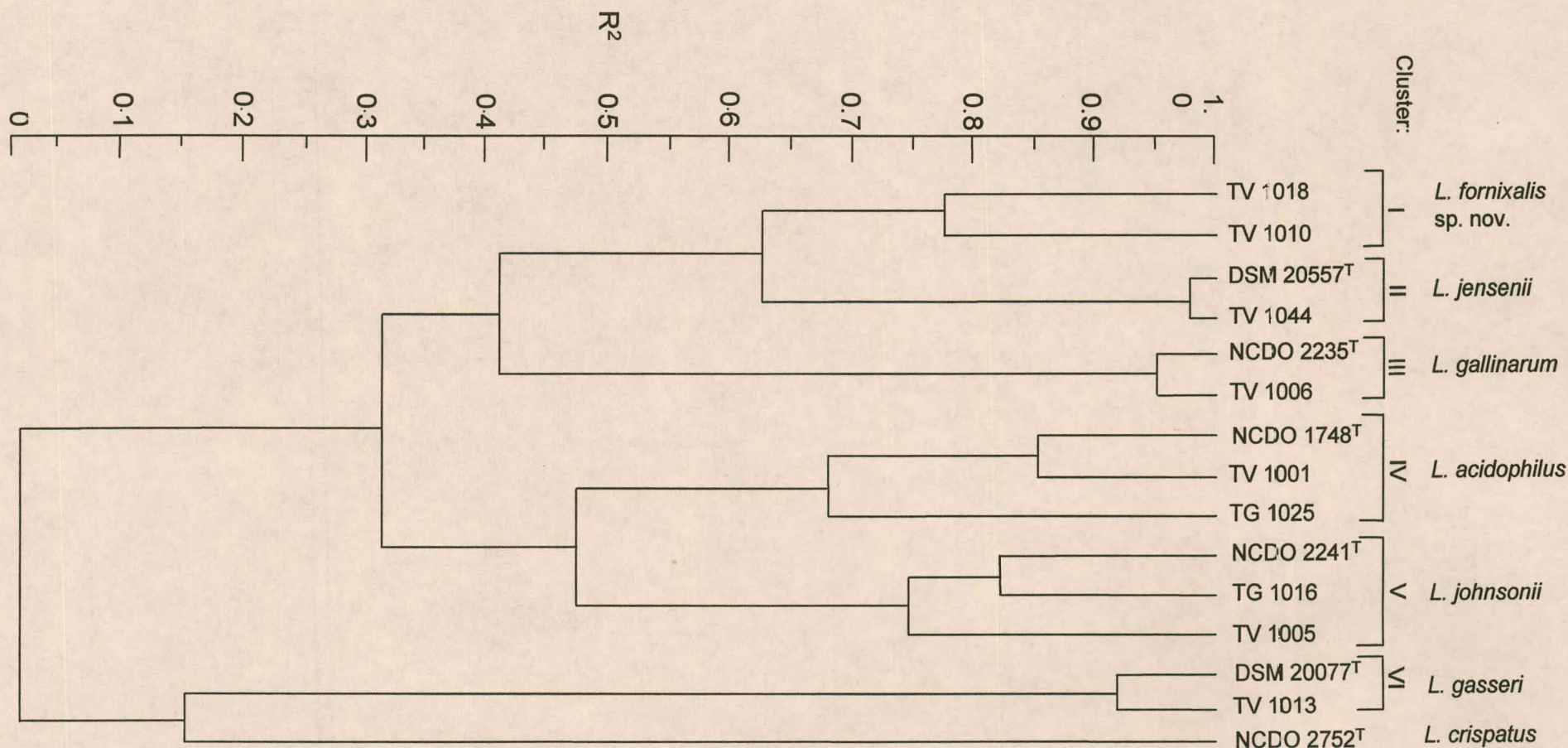
‡ Strains printed in bold were selected for RAPD-PCR analysis.

§ NC, did not cluster with the other strains.

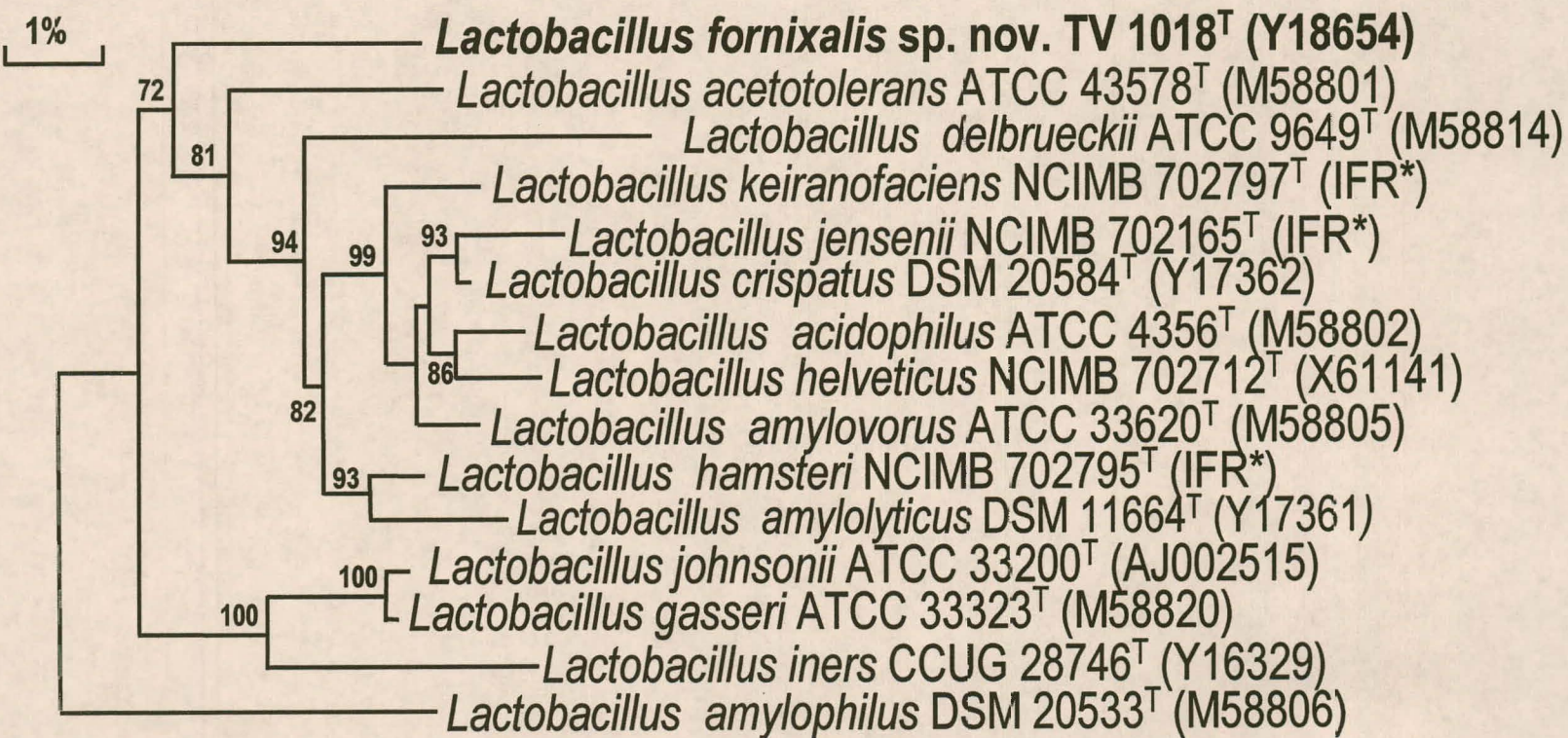




**Fig. 1.** Simplified dendrogram showing the clustering of *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. fornixalis* sp. nov. obtained by numerical analysis of total soluble cell protein patterns. Clustering was by UPGMA.



**Fig. 2.** Dendrogram showing the clustering of *L. fornixalis* sp. nov., *L. jensenii*, *L. gallinarum*, *L. acidophilus*, *L. johnsonii*, *L. gasseri* and *L. crispatus* obtained by numerical analysis of RAPD-PCR profiles. Clustering was by the normalized average linkage analysis. Distances between clusters are expressed in  $R^2$ -values.



**Fig. 3.** Unrooted tree showing the phylogenetic relationships of *L. fornixalis* sp. nov. and closely related lactic acid bacteria. The tree constructed using the neighbour-joining method was based on a comparison of approx. 1320 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points. \*Sequences from IFR data base.

**Table 2.** Differential carbohydrate fermentation reactions among strains of *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. fornixalis* sp. nov.\*

Characteristic	<i>L. johnsonii</i>	<i>L. acidophilus</i>	<i>L. gallinarum</i>	<i>L. crispatus</i>	<i>L. gasseri</i>	<i>L. jensenii</i>	<i>L. fornixalis</i> sp. nov.
Dextrin	d	-	+	d	+	ND	ND
Lactose	d	+	d	d	+	-	-
Mannitol	-	-	-	d	-	d	+
Melibiose	d	-	+	d	d	-	-
Melezitose	-	-	-	-	-	-	+
Raffinose	d	d	+	+	-	-	-
Ribose	-	-	-	d	-	-	+
Sorbitol	-	-	-	-	-	-	+
Starch	d	-	d	d	d	ND	-
Trehalose	d	+	-	d	+	+	+

\* +, 90% or more of the strains are positive; -, 90% or more of the strains are negative; d, 11 to 98% of the strains are positive; ND, not determined. Data for *L. johnsonii*, *L. acidophilus*, *L. gallinarum*, *L. crispatus* and *L. gasseri* from Fujisawa *et al.* (1992); data for *L. jensenii* from Kandler and Weiss (1986).

All strains fermented amygdalin, cellobiose, fructose, galactose, glucose, maltose, mannose, salicin and sucrose, and hydrolysed esculin.

None of the strains fermented arabinose, rhamnose and xylose.

All strains produced DL-lactic acid from glucose, except *L. jensenii* which produced the D-isomer.

## 6. GENERAL DISCUSSION AND CONCLUSIONS

Several studies have shown the presence of lactic acid bacteria in the urogenital tract of young and healthy patients. Lactobacilli, streptococci and staphylococci are the dominant microflora of the vagina and cervix. In healthy females lactobacilli dominates (Bartlett, 1977). Concentrations of  $10^7$ - $10^8$  *Lactobacillus* spp. per ml vaginal fluid have been reported (Redondo-Lopez *et al.*, 1990). Lactobacilli play a role in controlling of the vaginal microflora and maintenance of the normal state (Redondo-Lopez *et al.*, 1990). Lactobacilli produce lactic acid which maintains the pH of the vagina between 3.3 and 4.5 (Bokkenhauser, 1993). Pathogens such as *Gardnerella vaginalis*, *Bacteroides*, *Peptostreptococcus*, *Mobiluncus*, *Trichomonas vaginalis*, *Mycoplasma hominis* and *Chlamydia trachomatis* cause bacterial vaginosis, a condition where no or very few lactobacilli are present. Lactic acid bacteria protects the vagina against the overgrowth of these vaginosis-associated organisms (Skaren & Sylwan, 1986). The mechanisms of protection by lactobacilli are unknown, but possible control mechanisms are a low vaginal pH (Preti & Higgins, 1977), competition for adherence (Sobel *et al.*, 1981), production of hydrogen peroxide (Eschenbach *et al.*, 1989), broad-spectrum antimicrobial agents (Reid *et al.*, 1988) and stimulation of the immune system (Perdigon *et al.*, 1988, Bruce & Reid, 1988). The importance of the presence of lactobacilli in the genital tract of women are further demonstrated by the fact that pregnant women with a lactobacilli-predominant flora have a reduced risk of pre-term delivery (Martius *et al.*, 1988; Gravett *et al.*, 1986), amnionitis (Silver *et al.*, 1989) and postpartum endometritis (Watts *et al.*, 1990).

Rogosa and Sharpe (1960) isolated 35 vaginal *Lactobacillus* strains and studied 21 strains in detail by using sugar fermentations and other biochemical assays. The authors identified four *Lactobacillus* spp. in the vagina, namely *L. acidophilus* (14 strains), *L. casei* subsp. *rhamnosus* (currently *L. rhamnosus*, 2 strains), *L. fermentum* (4 strains) and *L. cellobiosus* (1 strain). Fagnant *et al.* (1982) developed an improved biochemical scheme for the identification of endocervical lactobacilli. Of the 106 strains isolated, 74% were identified, 18 strains were identified by a "best-fit" method to determine the most likely identification and for the remaining 10 strains the species identified were not previously isolated. *L. jensenii* (26 strains), *L. acidophilus* (22 strains), *L. leichmanii* (22 strains), *L. cellobiosus* (11 strains), *L. casei* subsp. *rhamnosus* (10 strains), *L. delbrueckii* (five strains), *L. brevis* (four strains), *L. fermentum* (three strains) and *L. salivarius* (three strains) were identified. *L. acidophilus* and

*L. fermentum* appeared to be the dominant species in the vagina according to classical taxonomic studies.

The conventional taxonomic techniques described left doubts as to correct classification of isolates. Giorgi *et al.* (1987) went a step further and did DNA-DNA hybridization of 19 vaginal lactobacilli strains. They identified one strain of *L. fermentum*, 1 strain of *L. crispatus*, two strains of *L. jensenii* and five strains of *L. gasseri*. Seven heterofermentative strains (which appeared to be two species according to the DNA reassociation statistics) and three homofermentative strains remained unidentified. The genetic heterogeneity of the *L. acidophilus*-group may explain why previous studies, using phenotypic tests, classified many homofermentative lactobacilli as *L. acidophilus*. From these studies it can be concluded that *L. gasseri*, *L. jensenii* and *L. crispatus* may be the dominant vaginal lactobacilli in healthy women.

Of the 100 strains isolated in this study, 36% were enterococci and 64% lactobacilli. Three strains remained unidentified. Of the known lactobacilli, *L. plantarum* was the most frequently isolated (19.7%), followed by *L. fermentum* (16.4%), *L. crispatus* (14.8%) and *L. jensenii* (13.1%). Strains of *L. fermentans* are currently regarded as *L. fermentum* and thus have the highest frequency after *L. plantarum*. The strains of *L. viridescens* are regarded as part of the genus *Weisella* and therefore there are actually only 11 strains of lactobacilli. Most lactobacilli isolated are homofermentative (47.5%) followed by facultative heterofermentative lactobacilli (29.5%) and obligate heterofermentative lactobacilli were the least isolated (23.0%).

Protein and DNA fingerprinting were used as taxonomic techniques. The results indicated that *L. plantarum* is the dominant *Lactobacillus* sp. in the vagina, followed by *L. fermentum*, *L. crispatus* and *L. jensenii* respectively. Two species namely *L. viridescens* (= *W. viridescens*) and *L. buchneri* were never before associated with the human vagina. The results obtained in this study corresponded to the results of a study done to determine the normal *Lactobacillus* flora of healthy human rectal and oral mucosa (Ahrne *et al.*, 1998). Previous studies using classical taxonomy referred to *L. acidophilus* and *L. casei* as the dominant lactobacilli of the human intestinal tract (Fingold *et al.*, 1983). DNA-DNA hybridization indicated that *L. plantarum* is the dominant species (52% of the cases), followed by *L. rhamnosus* and *L. paracasei* (26% and 17% respectively). The researchers

suggested that *L. plantarum* is a major colonizer of the human gastrointestinal tract and that its ability to adhere may be of some ecological importance.

Enterococci isolated from the vagina were classified as either *E. faecalis* or *E. faecium*. A number of strains did not cluster closely with either *E. faecalis* or *E. faecium*, which may indicate a new species within the genus *Enterococcus*. This merits further research. The high frequency of enterococci isolated in this study is particularly interesting. Enterococci are especially dominant in the urogenital tract of older women. Not much attention was given in the past to other lactic acid bacteria (with the exception of lactobacilli) of the female genital tract. It is not clear from the literature whether *Enterococcus* can be pathogenic in the urogenital tract and the role of *Enterococcus* in the female genital tract is unknown and totally ignored.

Confirmation for the identification by SDS-PAGE were given by RAPD-PCR and very reliable 16S rRNA sequencing, especially where relationship between the clinical isolate and the reference strain does not seem near enough or do not cluster closely together. An excellent example of this is the clinical strain TV 1005 and the corresponding reference strain *L. acidophilus*. SDS-PAGE gives accurate enough identification up to species level, but it is important to prove these results with additional numerical techniques like RAPD-PCR or 16S rRNA studies, especially where species are closely related as in the case of the *acidophilus*-group (*L. acidophilus*, *L. crispatus*, *L. johnsonii*, *L. gasseri*, *L. gallinarum* and *L. amylovorus*) (Johnson *et al.*, 1980) or *L. plantarum* and *L. pentosus* (Van Reenen & Dicks, 1996).

The microbial ecology of the female genital tract are certainly more complex than previously thought, especially since more sophisticated taxonomic techniques make identification more reliable. Since this study included a variety of women (symptomatic, asymptomatic, pregnant, non-pregnant, adolescents, premenopausal, postmenopausal) a wider perspective was gained into the microbial ecosystem of the female genital tract. Ten species of lactobacilli, one species of *Weisella* and two species of *Enterococcus* were isolated. This study indicated that *L. plantarum* may be one of the predominant species of the vaginal microbial flora. *W. viridescens* and *L. buchneri* were never associated with the vaginal region in the past. More studies into the vaginal microbial ecosystem are necessary, since this knowledge is important in the treatment of bacterial vaginosis and other vaginal infections, use of contraceptives and HIV infection.

A new species in the genus *Lactobacillus*, *Lactobacillus fornixalis*, was identified during this study. Protein fingerprinting suggested close relatedness to *L. gasseri* and *L. jensenii*. However, the results of 16S rRNA sequencing indicated separate species status for this strain.

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