A TAXONOMIC STUDY OF HIGH pH TOLERANT LACTIC ACID BACTERIA ASSOCIATED WITH VACUUM-PACKAGED MEAT PRODUCTS

by

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Submitted in fulfilment of the requirements for the degree
of Master of Science
M.Sc. (Microbiology)
in the faculty of Science,
Department of Microbiology and Plant Pathology,
at the University of Pretoria, Pretoria,
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December 1988

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I declare that this thesis hereby submitted to the University of Pretoria in fulfilment of the requirements for the M.Sc.(Microbiology) degree has not been submitted for a degree to any other university.

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ABSTRACT

A TAXONOMIC STUDY OF HIGH pH TOLERANT LACTIC ACID BACTERIA ASSOCIATED WITH VACUUM-PACKAGED MEAT PRODUCTS

by

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DEPARTMENT : Microbiology and Plant Pathology
DEGREE FOR WHICH THESIS IS SUBMITTED : M.Sc. (Microbiology)

December 1988

In the first part of this study the relationship between Lactobacillus divergens, Lactobacillus carnis and Lactobacillus piscicola and their relationship with other Lactobacillus species were investigated using numerical analysis of polyacrylamide gel electrophoresis (PAGE) patterns of their proteins and their physiological, morphological and biochemical characteristics. PAGE analysis of whole cell proteins indicated that L. divergens and L. carnis are separate species and that L. piscicola and L. carnis are the same species. Numerical analysis (Ssm) of the physiological, morphological and biochemical data could not distinguish between these organisms and Ssm did not have the same level of resolution as numerical analysis of PAGE data.

In the second part of this study DETA-MRS was used as a selective medium to investigate the bacterial population able to grow at pH 8,5 in vacuum-packaged meat and meat products. Some Lactobacillus sake and Leuconostoc mesenteroides subsp. mesenteroides strains were isolated. Two Leuconostoc and two lactic acid bacterial cultures were isolated that did not fit in with descriptions of any of the presently described species. DETA-MRS was also found not to be selective for L. divergens and L. carnis in vacuum-packaged meat products.

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SAMEVATTING

'N TAKSONOMIESE STUDIE VAN HOË-pH-TOLERANTE MELKSUURBAKTERIEË
GEASSOSIEER MET VAKUUMVERPAKTE VLEISPRODUKTE

deur

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In die eerste gedeelte van hierdie studie is die verwantskap tussen *Lactobacillus divergens*, *Lactobacillus carnis* en *Lactobacillus piscicola* en hulle verwantskap met ander *Lactobacillus*-spesies bepaal deur gebruik te maak van numeriese analise van poliakrielamied jel elektroferogramme (PAGE) van hulle proteïene en hulle fisiologiese, morfologiese en biochemiese eienskappe. Volgens PAGE van die heelsel-proteïene is bevind dat *L. divergens* en *L. carnis* aparte spesies is, maar dat *L. carnis* en *L. piscicola* dieselfde spesie is. Deur numeriese analise (Ssm) van die fisiologiese, morfologiese en biochemiese data kon nie onderskei word tussen hierdie organismes nie en Ssm het dus nie dieselfde resolusie as numeriese analise van PAGE data nie.

In die tweede gedeelte van hierdie studie is DETA-MRS gebruik as 'n selektiewe medium om die bakteriese populasie, wat by 'n pH van 8,5 kan groei, in vakuumverpakte vleis en vleisprodukte te ondersoek. Isolate van *Lactobacillus sake* en *Leuconostoc mesenteroides* subsp. *mesenteroides* is gevind. Twee *Leuconostoc*-species en twee melksuurbakterieë is geïsoleer wat geen ooreenkoms getoon het met bestaande spesiebeskrywings nie. DETA-MRS was ook nie selektief vir *L. divergens* en *L. carnis* in vakuumverpakte vleisprodukte nie.

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---oooOooo---

Aan God al die eer!

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CHAPTER 1

INTRODUCTION

Studies on vacuum-packaged fresh meat and meat products as a means of extending the shelf life, led to reports of lactobacilli domination in this environment (Gardner, 1968; Sharpe, 1982; Gerber, 1984). Although most of the strains reported corresponded to known species, a few atypical lactobacilli were reported (Cavett, 1963; Gardner, 1968; Mol, Hietbrink, Mollen & Van Tinteren, 1971; Hitchener, Egan & Rogers, 1982).

The first of these atypical lactobacilli to be described was Lactobacillus divergens (Holzapfel & Gerber, 1983), followed by the description of Lactobacillus carnis (Shaw & Harding, 1985). These two species do not differ very much from each other, but the biggest difference between them and other lactobacilli is their ability to grow at pH 8,5 whereas other lactobacilli grow in a neutral to acid medium. Hui, Holt, Sriranganathan, Seidler & Fryer (1984) described Lactobacillus piscicola, the cause of lactobacillosis in cutthroat trout. Although the habitat of this bacterium differed very much from that of L. divergens and L. carnis, the characteristics of the three species seem to be very similar. On the grounds of biochemical and chemical studies Collins, Farrow, Philips, Ferusu & Jones (1987) proposed that L. divergens and L. piscicola be reclassified in the genus Carnobacterium gen. nov.. Shaw & Harding (1985) using DNA-DNA hybridization techniques, were able to discern between L. divergens and L. carnis. However only a few biochemical tests can be used to distinguish between L. carnis and L. divergens, with some strains giving intermediate reactions.

The questions arose whether the two species are validly separable, and whether L. piscicola, even though from a different habitat, is not a member of any of the two abovementioned species. The null-hypothesis of this investigation therefore was formulated as follows: There is no sufficient reason for distinguishing between the three spp. L. carnis, L. divergens and L. piscicola.

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In this study the relationship between *L. carnis*, *L. divergens* and *L. piscicola* and their relationship with other *Lactobacillus* species and lactic acid bacteria were investigated using numerical analyses of PAGE patterns of their proteins and their morphological, physiological and biochemical data.

DETA-MRS, described as a selective medium for *L. divergens* and *L. carnis* by De Bruyn (1987) was only tested on reference strains and never applied to the investigation of the occurrence of these species in vacuum-packaged meat and meat products. In this study DETA-MRS was used to investigate the bacterial population, able to grow at pH 8,5, in vacuum packaged meat and meat products in order to determine its selectivity and usefulness in ecological studies.

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CHAPTER 2

LITERATURE SURVEY

2.1 INTRODUCTION

The first objective of this study was to establish the taxonomic position of *Lactobacillus divergens*, *Lactobacillus carnis* and *Lactobacillus piscicola*. Therefore the taxonomy of the lactic acid bacteria is given with special reference to these organisms' resistance to and ability to grow at a high pH.

Polyacrylamide gel electrophoresis (PAGE) and numerical analysis of morphological, physiological and biochemical properties were used in this study. These and other methods used in the identification and classification of the lactobacilli will be discussed briefly.

In the second part of the study, DETA-MRS described by De Bruyn (1987), was used in a study of the microbial ecology of vacuum-packaged meat and meat products. A review of the lactic acid bacteria associated with vacuum-packaged meat and meat products is given with special reference to atypical bacteria and those tolerant to high pH.

2.2 TAXONOMY OF THE LACTIC ACID BACTERIA

2.2.1 PRESENT STATUS OF THE LACTIC ACID BACTERIA

At present the lactic acid bacteria are represented in two different sections in Bergey's manual of systematic bacteriology Vol 2 (Sneath, Mair, Sharpe & Holt, 1986). The cocci are represented in Section 12: Gram-positive cocci, and the rods in Section 14: Regular nonsporing Gram-positive rods. Their taxonomic relationships, with other bacteria and among themselves, are uncertain and they are listed as separate genera and not part of an hierarchical taxonomic classification. On the basis of 16S rRNA oligonucleotide catalogue analysis the species of the

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genera Lactobacillus, Leuconostoc, Pediococcus and Streptococcus form a supercluster within the so-called clostridia sub-branch of the Gram-positive bacteria (Stackebrandt, Fowler & Woese, 1983).

The present taxonomic system for the lactic acid bacteria does not reflect these findings. Therefore the taxonomy of these bacteria should be revised to incorporate these phylogenetic relationships and give a better taxonomic system.

2.2.2 SECTION 12: GRAM-POSITIVE COCCI

This section consists of 15 different genera which are phylogenically and phenotypically quite diverse. On the basis of the presence or absence of catalase and cytochromes they are separated into two groups. All lactic acid bacteria are part of the catalase-negative group (Schleifer, 1986).

2.2.2.1 Streptococcus Rosenbach 1884

The streptococci are divided into six groups, the pyogenic haemolytic streptococci, the oral streptococci, the enterococci, the lactic acid streptococci, the anaerobic streptococci and the rest of the streptococci (Hardie, 1986).

Further studies led to the proposal to divide the genus Streptococcus into three genera as follows: the genus Streptococcus, encompassing the majority of known species, in particular the pyogenic and oral streptococci and the pneumococci (Hardie, 1986); the genus Enterococcus encompassing the enterococcal group (Schleifer & Kilpper-Bälz, 1984; Collins, Jones, Farrow, Kilpper-Bälz & Schleifer, 1984), and the genus Lactococcus to which all group N streptococci are transferred (Schleifer, Kraus, Dvorak, Kilpper-Bälz, Collins & Fischer, 1985).

Of all the streptococci only the enterococci are able to grow at pH 9,6. The ability of the enterococci to initiate growth in a broth at pH 9,6 is commonly used to segregate the enterococci from the rest of the

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streptococci. Streptococcus avium (Enterococcus avium) can grow at pH 10 with sorbose as energy source (Mundt, 1986). In 1979 Latham, Sharpe & Weiss reported that some Streptococcus bovis strains are able to grow at pH 9,6.

2.2.2.2 Leuconostoc van Tieghem 1878

Four species are described at present. The significance of the physiological similarities between Leuconostoc spp. and some heterofermentative lactobacilli require reassessing. The non-acidophilic leuconostocs appear to have more in common with Lactobacillus confusus and Lactobacillus viridescens than with Leuconostoc oenos (Garvie, 1986a). Stackebrandt et al. (1983) suggested that the description of the genus Lactobacillus should be expanded so as to accommodate the genera Leuconostoc and Pediococcus.

The Leuconostoc species are separated into non-acidophilic species that cannot grow at pH 4,8, but grow at pH 6,5 and Leuconostoc oenos which grows best in acid media (pH 4,2 - 4,8). None of the non-acidophilic leuconostocs was reported to grow at high pH (Garvie, 1986a).

2.2.3 Pediococcus Claussen 1903

Pediococci are unlikely to be confused with other lactic acid bacteria because they are morphologically distinct. They are undoubtedly lactic acid bacteria despite their cellular division along two planes. The separation of pediococci from aerococci requires further clarification (Garvie, 1986b).

Pediococcus damnosus and Pediococcus parvulus are classified as acid tolerant, but Pediococcus urinaeequi requires an initial pH of 7 - 8 for growth. The rest of the pediococci can grow at a neutral pH (Garvie, 1986b).

2.2.2.4 Aerococcus Williams Hirch and Cowan 1953

Aerococcus is a monospecific genus. This genus is differentiated from

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the other genera by testing negative for a wide range of characteristics that are positive for other genera. One of the positive characteristics is the formation of tetrads. This explains why they are sometimes confused with Pediococcus. Like the enterococci, Aerococcus viridans can initiate growth at pH 9,6 (Evans, 1986).

2.2.3 SECTION 14: REGULAR, NONSPORING GRAM-POSITIVE RODS

This section comprises a conglomerate of seven different genera which have only a few morphological and physiological characteristics in common. The largest genus is the genus Lactobacillus, which comprises the lactic acid bacteria in this section (Kandler & Weiss, 1986).

2.2.3.1 Lactobacillus Beijerinck 1901

The lactobacilli are arranged into the traditional three groups resembling Orla-Jensen's three genera without designating them as formal subgeneric taxa since they do not represent phylogenetically defined clusters. The new definitions do not include growth temperature and morphology, the classical characteristics of Orla-Jensen's subgenera (Kandler & Weiss, 1986).

Group One is the homofermentative lactobacilli and harbours the old subgenus Thermobacterium. Group Two is the facultatively heterofermentative lactobacilli and contains the old subgenus Streptobacterium. Group Three is the obligately heterofermentative lactobacilli and contains the old subgenus Betabacterium (Kandler & Weiss, 1986).

The lactobacilli are described as aciduric and grow best in slightly acid media with an initial pH of 4,5 to 6,4. Growth ceases when pH 4,0 to 3,6 is reached, depending on the species or strains (Kandler & Weiss, 1986).

In 1983 Holzapfel and Gerber described Lactobacillus divergens, a lactobacillus able to grow at a pH 8,5. This was the first lactobacillus recorded to grow at such a high pH. Hui et al. (1984) described Lactobacillus piscicola as being able to grow at pH 6 to 7, but it was later

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found to grow at pH 8,5 (Shaw, pers. comm.). In 1985 Shaw and Harding described Lactobacillus carnis, also able to grow at pH 8,5. The descriptions of these three species are given below.

2.2.3.1.1 Lactobacillus divergens Holzapfel and Gerber 1983

Lactobacillus divergens cells are Gram-positive, straight, slender and relatively short rods with rounded ends. They can occur singly, in pairs or in short chains. The species is nonmotile and does not form endospores. The colonies are cream-coloured to white, convex and shining (Holzapfel & Gerber, 1983).

Holzapfel and Gerber (1983) described L. divergens as a heterofermentative Lactobacillus species, producing L(+) lactic acid, CO_2, ethanol and acetate from hexoses. De Bruyn, Holzapfel, Visser & Louw (1988) found that it used mainly the Embden-Meyerhof-Parnas pathway for homolactic fermentation and suggested that it should be reclassified as a facultative heterofermentative organism. In addition to L(+) lactic acid L. divergens forms acetate as well as ethanol from ribose. No production of slime from sucrose or mannitol is observed. The organism does not produce gas from gluconate or malate (Holzapfel & Gerber, 1983).

The organism is catalase and benzidine negative, but can produce pseudocatalase on haeme-containing media. Nitrate is not reduced to nitrite and gelatinase, indole and H_2S are not produced. L. divergens grows at 10 but not at 45°C, nor at pH 3,9 or in 10% NaCl. It has a peptidoglycan of the m-Dpm-direct (diaminopimelic acid) type and the G+C content of the DNA is 34 mol%. The organism was first isolated from vacuum-packaged refrigerated meat (Von Holy, 1983). The type strain is DSM 20623 (Holzapfel & Gerber, 1983).

2.2.3.1.2 Lactobacillus piscicola Hui, Holt, Sriranganathan, Seidler and Fryer 1984

Lactobacillus piscicola is a Gram-positive rod which occurs singly or in pairs. It is nonmotile and does not form endospores. The colonies are usually white, convex, circular and not pigmented. The organism is

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facultatively anaerobic. It produces DL lactic acid homofermentatively. No gas is produced from glucose or gluconate. Acid is produced from glycerol, ribose, galactose, glucose, fructose, mannose, mannitol, amygdalin, arbutin, salicin, cellobiose, sucrose and trehalose. The organism does not form catalase or oxidase. It does not reduce nitrate or form H_2S. L. piscicola grows at a temperature range of 6 to 40°C, with an optimum temperature of approximately 30°C. The optimum pH range for growth is between pH 6,0 and 7,0. The cell wall peptidoglycan contains Dpm and the G+C content of the DNA is 34-36 mol%. The organism was first isolated from a diseased adult cutthroat trout. The organism causes lactobacillosis of fish. The type strain is ATCC 35586 (Hui et al., 1984).

2.2.3.1.3 Lactobacillus carnis Shaw and Harding 1985

Lactobacillus carnis cells are Gram-positive, straight slender rods usually occurring singly or in pairs but sometimes in short chains. It is non-sporeforming and nonmotile. The colonies are white, convex and shiny. It is a heterofermentative organism, producing L(+) lactic acid, ethanol and acetate from glucose. Gas production is weak and frequently not detectable. It does not produce slime from sucrose. It ferments amygdalin, cellobiose, glucose, glycerol, maltose, mannitol, ribose, salicin, sucrose and trehalose (Shaw & Harding, 1985).

L. carnis is catalase negative and nitrate is not reduced to nitrite. It produces a strong reducing reaction on tetrazolium agar. The organism grows at 4 and 25 but not at 45°C. No growth occurs at pH 3,9 or in 8% NaCl. The peptidoglycan is of the m-Dpm-direct type. The G+C content of the DNA is 34 - 36 mol%. It was first isolated from refrigerated vacuum-packaged meat. The type strain is NCDO 2764 (Shaw & Harding, 1985).

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2.3 METHODS FOR THE IDENTIFICATION OF LACTOBACILLUS SPECIES

2.3.1 CONVENTIONAL TAXONOMY

Historically, taxonomic investigation of the genus Lactobacillus have been limited to the phenotypic characteristics of these bacteria. Orla-Jensen, pioneer of lactic acid bacterial classification, deviced a classification system for these bacteria by using properties such as morphology, sugar fermentations, optimal growth temperatures, fermentation end products and optical isomers of the lactic acid produced. Briggs (1953) classified the lactobacilli on the basis of the results of six physiological tests, namely the production of gas from glucose and citrate, production of ammonia from arginine, growth at 15, 45 and 48°C, heat survival at 60 and 65°C and the tolerance of 4, 6 and 8% sodium chloride.

In 1962 Sharpe reviewed the taxonomy of the lactobacilli. Classification of the genus Lactobacillus was still based on morphological, physiological and biochemical tests, with growth temperature, the production of gas from glucose and the production of ammonia from arginine, as key characteristics. Serological reactions, chromatography of cell contents and the cell wall composition were mentioned but not used for identification purposes.

The API system for identification of lactobacilli is based on the ability of these bacteria to ferment fifty different substrates (Dolezil & Kirsop, 1977).

At present the lactobacilli are still differentiated using most of the above mentioned characteristics (Kandler & Weiss, 1986). Other methods are used to supply meaningful and reliable information to clarify the status of the groups and species within the genus. A problem with this system is that by using only a few characteristics the system can be subjective, ignoring some important characteristics.

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2.3.2 NUMERICAL TAXONOMY

The application of computers to process taxonomic data, using arithmetical methods, in the classification of organisms, has been one of the major advances in the field of classification. Numerical taxonomy was first applied to bacteria by Sneath in 1957.

The principles on which this method is based are that the more characters on which a study is based, the better a given classification will be; every character is of equal weight in creating a taxon, and the overall similarity between any two entities is a function of their individual similarities in each of the many characters in which they are compared. This correlation is used to create taxons (Sneath & Sokal, 1973). One of the biggest problems with this method is choosing the right similarity coefficient for a set of data because it can influence the outcome of the study. Two coefficients are mainly used, the simple matching coefficient and the Jaccard coefficient. The simple matching coefficient includes both positive and negative matches, the Jaccard coefficient does not include negative correlations (Austin & Colwell, 1977). Since 1957 this method had been used regularly to classify lactobacilli because it helped to arrange the bacteria in an objective manner (Seyfried, 1968; Shaw & Harding, 1984; Hastings & Holzapfel, 1987).

2.3.3 CELL WALL COMPOSITION

In 1956 Cummins & Harris published a paper on the possible value of cell wall composition of Gram-positive bacteria as a means of identifying these bacteria. The composition of the peptidoglycan seem to be constant among the Gram-negative bacteria (Kandler, 1970). Schleifer & Kandler (1972) suggested a classification system on the known peptidoglycan types based on their mode of cross-linkage. The amino acid composition of bacteria may be identical, but the amino acid sequence or the mode of cross linkage may be different. The amino acid sequence of the peptidoglycan can be determined by using an enzymatic or chemical method. They determined the amino acid composition of a pure cell wall preparation quantitatively with an amino acid analyzer, the N- and C-terminal amino acids by dinitrophenylation and the identification and

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separation of the amino acids and peptides by two-dimensional descending paper chromatography. Schleifer & Kandler (1972) divided the genus *Lactobacillus* into four subgenera according to their cell wall composition. However, this method is time consuming and is never used as the only method when bacteria are classified.

2.3.4 SEROLOGICAL METHODS

In 1955 Sharpe, using acid extracts and specific antisera, classified the lactobacilli into six serological groups, A to F. In 1959 Rogosa & Sharpe defined a further serological group G. The serological groupings were done by precipitin tests. In 1970 Sharpe described the location and chemical nature of the group antigens, some being cell walls, others cell membrane components and others polysaccharides. This method is not as regularly used for lactobacilli identification as for *Streptococcus* identification.

2.3.5 ASSAY OF KEY ENZYMES AND ENZYME ELECTROPHORESIS

Two major sugar fermentation pathways are followed by the lactobacilli. The homofermentative and facultative heterofermentative lactobacilli ferment sugars using the glycolitic pathway. The heterofermentative lactobacilli use this pathway as well as the hexose monophosphate pathway. The homofermentative and facultative heterofermentative lactobacilli contain fructose-1,6-diphosphate aldolase as their key enzyme. The facultative heterofermentative lactobacilli also contain glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The heterofermentative lactobacilli contain both these enzymes but no aldolase (Gasser, 1970). The presence of these enzymes are used to divide the lactobacilli into three groups of the genus *Lactobacillus*. This method cannot distinguish between species and is not widely used. The mobility of the glucose-6-phosphate dehydrogenase is used to distinguish between closely related species (Williams & Sadler, 1971). This method can be used as a preliminary screening method to ensure that appropriate strains are chosen for nucleic acid homology investigations.

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2.3.6 PYROLYSIS MASS SPECTROMETRY

Pyrolysis is the breakdown of the bacteria in an inert atmosphere to produce a series of volatile substances using heat alone. These fragments can be detected and quantified using mass spectrometry. Some researchers use only gas chromatography, but when combined with mass spectrometry, it yields more reproducible results faster, and can be more easily automated (Shute, Berkley, Norris & Gutteridge, 1985). Maezelaar & Kistemaker (1973) developed a pyrolysis gas spectrometer specifically for the "fingerprinting" of bacteria. The "fingerprints" of an organism are compared with spectra held in a data base, using computerized procedures. The data are used to study inter- and intra-group relationships. The disadvantages of this method are that it is a novel and complex method with the need for both microbiological and analytical chemistry expertise. The apparatus used is also very expensive (Shute et al., 1985). Not much work has been done on the lactobacilli using this technique because it is relatively new. However, Shaw, Puckey, MacFie & Bolt (1985) used it to classify lactic acid bacteria isolated from vacuum-packaged meat.

2.3.7 ELECTROPHORESIS OF PROTEINS

This method is based on the assumption that a bacterial strain growing under standardized conditions always produce the same proteins. These proteins can be separated because the proteins can differ in amino acid sequence, molecular weight and nett electrical charge. Electrophoresis of the proteins in an electrical field results in protein banding patterns which are typical for each bacterial species (Kersters & de Ley, 1980). Kersters & de Ley (1975) found that the patterns of genetically related bacterial strains are similar or nearly identical. These patterns can be compared by computing correlation coefficients from the normalized spectrophotometric tracings. These correlation coefficients are used in a cluster analysis to determine their interrelationships. Protein electrophoresis is relatively simple and inexpensive so that it can easily be used to study a large number of bacteria. The method is faster than DNA:DNA-hybridization but slower than pyrolysis GC-mass spectrometry. This method cannot be used in clinical bacteriology where

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an identification is needed within a few hours (Kersters & de Ley, 1980). Polyacrylamide gel electrophoresis (PAGE) has not been applied to the genus Lactobacillus as a whole, but only to certain selected groups (Dicks & van Vuuren, 1987).

2.3.8 DNA BASE COMPOSITION AND DNA:DNA HYBRIDIZATION

The more recently developed methods for the identification of bacteria are directed more towards the genetic composition of microorganisms. The two important methods based on DNA are base composition and reassociation.

Base composition can be determined directly by electrophoresis or indirectly by calculating the mole percent guanine and cytosine (%G+C) from the hyperchromic shift during thermal denaturation of DNA, the buoyant density in cesium chloride or the absorption differences at two wavelengths when purines are released during acid depurination of the DNA (Bradley, 1980). The method has limited utility in bacterial classification. It can only be used to exclude an organism from a taxon but not to include it, because the %G+C value of two biologically unrelated organisms may be the same, whereas the value for two related organisms cannot be markedly different (Owen & Pitcher, 1985).

In DNA reassociation, the entire genome of a bacterial strain is compared with that of another. The DNA can match exactly, partially or well in a certain region but other regions are unmatched. The degree of matching can be determined by measuring the stability of the reassociated DNA. It can also be monitored by radioactive labelling or direct visualization with the electron microscope (Bradley, 1980). It is doubtful that the method will be used for the identification of unknown strains, but it is used best in conjunction with other methods and can reflect the evolutionary development of the species tested. This method has been widely used in the classification of the entire genus Lactobacillus and for groups of different lactobacilli (Gasser & Mandel, 1965; Miller, Sandine & Elliker, 1971; Simonds, Hansen & Lakshamana, 1971; Dellagio, Bottatzi & Trovatelli, 1973; Dellagio, Bottatzi & Vescovo, 1975).

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2.3.9 CATALOGUING OF 16S rRNA

The 16S rRNA molecule has turned out to be the most suitable for phylogenetic taxonomic studies (Fowler, Ludwig & Stackebrandt, 1985). The ribosome is of very ancient origin and at least two of its RNA components, the 5S and 16S rRNA, have functionally equivalent forms over a wide range of the procaryotes. They contain some regions that are conserved and some that can vary, so that distant and close relationships can be examined. The 16S rRNA is used because it provides more information than the smaller 5S rRNA and is easier to experiment with than the larger 23S rRNA. This method is expensive and time consuming and requires considerable specialized expertise, and thus cannot replace the other more conventional taxonomic methods (Fox, Pechman & Woese, 1977). Strains must be carefully selected to avoid phylogenic conclusions based on data from atypical strains (Fowler et al., 1985). This method was used in a phylogenic analysis of Lactobacillus, Pediococcus and Leuconostoc species (Stackebrandt et al., 1983). They found that Pediococcus and Leuconostoc clustered with some of the lactobacilli and that the groupings that were formed did not correspond entirely to the classical division of Lactobacillus into the three groups still given in Bergey's Manual of Systematic Bacteriology Volume 2 (Kandler & Weiss, 1986).

2.4 LACTIC ACID BACTERIA ASSOCIATED WITH VACUUM-PACKAGED MEAT PRODUCTS

Vacuum-packaging is a method that is increasingly used to extend the shelf life of meat and meat products. It creates a favourable environment for lactic acid bacteria because the lower redox potential encourages their growth and supresses that of strictly aerobic organisms, particularly at refrigeration temperatures (Sharpe, 1982).

The lactic acid bacteria associated with vacuum-packaged meat products have been studied by a number of researchers. Most of the strains isolated corresponded to known species and the lactobacilli were the dominant group (Allen & Foster, 1960; Cavett, 1963; Gardner, 1968; Mol et al., 1971; Hitchener et al., 1982; Egan, 1983; Gerber, 1984; Shaw & Harding, 1984; Shaw et al., 1985). The incidence of Leuconostoc and

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Pediococcus strains were also reported (Cavett, 1963; Hitchener et al., 1982; Egan, 1983).

Heterofermentative lactobacilli were mainly assigned to the species Lactobacillus cellobiosus, L. viridescens, L. brevis, L. fermentum and L. buchneri. The facultative heterofermentatives included L. plantarum, L. sake and L. curvatus (Egan, 1983; Holzapfel & Gerber, 1983; Shaw et al., 1985). Hitchener et al. (1982) identified the Leuconostoc strains as Leuconostoc mesenteriodes.

Problems were experienced in the identification of some of the lactobacilli. They were referred to as atypical lactobacilli (Hitchener et al., 1982; Shaw & Harding, 1985). Most were described as atypical streptobacteria (facultative heterofermentatives) (Cavett, 1963; Gardner, 1968; Mol et al., 1971).

In 1983 Holzapfel & Gerber described L. divergens, a new heterofermentative lactobacillus isolated from vacuum-packaged minced beef (Von Holy, 1983). De Bruyn (1987) suggested that it should be reclassified as a facultative heterofermenter. The description of some of the atypical lactobacilli by Mol et al. (1971) is very similar to that of L. divergens and they probably could be the same or a closely related species.

In 1985 Shaw & Harding described L. carnis and presented evidence that it is closely related to L. divergens. Both of these bacteria are able to grow at pH 8,5, a characteristic never before described for any lactobacillus. De Bruyn (1987) made use of this property when a selective medium was devised for studying the ecology of these bacteria.

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CHAPTER 3

RELATIONSHIP BETWEEN *LACTOBACILLUS DIVERGENS*, *L. CARNIS* AND *L. PISCICOLA* AND THEIR RELATIONSHIP WITH OTHER LACTIC ACID BACTERIA

3.1 INTRODUCTION

The predominance of lactobacilli in the microbial population of vacuum-packaged meat and meat products has been frequently reported (Reuter, 1970; Sutherland, Patterson & Murray, 1975; Egan & Shay, 1982). Most of the species isolated corresponded well with known *Lactobacillus* species (Reuter, 1970), but various workers reported the isolation of atypical betabacteria and streptobacteria (Cavett, 1963; Gardner, 1968; Reuter, 1970; Mol *et al*., 1971; Hitchener *et al*., 1982; Holzapfel & Gerber, 1983; Shaw & Harding, 1985).

Holzapfel & Gerber (1983) were the first to describe an atypical beta-bacterium, isolated by Von Holy (1983) as *Lactobacillus divergens*. De Bruyn *et al*. (1988) studied the metabolism of *L. divergens* and proposed that it should be classified as a facultative heterofermentative lacto-bacillus and not as a obligately heterofermentative lactobacillus.

In 1985 Shaw & Harding studied 20 strains of atypical lactobacilli isolated from vacuum-packaged meats in the United Kingdom, South Africa and Australia. Deoxyribonucleic acid hybridization revealed that these bacteria represented two closely related species, the one species *L. divergens* and the other *Lactobacillus carnis spec. nov*. These two species can only be distinguished from each other by the ability of *L. carnis* to ferment mannitol, the only invariable distinguishing charac-teristic among the isolates investigated.

Hui *et al*. (1984) described *Lactobacillus piscicola*, isolated from diseased cutthroat trout. Although the habitat differed markedly from that of *L. divergens* and *L. carnis*, certain characteristics of this species closely resembled those of the previous two *Lactobacillus* spe-cies.

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The aim of this part of the study was to clarify the taxonomic position of *L. divergens*, *L. carnis* and *L. piscicola* by means of polyacrylamide gel electrophoresis (PAGE) of their whole cell proteins. These results were compared with a numerical taxonomic analysis of their morphological, physiological and biochemical characteristics. Certain reference strains were included to give an indication of their relationship to either *Lactobacillus* or *Enterococcus* species.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIA

3.2.1.1 BACTERIAL STRAINS

The *Lactobacillus* and *Enterococcus* strains included in this study are listed in Table 1.

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TABLE 1 List of strains and their origin

Species	Strain number	Origin (a)
L. divergens	20623	DSM
L. divergens	60	Holzapfel and Gerber, 1983
L. carnis	83	Holzapfel and Gerber, 1983
L. carnis	2764	NCDO
L. divergens	LV6	Shaw & Harding, 1985
L. divergens	LV60	Shaw & Harding, 1985
L. carnis	LV14	Shaw & Harding, 1985
L. divergens	M2	Hitchener *et al*., 1982
L. divergens	L90	Hitchener *et al*., 1982
L. carnis	N3	Hitchener *et al*., 1982
L. carnis	N61	Hitchener *et al*., 1982
L. piscicola	2762	NCDO
L. curvatus	20019	DSM
L. sake	20017	DSM
L. brevis	20054	DSM
L. farciminis	20184	DSM
L. plantarum	8014	ATCC
L. vaccinostercus	1092	UP
E. faecalis	20409	DSM
E. faecium	20160	DSM

(a) DSM Deutsche Sammlung von Microorganismen, West Germany
NCDO National Collection of Dairy Organisms, U.K.
ATCC American Type Culture Collection
UP University of Pretoria Culture Collection

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3.2.1.2 PURIFICATION AND MAINTENANCE OF CULTURES

All freeze-dried cultures of strains of L. divergens, L. carnis, L. piscicola, Enterococcus faecium and E. faecalis were transferred to D-MRS broth (De Bruyn, 1987) (Appendix 7.1). All freeze-dried cultures of other reference strains of Lactobacillus species were transferred to MRS broth (De Man, Rogosa & Sharpe, 1960) (Appendix 7.2). All isolates were cultured aerobically at 30°C for 2 d and streaked on either D-MRS or MRS agar. The agar plates were incubated in anaerobic jars using Anaerocult A (Merck Darmstadt, West Germany) to produce CO_2. Incubation was at 30°C for 2 d. Agar plates were then stored at 4°C and subcultures made at monthly intervals.

3.2.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

3.2.2.1 PREPARATIONS OF BACTERIAL PROTEIN EXTRACTS

Ten mℓ of MRS broth or D-MRS broth were inoculated and incubated at 30°C for 1 d. These actively growing cultures were used to inoculate 190 mℓ of the corresponding broths. After 2 d of growth at 30°C the cultures were harvested by centrifugation, using a Sorvall RC-SB refrigerated Superspeed centrifuge at 8000 rpm for 15 min, resuspended in 100 mℓ of sterile distilled water and centrifuged again. Fifteen mℓ of the sample buffer (Appendix 7.4) were added to the approximately 0,5 g (wet mass) of bacteria.

The bacteria were disrupted in a French pressure cell (Aminco, SLM Instruments, Urbana, Illinois, USA) at 113 MPa in the liquid. These suspensions were stored at -20°C and were used within four weeks.

3.2.2.2 PREPARATION OF GELS AND ELECTROPHORESIS

The discontinuous buffer system of Laemmli (1970) (Appendix 7.4) was used in slab gels as first described by Raymond, Nakamichi & Aurell (1962). The separating gels had a total acrylamide concentration of 10% and the stacking gel a concentration of 4%. With each 30 μℓ of sample ten μℓ of a glycerol-bromophenol blue mixture was loaded onto the gel.

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Standard proteins (Kersters & De Ley, 1975) were run separately on the same gel and were not included in each sample.

Electrophoresis was performed in a Protean TM II Slab Gel electrophoresis apparatus (Biorad Laboratories, Richmond, CA) as specified by Biorad in the instruction manual. The run was terminated when the bromophenol dye marker was 1 cm from the bottom of the gel. The gels were fixed and stained using Anderson's Brilliant Blue R staining procedure (Anderson & Anderson, 1977) (Appendix 7.5).

3.2.2.3 SPECTROMETRY

The stained gels were placed between two glass plates and scanned in a Beckman DU-8 spectrophotometer. The gel scan module was adjusted according to the settings given in Appendix 7.9.

3.2.2.4 COMPUTER ASSISTED ANALYSIS

The spectrophotometric tracings were normalized as described by Kersters & De Ley (1975) and divided into a sequence of 130 equal parts. A value expressed in mm height was assigned to each position representing the optical densities of each position on the scan.

The Pearson-product moment correlation coefficient (Sokal & Sneath, 1963) between any pair of densitometric tracings was computed for the values of position 11 to position 130. These values were clustered, by unweighted average linkage (Sokal & Sneath, 1963). All calculations were done using a program written by J. Maritz and Elritha van Zyl of the University of Pretoria and making use of the IBM main frame computer of the University of Pretoria, Pretoria.

3.2.2.5 REPRODUCIBILITY

The reproducibility of the technique was studied using *Lactobacillus divergens* strain DMS 20623. Protein extracts were prepared from three separately grown cultures of DSM 20623 as described. The samples were run on the same gel and the correlation determined as described.

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3.2.3 MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TESTS AND NUMERICAL ANALYSIS OF THE DATA

3.2.3.1 MORPHOLOGY

Twenty four h cultures grown in D-MRS or MRS broth were examined under a phase-contrast light microscope to determine their morphological characteristics and motility.

3.2.3.2 GRAM REACTION

Gram reaction was tested according to the method described by Bartholomew (1962) using 24 h cultures grown on D-MRS or MRS agar. *Escherichia coli* served as Gram-negative control organism.

3.2.3.3 MOTILITY

Stab inoculations were made in semi-solid MRS agar (0,3% m/v agar). Spreading of the cultures was recorded after 2 d of growth.

3.2.3.4 GROWTH AT VARIOUS TEMPERATURES

MRS broth with the pH adjusted to 7 was inoculated and incubated at 10, 15 and 45°C for 5 d.

3.2.3.5 GROWTH AT pH 3,9

1N HCl was used to adjust the final pH of MRS broth to 3,9. Tubes were inoculated and incubated at 30°C for 4 d.

3.2.3.6 GROWTH IN THE PRESENCE OF 10% NaCl

MRS broth containing 10% (m/v) NaCl was inoculated and incubated for 4 d.

3.2.3.7 CATALASE AND BENZIDINE REACTIONS

The catalase and benzidine reactions were tested as described by Deibel

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& Evans (1960) using cultures grown aerobically for 2 d on D-MRS or MRS agar.

3.2.3.8 LACTIC ACID CONFIGURATION

The enzymatic method described by Bergmeyer (1965) using the enzymes D-LDH and L-LDH (Boehringer Mannheim, West Germany) was followed.

3.2.3.9 PRODUCTION OF GAS FROM GLUCOSE

A modification of the hot tube method (Sperber & Swan, 1976) as described by Dicks (1985) was used.

3.2.3.10 PRODUCTION OF GAS FROM GLUCONATE

The method of Dicks (1985) was followed using MRS broth where the glucose was replaced by 2% (m.v) sodium gluconate.

3.2.3.11 NITRATE REDUCTION

Cultures were grown in MRS broth, to which 2% (m/v) KNO_3 was added, for 2 d. The presence of nitrite was determined as described by Neyra & Dobereiner (1977).

3.2.3.12 AMMONIA FROM ARGININE

Cultures were grown for 2 d in modified MRS broth (without ammonium citrate) with 0,2% (m/v) arginine added. The presence of ammonia was determined with Nessler's reagent (Merck, Darmstadt, West Germany).

3.2.3.13 PRODUCTION OF GELATINASE

Stab inoculations were made in semi-solid MRS (3% m/v gelatin). After 2 d of incubation the cultures were put at 4°C for 2 h. All cultures where the gelatin remained liquid were taken as gelatinase positive.

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3.2.3.14 m-Dpm (MESO-DIAMINOPIMELIC ACID) IN CELL WALLS

The presence of m-Dpm in the cell walls of cultures was determined using the chromatographic method described by Harper & Davis (1979). Cells harvested from 5 mℓ MRS broth were prepared by hydrolysis at 100°C in 6N HCl for 16 h.

3.2.3.15 API 50 CHL CARBOHYDRATE FERMENTATIONS

The API 50 CHL galleries (API Systems S.A., Montalieu, France), containing the following 49 different dehydrated carbon sources were used: Glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, galactose, glucose, fructose, D-mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-mannoside, α methyl-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate and 5-keto-gluconate. Preparation of inocula and galleries and incubation were done according to the manufacturers' instructions. Carbohydrates fermented were recorded after 24 h of incubation at 30°C.

3.2.3.16 NUMERICAL ANALYSIS

3.2.3.16.1 CODING OF DATA

The data from the morphological, physiological and biochemical tests were coded for computation by recording positive reactions as 1 and negative reactions as 0. Tests not performed were coded as 2 and ignored in the analysis.

3.2.3.16.2 COMPUTATION

The overall similarity between the cultures was calculated by using the simple matching coefficient (Sokal & Sneath, 1963).

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$$S_{sm} = n_{sp} + n_{sn} / n_{sp} + n_{sn} + n_d$$

in which negative similarities are included. S_{sm} is the similarity coefficient expressed as a percentage, n_{sp} and n_{sn} the number of positive and negative similarities respectively and n_d the number of non-matching characters.

The unweighted pair-group average linkage analysis (Sokal & Sneath, 1963) was used to arrange the organisms into groups.

The data was analysed by using a numerical taxonomical programme written by Bezuidenhout (pers. comm.) and making use of a IBM personal computer of the University of Pretoria.

3.3 RESULTS

3.3.1 POLYACRYLAMIDE GEL ELECTROPHORESIS

3.3.1.1 RELATIONSHIP OF ORGANISMS

A dendrogram showing the taxonomic relationship between the various bacterial cultures is presented in Fig. 3.1 (correlation coefficient calculated for the bacteria are given in Appendix 7.6). All 20 bacterial cultures tested clustered at a correlation coefficient of r = 0,35. At a correlation level of r = 0,80 two distinct clusters were discernable.

All the Lactobacillus divergens strains (20623, 60, L90, M2, LV6, LV60) (Fig. 3.2) were grouped in the first cluster. The second cluster consisted of all the L. carnis strains (2764, LV14, 83, N61, N3) and the reference strain of L. piscicola (Fig. 3.3). The other reference strains were not included at this level.

The reference strain of L. curvatus (20019) and L. sake (20017) appeared with cluster 1 at correlation coefficients r = 0,78 and r = 0,75 respectively to form sub-group 1A. Sub-group 1B was formed by the asso-

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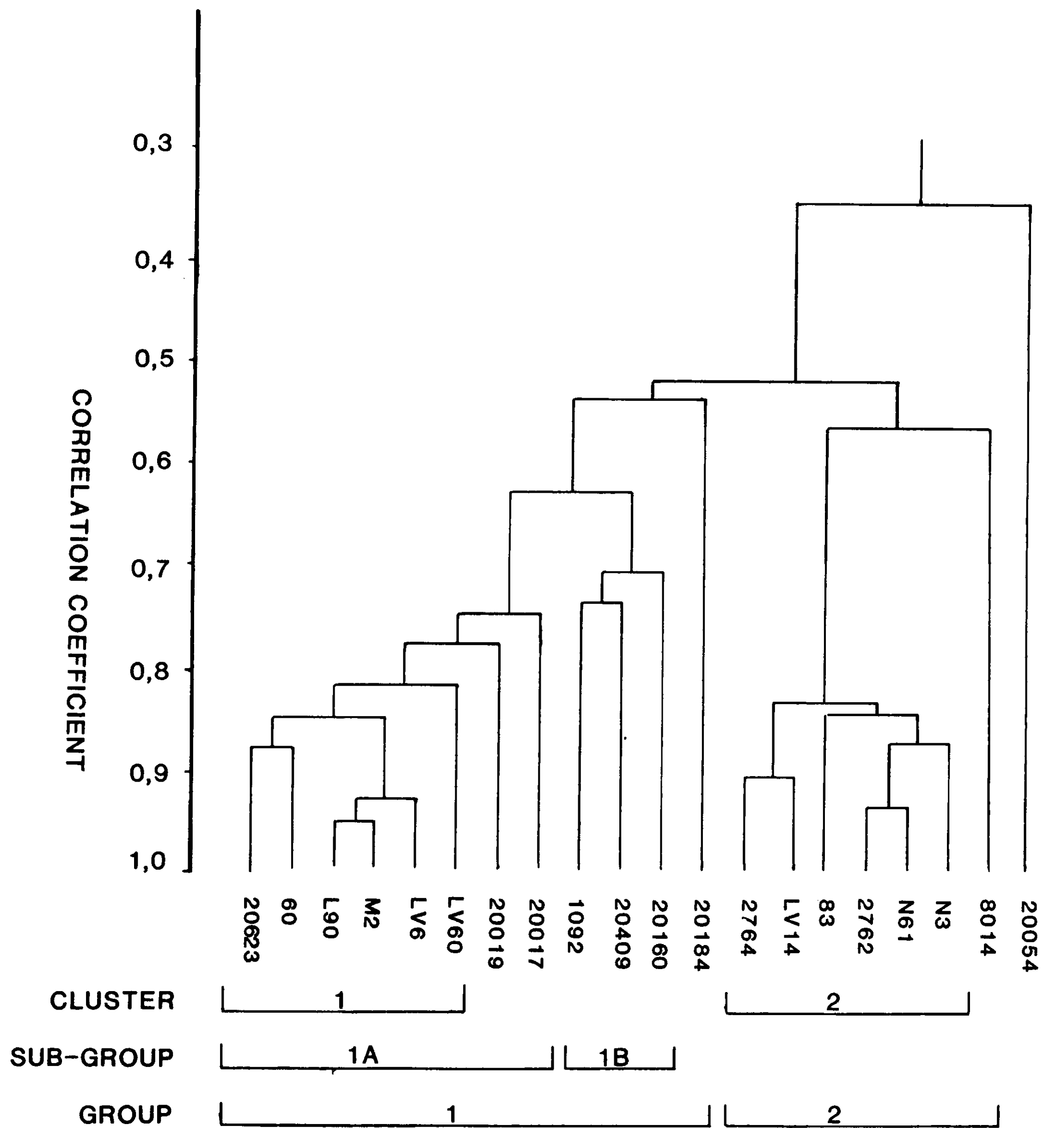

FIGURE 3.1 Dendrogram showing the taxonomic relationships between L. divergens, L. carnis and other lactic acid bacteria reference strains as based on the correlation coefficient (r), determined by unweighted average linkage cluster analysis of PAGE data.

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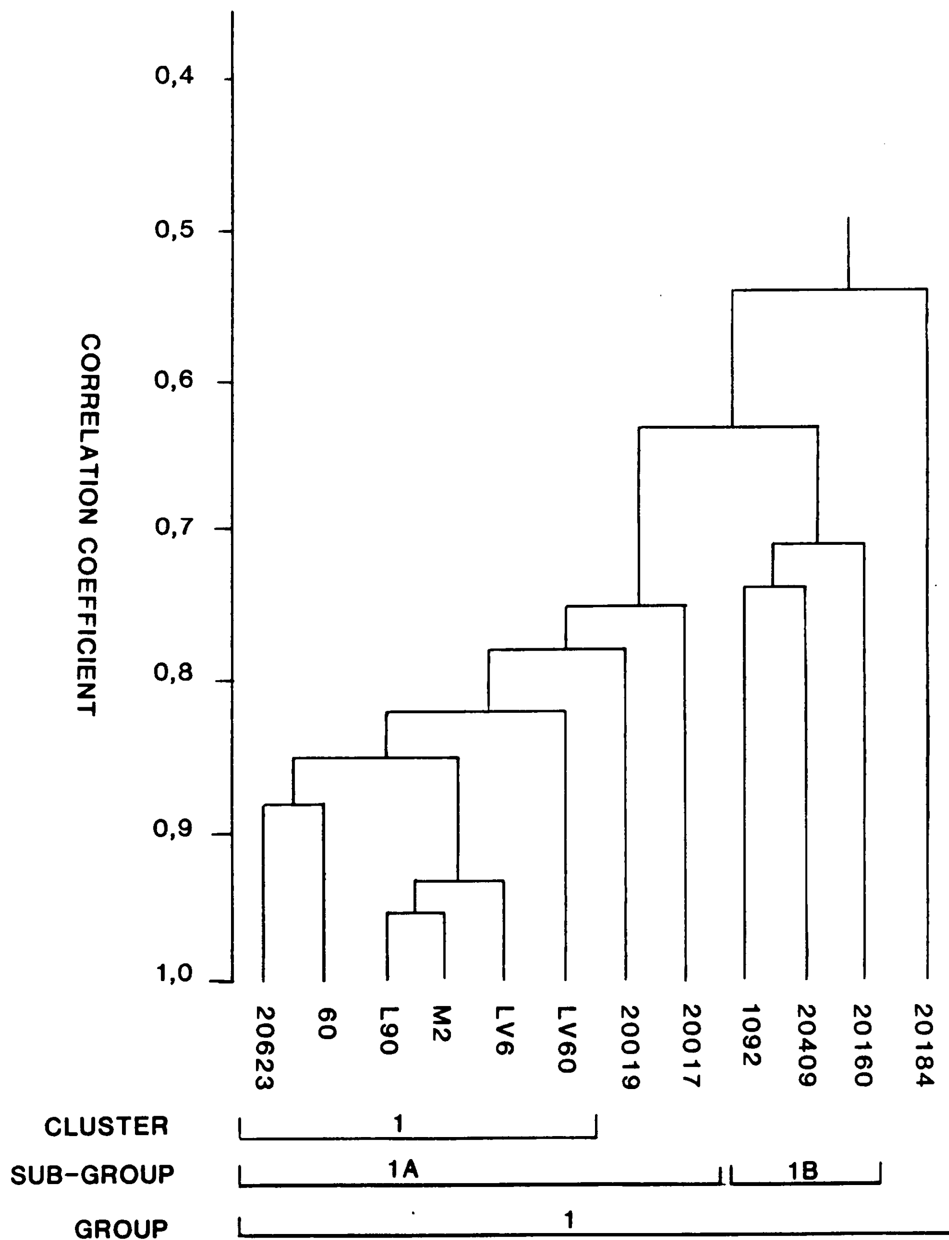

FIGURE 3.2 Dendrogram showing the taxonomic relationships between L. divergens and other lactic acid bacteria reference strains as based on the correlation coefficient (r), determined by unweighted average linkage cluster analysis of PAGE data.

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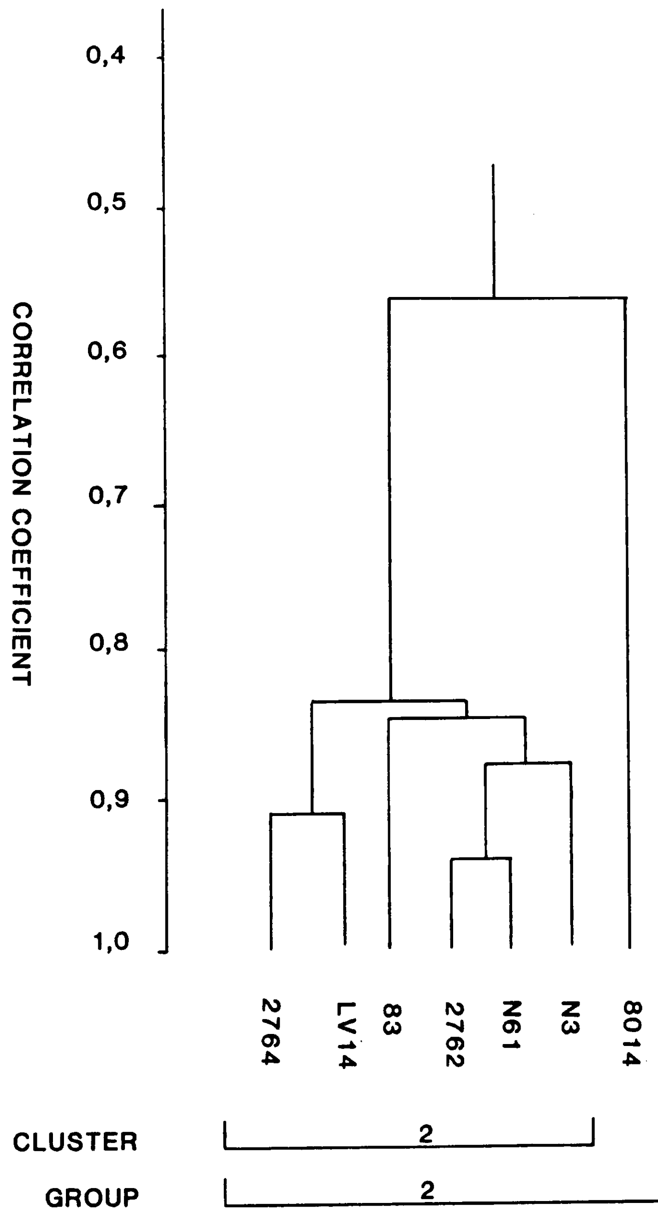

FIGURE 3.3 Dendrogram showing the taxonomic relationships between L. carnis strains and the reference strains of L. piscicola and L. plantarum as based on the correlation coefficient (r), determined by unweighted average linkage cluster analysis of PAGE data.

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ciation of the L. vaccinostercus strain (1092) with the Enterococcus faecalis strain (20409) at a level of r = 0,74 and these two in turn associated with Enterococcus faecium (20160) at a level of r = 0,71. Sub-group 1A and 1B clustered at a level of r = 0,63 and together with Lactobacillus farciminis (20184) they formed Group 1 at a correlation coefficient of r = 0,54 (Fig. 3.2).

Group 2 was formed by the association of Cluster 2 with the L. plantarum strain (8014) at r = 0,57 (Fig. 3.3).

The overall association of Groups 1 and 2 clustered at r = 0,52 and a strain of Lactobacillus brevis (20054) clustered with all the other bacteria at r = 0,35.

3.3.1.2 REPRODUCIBILITY

The three strains of L. divergens (DSM 20623) grouped at a correlation coefficient of r = 0,97.

3.3.2 NUMERICAL TAXONOMY

3.3.2.1 RELATIONSHIP OF ORGANISMS

The dendrogram, based on data in Table 3.2, showing the similarities between the bacteria is given in Fig. 3.4 (the similarity coefficients calculated for the bacteria are given in Appendix 7.7).

At a similarity coefficient of 91% all the strains of L. divergens, L. carnis and the L. piscicola strain grouped together to form Cluster 1 (Fig. 3.5). L. divergens and L. carnis strains did not form two separate sub-clusters. Although sub-cluster 1A consisted only of L. divergens strains, sub-cluster 1B comprised the L. carnis strains, a L. divergens strain (L90) as well as the L. piscicola reference strain.

The strains of L. sake and L. curvatus were grouped at a similarity coefficient of 83% as were the strains of Enterococcus faecium and E. faecalis.

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TABLE 3.2 Characteristics of Lactobacillus divergens and L. carnis strains and some reference strains of Lactobacillus and Enterococcus

Property	Reaction of isolates																			
	20623	2764	2762	20019	20017	20184	20054	8014	1092	20409	20160	60	83	LV6	LV60	LV14	M2	L90	N3	N61
Rod shaped	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Cocci	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Lactic acid produced	DL	L	L	DL	DL	L	DL	DL	DL	L	L	DL	DL	L	L	L	L	L	L	L
Growth at 10°C	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Growth at 15°C	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-
Gas from glucose	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
Gas from gluconate	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
DAP in the cell wall	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+
Growth in 10% NaCl	-	-	-	-	-	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-
Growth at pH 3,9	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of																				
glycerol	+	+	+	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+
L-arabinose	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
ribose	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
D-xylose	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
β-methyl-xyloside	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
galactose	-	+	+	+	+	+	-	+	-	+	+	-	-	-	-	+	-	+	-	-
D-fructose	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
D-mannose	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
mannitol	-	+	+	-	-	+	-	+	-	+	-	-	+	-	+	+	-	+	+	+
sorbitol	-	-	-	-	-	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-
α methyl-D-mannoside	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
α methyl-D-glucoside	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl-glucosamine	+	+	+	+		+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
amygdalin	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
arbutin	+	+	+	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
aesculin	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
salicin	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
cellobiose	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
lactose	-	-	-	+	+	+	-	+	-	-	+	-	+	-	-	+	-	-	-	-
melibiose	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-
saccharose	+	-	+	+		+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
trehalose	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
inulin	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+
melezitose	+	+	-	-	-	+	-	+	-	+	-	+	-	+	+	+	+	-	-	-
D-raffinose	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
amidon	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β gentibiose	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+
D-turanose	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-lyxose	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-tagatose	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-
D-arabitol	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
gluconate	+	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	+	+	+
2-keto-gluconate	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-

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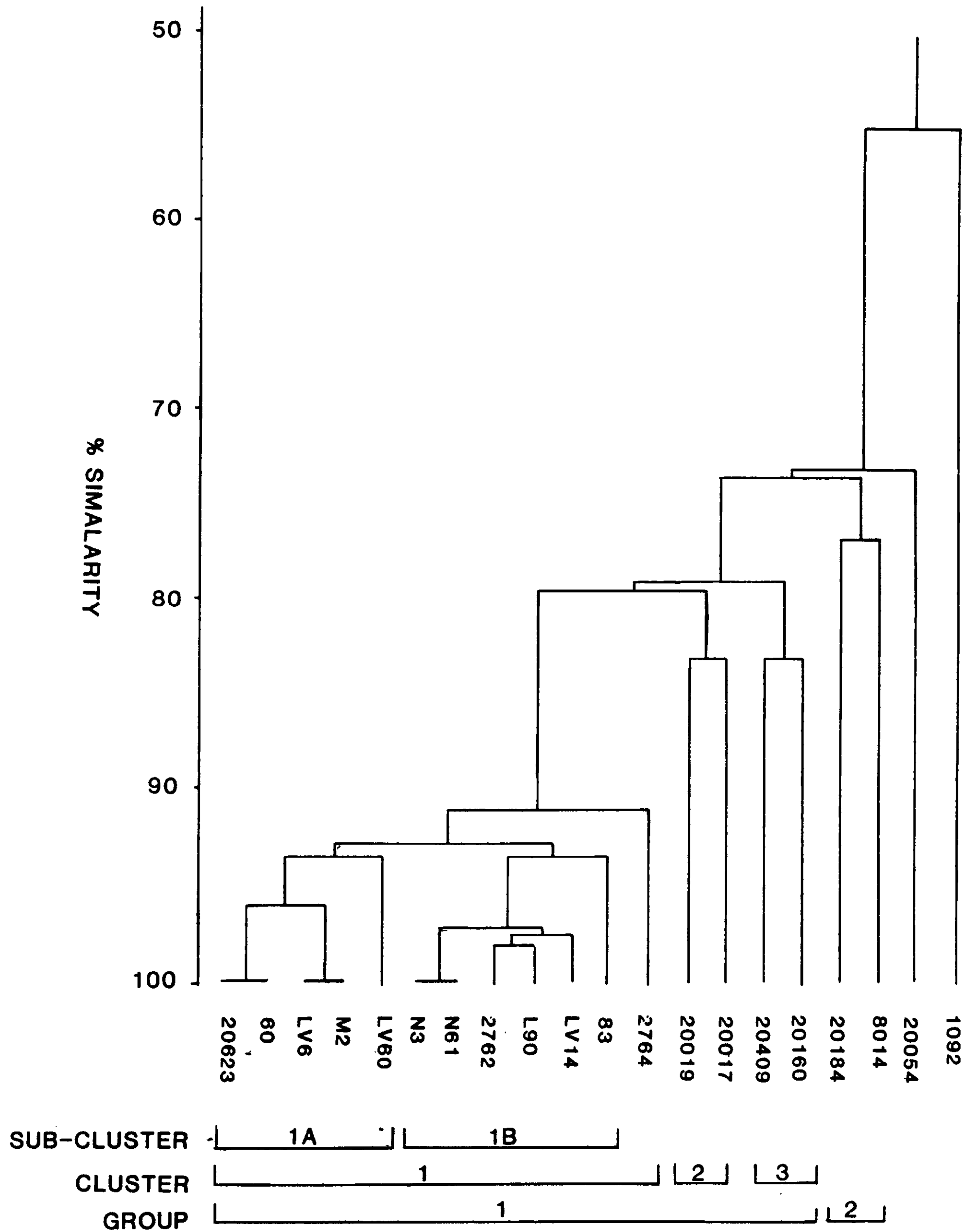

FIGURE 3.4 Dendrogram showing the taxonomic relationships between L. divergens, L. carnis and other lactic acid bacteria reference strains as based on the similarity coefficient, S_{sm}, determined by unweighted average linkage cluster analysis of morphological, physiological and biochemical data.

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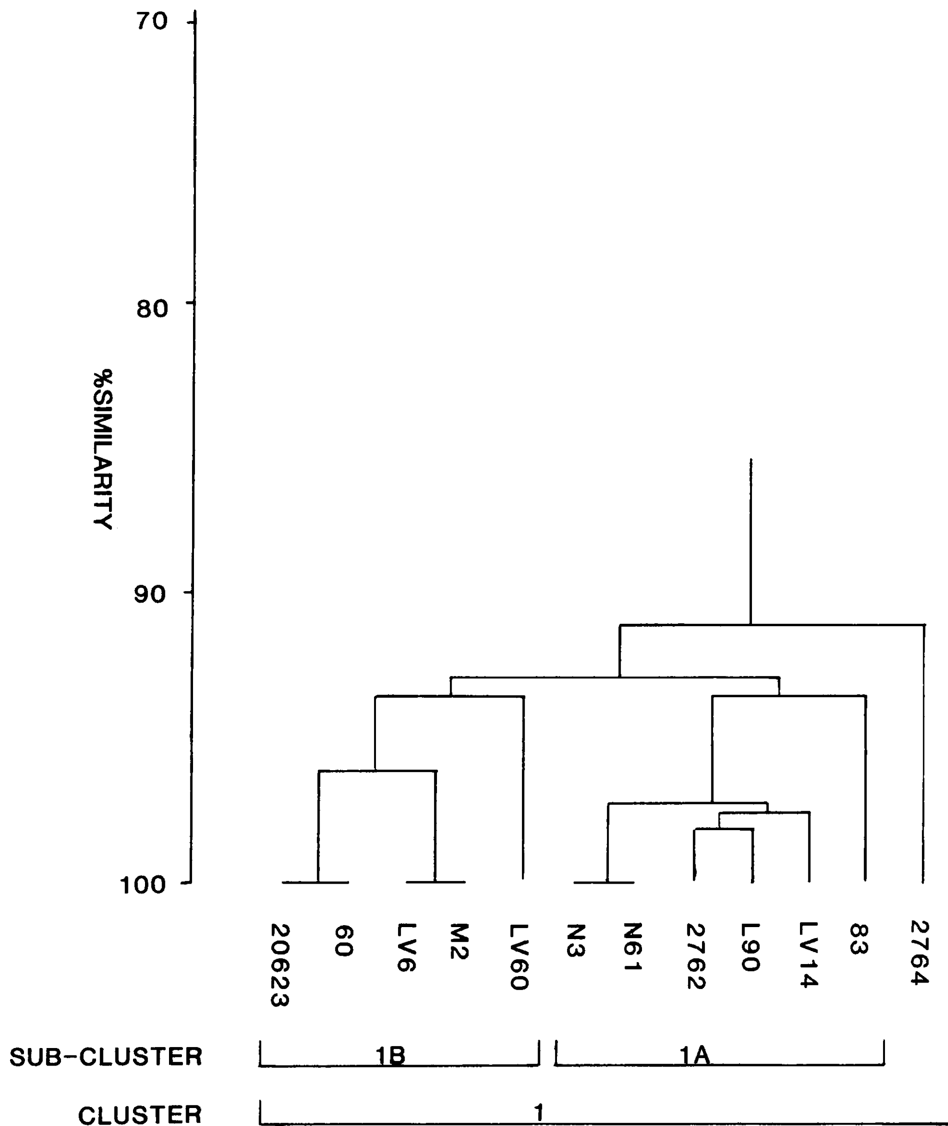

FIGURE 3.5 Dendrogram showing the taxonomic relationships between *L. divergens*, *L. carnis* and *L. piscicola* as based on the similarity coefficient, S_{sm}. determined by unweighted average linkage cluster analysis of morphological, physiological and biochemical data.

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The L. sake / L. curvatus group associated with Cluster 1 at a level of 79,7% and the enterococci grouped with the latter at a similarity coefficient of 79,3% to form Group 1.

Group 2 was comprised of L. farciminis and L. plantarum at a similarity coefficient of 76%. They in turn clustered with group 1 at a level of 73,6%. L. brevis associated with this group at a level of 72,9%. L. vaccinostercus was the last strain to cluster with the other bacterial strains at a similarity coefficient of 55%.

3.3.2.2 CHARACTERISTICS OF THE BACTERIA

All the properties of the strains are shown in Table 3.2. All strains gave similar results for 22 of the tests. They all gave negative results for the following tests: production of only D(-) lactic acid, the presence of catalase, benzidine reaction, motility, production of gelatinase and the fermentation of erythritol, D-arabinose, L-xylose, adonitol, L-sorbose, rhamnose, dulcitol, inositol, glycogen, xylitol, L-arabitol, L-fucose and 5-keto-gluconate. All strains gave positive results for the following tests: Gram reaction and the fermentation of glucose, D-fructose and maltose.

3.3.2.3 CHARACTERISTICS OF THE GROUPS

3.3.2.3.1 GROUP 1

Group 1 consisted of clusters 1, 2 and 3.

3.3.2.3.1.1 CLUSTER 1

Sub-cluster 1A was comprised of L. divergens strains. Strain 60 and 20623 reacted identically as did strain 60, LV6 and M2. Strains 60 and 20623 differed from strains LV6 and M2 in that the latter two strains formed only L(+) lactic acid and strain 60 and 20623 formed a mixture of L(+) and D(-) lactic acid, with the D(-) lactic acid forming a small percentage of the total amount of lactic acid produced. Strain LV60 differed from these bacterial strains because it was able to ferment

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mannitol and sorbitol.

Sub-cluster 1B was comprised of all the L. carnis strains, a L. divergens strain and the reference strain of L. piscicola. Strain N3 and N61 reacted identically, but were unable to ferment galactose. The reference strain of L. piscicola (2762) differed from the L. divergens strain L90 only in its ability to ferment amidon. The L. carnis strain LV14 differed from the latter two by its ability to ferment lactose. Strain 83 fermented lactose but formed a mixture of D(-) and L(+) lactic acid with D(-) lactic acid forming only a small percentage of the total lactic acid production (18%).

The two sub-clusters differed from each other in that sub-cluster 1A fermented melezitose but was unable to ferment inulin. The reference strain of L. carnis (2764) differed from the rest of the strains in Cluster 1 in its ability to ferment saccharose and gluconate.

3.3.2.3.1.2 CLUSTER 2

The two species in Cluster 2 included L. sake and L. curvatus. They differed from each other in the fermentation of amygdalin, melibiose, trehalose, D-lyxose and gluconate. L. sake was able to ferment these sugars whereas L. curvatus was not.

3.3.2.3.1.3 CLUSTER 3

Cluster 3 consisted of two species namely E. faecium and E. faecalis. They differed from each other in the ability of E. faecalis to ferment glycerol, mannitol, sorbitol, melezitose and gluconate whereas E. faecium only fermented lactose and melibiose.

3.3.2.3.2 GROUP 2

Group 2 consisted of two species namely L. farciminis and L. plantarum. The sugar fermentation patterns differed considerably from each other and from the rest of the strains as can be seen in Table 3.2.

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3.3.2.3.3 REMAINDER OF THE STRAINS

The strains of L. brevis and L. vaccinostercus differed in many ways from the rest. The similarity coefficient between L. vaccinostercus and the rest was very low (55%).

3.4 DISCUSSION

The results obtained from polyacrylamide gel electrophoresis (PAGE) of whole cell proteins showed clearly that L. divergens and L. carnis are two separate species. This corresponded with the results of DNA-DNA hybridization performed by Shaw & Harding (1985). They found that the percentage homology between DNA from L. carnis strains and from L. divergens strains was never higher than 41%. The percentage homology between different L. carnis strains was never lower than 78%. These data also agreed with the data obtained from direct mass spectrometry of these bacteria (Shaw et al., 1985).

The whole cell protein PAGE patterns of L. piscicola had a high correlation coefficient with the L. carnis strains. It was therefore impossible to designate these as separate species, even though their habitats differed markedly. This observation concurs with results obtained from DNA-DNA hybridization studies done at the Food Research Institute, Bristol, U.K. (Shaw, pers. comm., 1987), as well as work done by Collins et al. (1987). The specific name L. piscicola should be retained because L. piscicola was first described in 1984 (Hui et al., 1984) and L. carnis subsequently described in 1985 (Shaw & Harding, 1985).

Numerical analysis of the morphological, physiological and biochemical data of these bacterial strains failed to distinguish L. carnis, L. divergens and L. piscicola as separate species or to confirm that L. carnis and L. piscicola are the same species. The above strains clustered at a similarity coefficient of 91% (Fig. 3.7). The similarity coefficient between the L. divergens strain L90 and the L. piscicola strain was 97,7% and their similarity coefficient with the L. carnis strain LV14 was 97,45%.

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Groupings obtained by numerical analysis of the morphological, physiological and biochemical data did not correspond well to those obtained by analysis of the whole cell protein patterns by PAGE. The PAGE data corresponded well to that of DNA-DNA hybridization (Shaw & Harding, 1985) and direct mass spectrometry (Shaw *et al*., 1985). It appears that relationships based on the numerical analysis of phenotypic data of these bacteria do not reflect the genetic relatedness of these species to each other. PAGE of whole cell proteins gave a better correlation with data obtained from DNA-DNA hybridization probably because proteins are the direct translational products of DNA, whereas the phenotypic characteristics are based on the reactions of these proteins in the bacterial cell. Although the tests are conducted under standardized conditions, the activity of proteins may nevertheless be affected by varying environmental conditions, giving rise to differing results. Proteins can be accepted to be the more basic representatives of the genotype. Although different proteins may have the same electrophoretic mobility under the prevailing conditions, the chances are slim that this will affect the results significantly.

The data obtained from PAGE of the whole cell proteins and the numerical analysis (S_{sm}) of their morphological, physiological and biochemical properties indicated that the facultative heterofermentative lactobacilli are a heterogenous group. Contrary to expectations the genus *Lactobacillus* did not appear as a homogeneous group, with the *Enterococcus* species appearing in the centre of the *Lactobacillus* spp. cluster.

The PAGE results showed that *L. divergens*, *L. sake* and *L. curvatus*, all facultative heterofermentative lactobacilli, were grouped with *L. vaccinostercus*, a heterofermentative *Lactobacillus*, *Enterococcus faecium*, *E. faecalis* and *L. farciminis*, a homofermentative *Lactobacillus* species, before all these bacteria clustered with *L. carnis*, *L. piscicola*, *L. plantarum* and *L. brevis*, the remaining facultative heterofermentative lactobacilli (Fig. 3.1).

The numerical analysis of their morphological, physiological and biochemical data showed that *L. carnis*, *L. divergens*, *L. piscicola*, *L. sake* and *L. curvatus* grouped with *E. faecalis* and *E. faecium* before grouping

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with L. farciminis, a homofermentative Lactobacillus, and L. plantarum, a facultative heterofermentative lactobacillus and L. brevis, a heterofermentative lactobacillus (Fig. 3.6).

Stackebrandt et al. (1983) concluded, after characterizing certain Lactobacillus species by oligonucleotide cataloguing of their 16S ribosomal RNA, that his data did not indicate a subdivision of the genus Lactobacillus into three groups corresponding to Orla Jensen's genera Thermobacterium (obligate homofermentative), Streptobacterium (facultative heterofermentative) and Betabacterium (obligate heterofermentative). The way that homofermentative and facultative heterfermentative Lactobacillus strains were grouped together by both approaches used in this study concurs with the data of Stackebrandt et al. (1983).

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CHAPTER 4

ISOLATION OF HIGH pH TOLERANT LACTIC ACID BACTERIA FROM VACUUM-PACKAGED MEAT PRODUCTS

4.1 INTRODUCTION

The predominance of the lactobacilli in the microbial population of vacuum-packaged meat products has been reported by Allen & Foster (1960), Cavett (1963), Gardner (1968) and Mol *et al*. (1971). The more frequent use during the last decade of vacuum-packaging of fresh meat as a means of extending the shelf life, also led to a number of reports of lactobacilli domination in this environment (Patterson & Gibbs, 1977; Egan & Shay, 1982; Hitchener *et al*., 1982; Von Holy, 1983 and Shaw & Harding, 1985).

Various workers have studied these lactic acid bacteria and although most of the strains corresponded to known species, a few atypical lactobacilli were reported (Cavett, 1963; Gardner, 1968; Mol *et al*., 1971; Hitchener *et al*., 1982; Holzapfel & Gerber, 1983; Shaw & Harding, 1985). Holzapfel & Gerber (1983) were the first to describe one of these atypical lactobacilli as *Lactobacillus divergens* spec. nov. In 1985 Shaw & Harding described a similar bacterial species as *L. carnis*.

Acetate agar (Rogosa, Mitchell & Wiseman, 1951) and MRS agar (De Man *et al*., 1960) have been regarded as selective media for lactobacilli. These media had to be modified to allow the growth of *L. divergens* and *Lactobacillus carnis* which are non-aciduric and acetate sensitive. They are able to grow at a pH of 8,5. In 1987 De Bruyn described DETA-MRS, a selective medium for these bacteria to be used in ecological studies. She reported that the combination of a high pH (8,5) and the presence of 0,2% (m/v) thallium acetate precluded the growth of all organisms except *L. divergens*, *L. carnis*, *Enterococcus faecalis* and *E. faecium*. Eriochrome black was included to distinguish between the lactobacilli and the enterococci.

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The aim of this study was to investigate the incidence of *L. divergens* and *L. carnis* in vacuum-packaged meat, using DETA-MRS as a selective medium.

4.2 MATERIALS AND METHODS

4.2.1 ISOLATION OF CULTURES

Isolations were made from vacuum-packaged fresh mince, smoked fish, processed meat and processed chicken. Standard procedures for plate counts were followed using DETA-MRS (De Bruyn, 1987) (Appendix 7.3). The plates were incubated in anaerobic jars using Anaerocult A (Merck, Darmstadt, West Germany) to produce CO_2. Incubation was performed at 30°C for 2 d. Representative colonies were mostly isolated from the 10^3 dilution plates and purified on D-MRS (De Bruyn, 1987) (Appendix 7.1).

4.2.2 MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TESTS

4.2.2.1 MORPHOLOGY

As described in 3.2.3.1.

4.2.2.2 GRAM REACTION

As described in 3.2.3.2.

4.2.2.3 MOTILITY

As described in 3.2.3.3.

4.2.2.4 GROWTH AT VARIOUS TEMPERATURES

As described in 3.2.3.4.

4.2.2.5 GROWTH AT pH 3,9

As described in 3.2.3.5.

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4.2.2.6 GROWTH IN THE PRESENCE OF 10% NaCl

As described in 3.2.3.6.

4.2.2.7 SENSITIVITY TO ANTIBIOTICS

Antibiotic assay discs (Mast Laboratories) were placed on spread plates of the isolates on D-MRS. The following antibiotics were tested: Cefalotin (30 μg); Lincocin (10 μg); Kantrex (30 μg); Ceparex (10 μg); Chlortetracyclin (25 μg); Erythromycin (10 μg); Colistin sulphate (100 μg); Chloramphenicol (30 μg); Neomycin (30 μg) and ampicillin (25 μg). The plates were incubated anaerobically at 30°C. Zones of inhibition were noted after 3 d.

4.2.2.8 CATALASE AND BENZIDINE REACTIONS

As described in 3.2.3.7.

4.2.2.9 LACTIC ACID CONFIGURATION

As described in 3.2.3.8.

4.2.2.10 PRODUCTION OF GAS FROM GLUCOSE

As described in 3.2.3.9.

4.2.2.11 PRODUCTION OF GAS FROM GLUCONATE

As described in 3.2.3.10.

4.2.2.12 PRODUCTION OF SLIME

Isolates were streaked out on D-MRS plates containing 20% (m/v) sucrose. The plates were incubated anaerobically at 30°C for 3 d.

4.2.2.13 NITRATE REDUCTION

As described in 3.2.3.11.

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4.2.2.14 AMMONIA FROM ARGININE

As described in 3.2.3.12.

4.2.2.15 PRODUCTION OF GELATINASE

As described in 3.2.3.13.

4.2.2.16 m-Dpm (MESO-DIAMINOPIMELIC ACID) IN CELL WALLS

As described in 3.2.3.14.

4.2.2.17 SUGAR FERMENTATION TESTS

Two drops of each culture were inoculated into 5 mℓ MRS broth prepared by replacing glucose and meat extract with 0,5% (m/v) of the various carbon sources. Chlorophenol red, at a concentration of 0,004% (m/v) was used as indicator. The pH was adjusted to pH 7. The carbon sources tested included: glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, galactose, glucose, fructose, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol and gluconate. For esculin the chlorophenol red was omitted and 0,05% (m/v) $FeCl_3$ was added. Tubes were incubated at 30°C for 3 days. Colour change of the indicator was taken as a positive reaction indicating that the isolate was able to ferment that specific carbon source.

4.2.3 NUMERICAL ANALYSIS

4.2.3.1 CODING OF DATA

As described in 3.2.3.16.1.

4.2.3.2 COMPUTATION

As described in 3.2.3.16.2.

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4.3 RESULTS

4.3.1 RELATIONSHIP OF ORGANISMS

The sources from which the isolates were obtained are given in Table 4.1. The inter-relationships of these isolates (computed from their characteristics as given in Table 4.2), are shown in the dendrogram (Fig. 4.1) (Appendix 7.8). Eleven (32%) of the isolates had a similarity coefficient of 90% and 28 (82%) of the isolates a similarity coefficient of 85%. The results indicated three main clusters, of which cluster three consisted of two clearly defined sub-clusters, namely 3a and 3b. The reference strain of *L. sake* fell in the sub-cluster 3b. Cluster 1 grouped at 85,4%, cluster 2 at 86,7% and cluster 3 at 85,7%. Cluster 1 and 2 grouped together at 84,7% and cluster 3 and the reference strains of *L. divergens* and *L. carnis* at 83,4%. The overall relationship between Groups 1 and 2 was 83%. Only two isolates (F34 and F43) did not fall into the two main groups with all the other isolates. The strains of *Enterococcus faecium* and *E. faecalis* clustered at 83,3% and finally grouped together with the rest of the strains at 81%.

4.3.2 CHARACTERISTICS OF THE ISOLATES

All isolates gave similar results for 28 of the tests. They all gave a negative result for the following tests: production of ammonia from arginine, presence of m-Dpm in the cell walls, motility, nitrate reduction, production of gelatinase, the presence of catalase, benzidine reaction, resistance to 25 μg ampicillin and fermentation of erythritol, D-arabinose, L-xylose, adonitol, β-methyl-xyloside, dulcitol, inositol, mannitol, α-methyl-D-mannoside, xylitol, D-fucose, L-fucose, D-arabitol and L-arabitol. All isolates gave a positive result for the following tests: Gram reaction, growth at 15°C and the fermentation of D-glucose and sucrose.

4.3.3 CHARACTERISTICS OF THE GROUPS

In discussing the characteristics of these groups attention will be given to the distinguishing sugars, indicating which were most relevant in the identification of the various isolates.

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TABLE 4.1 Isolates obtained in this study and their sources

Isolate	Sample	Spoilage
F2	Vienna	Gas formation, Exudate
F3	Vienna	Gas formation, Exudate
F5	Vienna	Gas formation, Exudate
F7	Smoked Vienna	Exudate
F8	Vienna	Gas formation, Exudate
F9	Vienna	Gas formation, Exudate
F11	Smoked Vienna	Exudate
F12	Vienna	Gas formation, Exudate
F28	Bacon	None
F29	Mince	None
F30	Chicken Vienna	None
F31	Chicken Vienna	None
F32	Chicken Vienna	None
F33	Ham + Tongue Polony	None
F34	Ham + Tongue Polony	None
F35	Ham + Tongue Polony	None
F36	Ham + Tongue Polony	None
F37	Ham + Tongue Polony	None
F38	Chicken Vienna	None
F39	Mini Salami	None
F40	Mini Salami	None
F41	Mini Salami	None
F42	Chicken Vienna	None
F43	Smoked Trout	None
F44	Smoked Trout	None
F46	Smoked Viennas	Gas formation, Exudate
F47	Smoked Viennas	Gas formation, Exudate
F49	Viennas	Gas formation, Exudate
F22	Russians	Exudate

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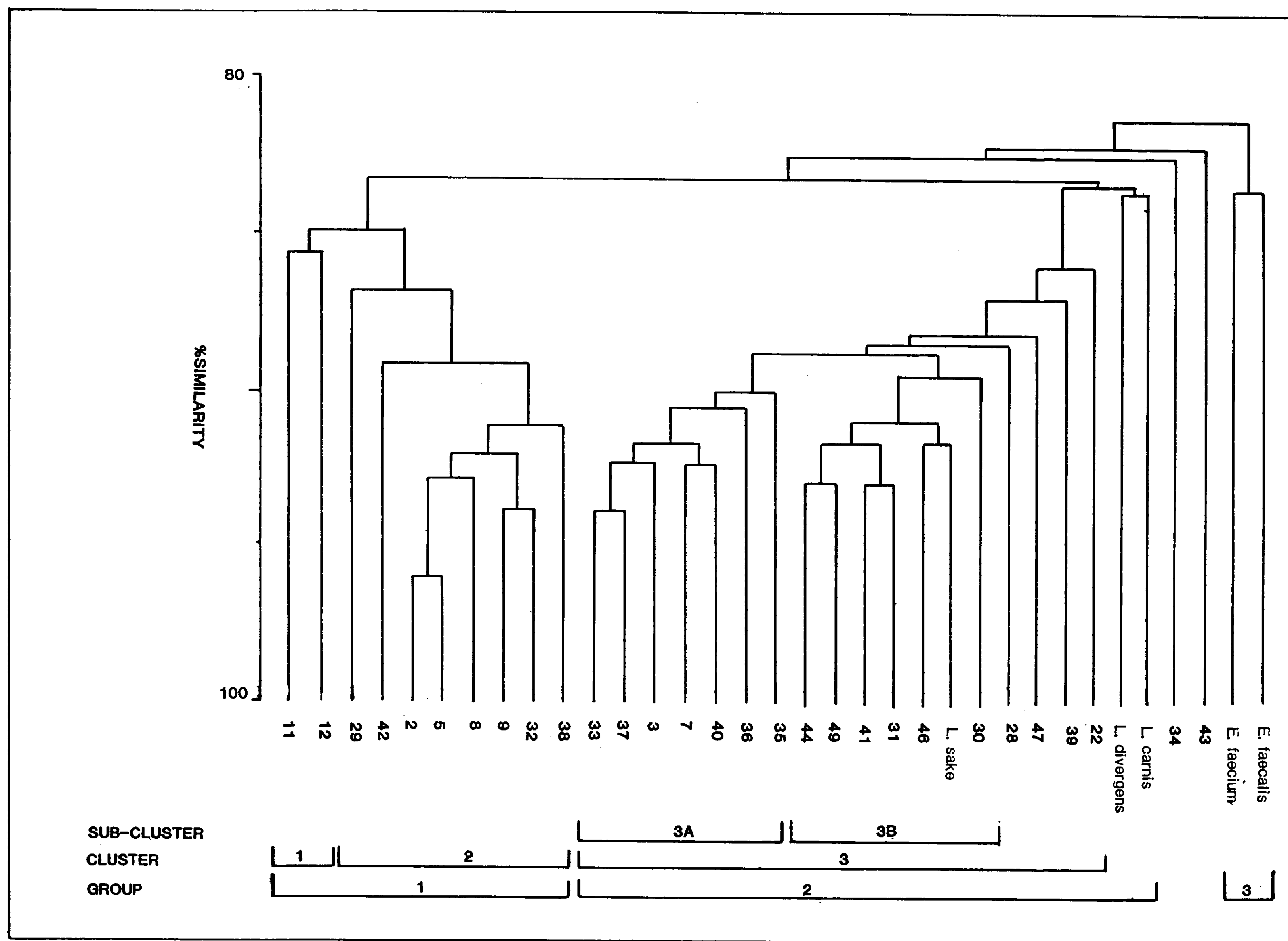

FIGURE 4.1 Dendrogram showing the taxonomic relationships between the isolates and other lactic acid bacteria reference strains as based on the similarity coefficient, S_{sm}, determined by unweighted average linkage cluster analysis of morphological, physiological and biochemical data.

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TABLE 4.2 Characteristics of the lactic acid bacteria isolated on DETA-MRS from vacuum-packaged meat products

Tests	ISOLATES																												
	F2	F3	F5	F7	F8	F9	F11	F12	F28	F29	F30	F31	F32	F33	F34	F35	F36	F37	F38	F39	F40	F41	F42	F43	F44	F46	F47	F49	F22
Rod shape	-	+	-	+	-	-	-	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	+
Cocci	+	-	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-
Lactic acid produced	D	DL	D	L	DL	D	D	D	DL	D	DL	DL	D	DL	DL	DL	DL	DL	D	DL	L	DL	D	L	DL	DL	DL	DL	DL
Growth at 10°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Growth at 45°C	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
Gas from glucose	+	-	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Gas from gluconate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+
Slime from sucrose	+	-	+	-	+	+	-	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Growth in 10% NaCl	-	+	-	+	+	-	-	-	+	-	+	-	-	-	+	-	-	+	-	+	-	-	-	-	+	-	+	-	-
Growth at pH 3,9	+	-	+	+	+	-	+	+	-	+	+	+	-	+	-	+	-	+	+	+	+	+	+	-	-	-	+	-	-
Inhibition by:																													
Colistin sulphate	-	+	-	+	-	-	+	+	-	+	-	-	-	-	-	+	-	+	+	-	+	-	-	+	-	+	+	-	+
Chloramphenicol	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Chlortetracycline	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ceparex	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Erythromycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Lincocin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Cefalotin	-	+	-	+	-	-	-	-	-	-	-	+	-	+	+	-	-	+	+	+	-	+	+	-	-	+	+	+	+
Kantrex	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:																													
glycerol	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	-	+	-	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+	-	+	+	+	+	-	+	+	-
ribose	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-xylose	+	+	+	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-
galactose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
rhamnose	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
arbutin	-	+	+	+	-	-	+	+	-	+	+	+	-	+	-	+	+	+	-	-	+	+	+	+	+	+	+	+	-
esculin	+	+	+	+	+	-	+	+	-	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
salicin	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
cellobiose	-	+	+	+	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
maltose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
lactose	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
melibiose	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
trehalose	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
inulin	-	+	-	+	-	-	-	-	+	-	+	-	-	+	+	+	+	+	-	+	+	-	-	+	-	-	+	-	+
melezitose	-	+	-	+	-	-	-	-	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	+	-	-	+	-	-
D-raffinose	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-	+	-	+
glycogen	-	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
β-gentibiose	-	+	-	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+
D-turanose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+
D-lyxose	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	+
gluconate	-	+	-	+	-	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+

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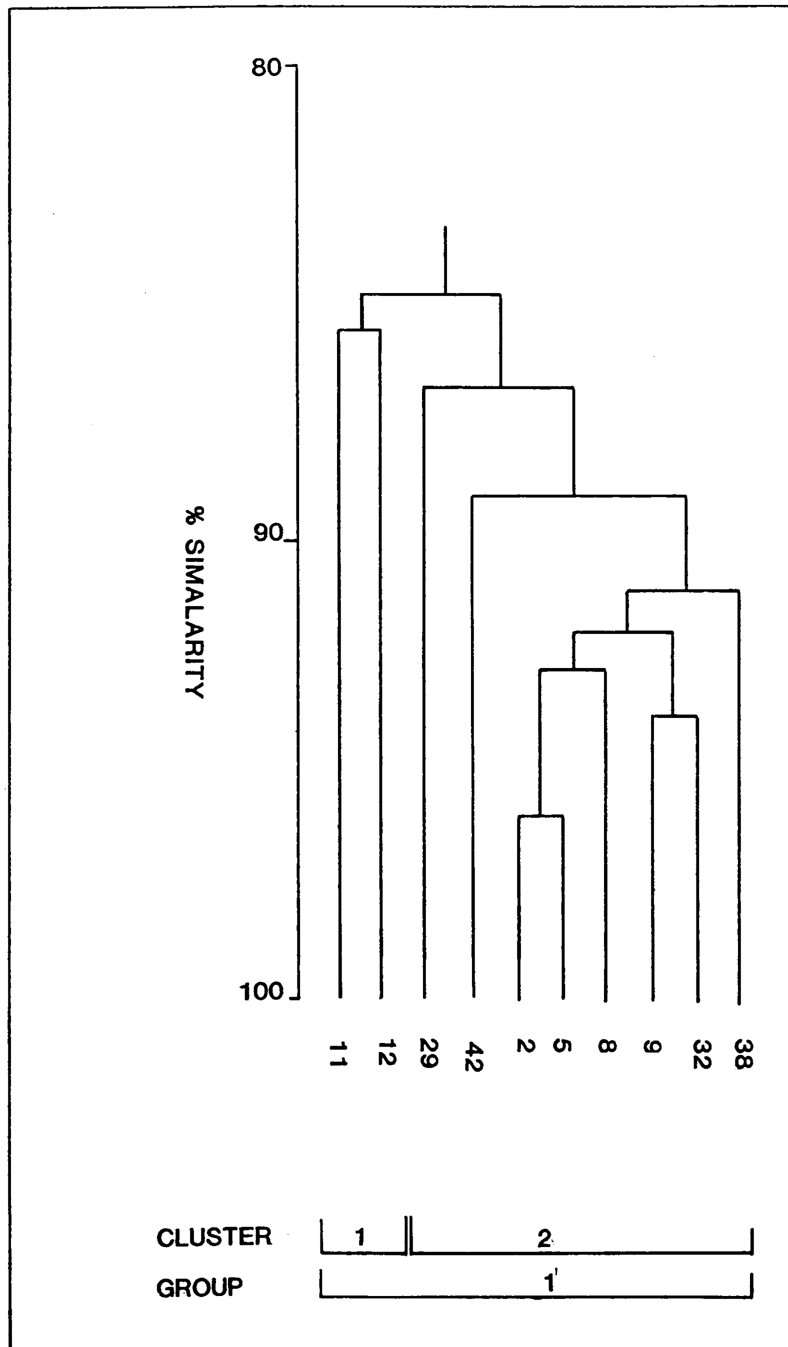

FIGURE 4.2 Dendrogram showing the taxonomic relationships between the *Leuconostoc* isolates as based on the similarity coefficient, S_{sm}, determined by unweighted average linkage cluster analysis of morphological, physiological and biochemical data.

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4.3.3.1 GROUP 1

This group consisted of cluster 1 and cluster 2 (Fig. 4.2). The distinguishing characteristics of this group include the shape (all isolates were cocci) and the production of D(-) lactic acid, except isolate F8 which produced a mixture of D(-) and L(+) lactic acid of which 87% consisted of D(-) lactic acid. All the isolates produced slime in the presence of sucrose in the medium, except isolate F11. The isolates also produced CO_2 from glucose as well as from gluconate and were able to ferment fructose, maltose, mannose, sucrose and trehalose. Cluster 1 differed from cluster 2 in that the isolates were not able to ferment ribose.

4.3.3.2 GROUP 2

Group 2 (Fig. 4.3) consisted of cluster 3 and the reference strains of *L. divergens* and *L. carnis*. The distinguishing characteristics of this cluster were that most of these rods produced a mixture of L(+) and D(-) lactic acid with the percentage of L(+) lactic acid always higher than 50%. Two isolates F7 and F40 produced only L(+) lactic acid. All were able to ferment galactose, lactose, melibiose, ribose and sucrose. Not one of the isolates fermented mannitol.

All the isolates in cluster 3a were able to ferment cellobiose, maltose, melezitose, raffinose, trehalose and salicin, but not L-arabinose, whereas isolates of cluster 3b, including the reference strain of *L. sake* fermented salicin, but were unable to ferment melezitose, raffinose, and D-xylose. Isolates F28 and F47 differed from the rest of this cluster by their ability to ferment rhamnose. Isolate F22 was unable to ferment salicin. Isolate F39 fermented sorbose while the rest of the group were unable to ferment this sugar.

4.3.3.3 GROUP 3

The two strains in group 3 were the authentic strains of *E. faecalis* (20409) and *E. faecium* (20160).

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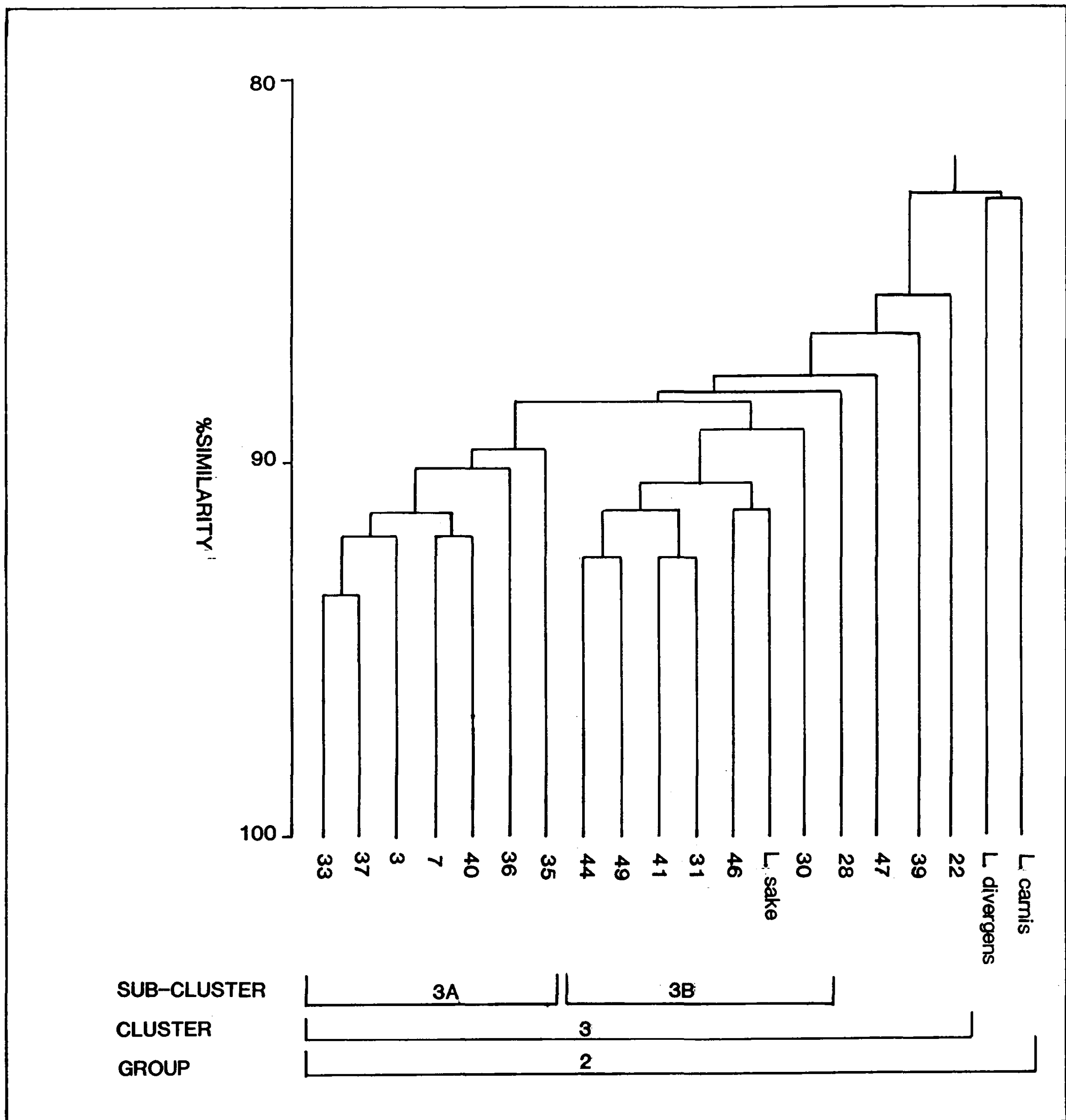

FIGURE 4.3 Dendrogram showing the taxonomic relationships between the Lactobacillus isolates and the reference strains of L. sake, L. divergens and L. carnis as based on the similarity coefficient, S_{sm}, as determined by unweighted average linkage cluster analysis of morphological, physiological and biochemical data.

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4.3.3.4 UNCLUSTERED ISOLATES

The two unclustered isolates were F43 and F34. F43, a coccus, fermented sorbitol, produced L(+) lactic acid and did not produce gas from glucose. Isolate F34, a rod, was unable to ferment galactose and fructose, but could however utilize rhamnose.

4.4 DISCUSSION

Results from the sugar fermentation tests were used to identify the isolates. The two isolates in cluster 1 (Fig. 4.2) F11 and F12 were classified as belonging to the genus *Leuconostoc*, but they do not fit the description of any of the described species (Garvie, 1986a). The isolates in cluster 2 (Fig. 4.2) F29, F42, F4, F5, F8, F9, F32 and F8 were identified as *Leuconostoc mesenteroides* subsp. *mesenteroides*.

The isolates in cluster 3 (Fig. 4.3) F33, F37, F3, F7, F40, F36, F35, F44, F49, F41, F31, F46 and F30 were all classified as *Lactobacillus sake*, although their sugar fermentation patterns differed. The reference strain of *Lactobacillus sake* 20017 also grouped with these bacteria.

The reference strains of *L. divergens* (20623) and *L. carnis* (2762) grouped together, but none of the isolates grouped with them. This confirmed the fact that none of the isolates fitted the description of these two lactobacilli.

Isolates F34 and F43 could not be identified as any of the described lactic acid bacteria.

Hitchener *et al.* (1982) reported the isolation of *Leuconostoc mesenteroides* from vacuum-packaged beef. The fact that most of the isolates were identified as *L. sake* is of interest because reports of the presence of *L. sake* / *L. curvatus* group as predominant species in the spoilage of vacuum-packaged meat products are regularly published (Hitchener *et al.*, 1982; Egan, 1983; Shaw & Harding, 1984; Hastings & Holzapfel, 1987).

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Most lactobacilli have an optimum pH of between 5,5 and 5,8 and a maximum of pH 7,2 (Kandler & Weiss, 1986). The only exceptions known are L. divergens, L. carnis and L. piscicola. The non-acidophilic leuconostocs like Leuconostoc mesenteroides subsp. mesenteroides cannot grow at a pH 4,8 but can grow at a pH 6,5 (Garvie, 1986a). The growth of L. sake, described as aciduric, and Leuconostoc mesenteroides subsp. mesenteroides at a pH 8,5, have not previously been reported. The isolation of these bacteria is an indication of their ability to adapt to a change in the environment.

The similarity coefficient of the bacterial strains studied was very high, as all isolates clustered above 80%. This can be ascribed to the fact that these organisms were isolated from a very specific environment and by means of a medium which was very selective with regard to pH and growth factors.

The fact that L. divergens and L. carnis were not isolated was contrary to expectations. This could be due to the fact that L. sake, normally a predominant species in vacuum-packaged meat products, was adapted to growth at pH 8,5. This resulted in L. sake being present in higher numbers making it difficult to isolate other lactic acid bacteria present in smaller numbers.

DETA-MRS had been developed as a selective medium for L. carnis and L. divergens. However, the selectivity was tested only with reference strains of lactic acid bacteria. In this study it was found that this medium was not selective for these two species only when applied to vacuum-packaged meat and meat products.

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CHAPTER 5

CONLCUSIONS

The following general conclusions were reached at completion of this study:

* PAGE of whole cell proteins indicated that Lactobacillus divergens and Lactobacillus carnis are two separate species and that L. carnis and L. piscicola are the same species.

* The specific name L. piscicola should be retained because L. piscicola was first described in 1984 (Hui et al., 1984) and L. carnis subsequently in 1985 (Shaw & Harding, 1985).

* Numerical analysis (S_{sm}) of the morphological, physiological and biochemical data of L. divergens, L. carnis and L. piscicola could not be used to distinguish between these organisms. Numerical analysis of PAGE has a higher resolution than S_{sm} in distinguishing between the above organisms.

* PAGE of whole cell proteins showed that the facultative heterofermentative lactobacilli are a heterogeneous group. More species and strains of each species of the facultative heterofermentative lactobacilli should be subjected to PAGE analysis to elucidate the taxonomic relationships within this group.

* Some Lactobacillus sake and Leuconostoc mesenteroides subsp. mesenteriodes strains can grow at pH 8,5.

* The observation that some Leuconostoc species are able to grow at pH 8,5 does not fit in with the description of the species. These isolates should be studied further.

* Two lactic acid bacterial isolates able to grow at pH 8,5, do not fit descriptions of any of the presently described lactic acid

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bacteria and should be further investigated to clarify their taxonomic position.

* DETA-MRS cannot be used as a selective medium for *Lactobacillus divergens* and *Lactobacillus carnis* in vacuum-packaged meat products.

* PAGE analysis of whole cell proteins proved to be a powerful tool for rapidly distinguishing between *L. sake* and *L. curvatus*.

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CHAPTER 6

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APPENDIX

7.1 DIVERGENS MRS (D-MRS)

Universal peptone	10,0 g
Meat extract	5,0 g
Yeast extract	5,0 g
Sucrose	20,0 g
Di-potassium hydrogen phosphate	2,0 g
Tween 80	1,0 g
Di-ammonium hydrogen citrate	2,0 g
Magnesium sulfate	0,1 g
Manganese sulfate	0,05 g
Distilled water	1,0 ℓ

The pH was adjusted to pH 8,5 with 10N NaOH.

7.2 MRS-BROTH (pH 6,5)

Universal peptone	10,0 g
Meat extract	5,0 g
Yeast extract	5,0 g
D(+) glucose	20,0 g
Di-potassium hydrogen phosphate	2,0 g
Tween 80	1,0 g
Di-ammonium hydrogen citrate	2,0 g
Sodium acetate	5,0 g
Magnesium sulfate	0,1 g
Manganese sulfate	0,05 g
Distilled water	1,0 ℓ

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7.3 DETA-MRS

DETA-MRS was prepared by adding 0,2 % thallium acetate to D-MRS, pH 8,5. This medium was then poured in 15 mℓ quantities into Petri dishes containing 0,15 mℓ of a 0,4% Eriochrome black solution.

7.4 DISCONTINUOUS BUFFER SYSTEM OF LAEMMLI

7.4.1 Stock solutions

A. Acrylamide/Bis

Acrylamide	146 g
N'N'-Bis-methylene-acrylamide	4 g

Make to 500 mℓ with distilled water. Store at 4°C in the dark.

B. 1,5M Tris-HCl, pH 8,8

Tris base	54,45 g
Distilled water	150,0 mℓ

Adjust to pH 8,8 with 1N HCl. Make to 300 mℓ with distilled water and store at 4°C.

C. 0,5M Tris-HCl, pH 6,8

Tris base	6,0 g
Distilled water	30,0 mℓ

Adjust to pH 6,8 with 1N HCl. Make to 100 mℓ with distilled water and store at 4°C.

D. 10% SDS

Dissolve 10 g SDS in water with gentle stirring and bring to 100 mℓ with distilled water.

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E. Sample buffer

0,5M Tris-HCl, pH 6,8	18,75 mℓ
Glycerol	15,0 mℓ
10% SDS	30,0 mℓ
Mercaptoethanol	7,5 mℓ
0,05% Bromophenol blue	37,5 mℓ
Distilled water	75,0 mℓ

Dilute the sample at least 1:4 with sample buffer, and heat at 95°C for 4 minutes.

F. 5X Electrode buffer, pH 8,3

Tris base	45 g
Glycine	216 g
SDS	15 g

Make up to 3 ℓ with distilled water and store at 4°C. Dilute 300 mℓ 5X stock with 1200 mℓ distilled water.

7.4.2 Separating gel (10%)

1,5M Tris-HCl, pH 8,8	25,0 mℓ
10% SDS	1,0 mℓ
Acrylamide/Bis	33,3 mℓ
Distilled water	40,2 mℓ
10% Ammonium persulfate	500 μℓ
TEMED	50 μℓ

7.4.3 Stacking gel (4%)

0,5M Tris-HCl, pH 6,8	2,5 mℓ
10% SDS	100 μℓ
Acrylamide/Bis	1,3 mℓ
Distilled water	6,1 mℓ
10% Ammonium persulfate	50 μℓ
TEMED	10 μℓ

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7.5 ANDERSON'S BRILLIANT BLUE R STAINING

7.5.1 SOLUTIONS

A Staining concentrate

Dissolve 2,5 g of Brilliant Blue R in 1 ℓ of 95% ethanol. Stir for 1h and filter before use.

B 10% Acetic acid

Dilute 100 mℓ of glacial acetic acid to 1 ℓ with distilled water.

C 5% Acetic acid

Dilute 50 mℓ of glacial acetic acid to 1 ℓ with distilled water.

7.5.2 FIXING AND STAINING

Mix 100 mℓ of 10% acetic acid with 100 mℓ of the stain concentrate.
Staining time : overnight

7.5.3 DESTAINING

A Mix 200 mℓ of 95% ethanol and 300 mℓ of 5% acetic acid.
Destaining time : 1 - 2 h

B Mix 150 mℓ of 95% ethanol and 350 mℓ of 5% acetic acid.
Destaining time : 1 - 2 h

C Repeat B

D Mix 100 mℓ of 95% ethanol and 400 mℓ of 5% acetic acid.
Destaining time : 2 h

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E If destained enough.
Mix 350 mℓ of 5% acetic acid and 150 mℓ water.

F If more destaining is required
Mix 350 mℓ of 5% acetic acid, 25 mℓ of ethanol and 125 mℓ of water.
Destaining time : overnight.

7.6 CORRELATION COEFFICIENTS CALCULATED FOR SPECTROPHOTOMETRIC TRACINGS OF WHOLE CELL PROTEIN PATTERNS OF BACTERIA

Cluster number 1
Number of isolates 2
Correlation coefficient 0,955
Isolates 2 3

Cluster number 2
Number of isolates 2
Correlation coefficient 0,942
Isolates 8 9

Cluster number 3
Number of isolates 3
Correlation coefficient 0,935
Isolates 2 3 4

Cluster number 4
Number of isolates 2
Correlation coefficient 0,912
Isolates 6 11

Cluster number 5
Number of isolates 2
Correlation coefficient 0,903
Isolates 1 5

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Cluster number	6
Number of isolates	3
Correlation coefficient	0,885
Isolates	8 9 10

Cluster number	7
Number of isolates	4
Correlation coefficient	0,854
Isolates	7 8 9 10

Cluster number	8
Number of isolates	5
Correlation coefficient	0,854
Isolates	1 5 2 3 4

Cluster number	9
Number of isolates	6
Correlation coefficient	0,843
Isolates	6 11 7 8 9 10

Cluster number	10
Number of isolates	6
Correlation coefficient	0,820
Isolates	1 5 2 3 4 12

Cluster number	11
Number of isolates	7
Correlation coefficient	0,782
Isolates	1 5 2 3 4 12 13

Cluster number	12
Number of isolates	8
Correlation coefficient	0,755
Isolates	1 5 2 3 4 12 13 14

Cluster number	13
Number of isolates	2
Correlation coefficient	0,741
Isolates	15 20

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Cluster number	14
Number of isolates	3
Correlation coefficient	0,711
Isolates	15 20 19

Cluster number	15
Number of isolates	11
Correlation coefficient	0,630
Isolates	1 5 2 3 4 12 13 14 15 20 19

Cluster number	16
Number of isolates	7
Correlation coefficient	0,577
Isolates	6 11 7 8 9 10 17

Cluster number	17
Number of isolates	12
Correlation coefficient	0,539
Isolates	1 5 2 3 4 12 13 14 15 19 20 16

Cluster number	18
Number of isolates	19
Correlation coefficient	0,520
Isolates	1 5 2 3 4 12 13 14 15 20 19 16 6 11 7 8 9 10 17

Cluster number	19
Number of isolates	20
Correlation coefficient	0,350
Isolates	1 5 2 3 4 12 13 14 15 20 19 16 6 11 7 8 9 10 17 18

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NUMBER	ISOLATE
1	DSM 20623
2	L90
3	M2
4	LV6
5	60
6	NCDO 2764
7	83
8	NCDO 2762
9	N61
10	N3
11	LV14
12	LV60
13	DSM 20019
14	DSM 20017
15	UP 1092
16	DSM 20184
17	ATCC 8014
18	DSM 20054
19	UP 20160
20	UP 20409

7.7 SIMILARITY COEFFICIENTS CALCULATED FOR MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL DATA OF BACTERIA USED IN PAGE EXPERIMENT

Cluster number 1
Number of isolates 2
Average similarity 1
Isolates 1 12

Cluster number 2
Number of isolates 2
Average similarity 1
Isolates 14 17

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Cluster number	3
Number of isolates	2
Average similarity	1
Isolates	19 20

Cluster number	4
Number of isolates	2
Average similarity	0,9792
Isolates	3 18

Cluster number	5
Number of isolates	3
Average similarity	0,9745
Isolates	3 18 16

Cluster number	6
Number of isolates	5
Average similarity	0,9740
Isolates	3 18 16 19 20

Cluster number	7
Number of isolates	4
Average similarity	0,9583
Isolates	1 12 14 17

Cluster number	8
Number of isolates	6
Average similarity	0,9367
Isolates	3 18 16 19 20 13

Cluster number	9
Number of isolates	5
Average similarity	0,9439
Isolates	1 12 14 17 15

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Cluster number 10
Number of isolates 11
Average similarity 0,9282
Isolates 1 12 14 17 15 3 18 16 19 20
13

Cluster number 11
Number of isolates 12
Average similarity 0,9105
Isolates 1 12 14 17 15 3 18 16 19 20
13 2

Cluster number 12
Number of isolates 2
Average similarity 0,8333
Isolates 4 5

Cluster number 13
Number of isolates 2
Average similarity 0,8333
Isolates 10 11

Cluster number 14
Number of isolates 14
Average similarity 0,7972
Isolates 1 12 14 17 15 3 18 16 19 20
13 2 4 5

Cluster number 15
Number of isolates 16
Average similarity 0,7928
Isolates 1 12 14 17 15 3 18 16 19 20
13 2 4 5 10 11

Cluster number 16
Number of isolates 2
Average similarity 0,7679
Isolates 6 8

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Cluster number	17
Number of isolates	18
Average similarity	0,7362
Isolates	1 12 14 17 15 3 18 16 19 20 13 2 4 5 10 11 6 8

Cluster number	18
Number of isolates	19
Average similarity	0,7292
Isolates	1 12 14 17 15 3 18 16 19 20 13 2 4 5 10 11 6 8 7

Cluster number	19
Number of isolates	20
Average similarity	0,5516
Isolates	1 12 14 17 15 3 18 16 19 20 13 2 4 5 10 11 6 8 7 9

Number	Isolate
1	DSM 20623
2	NCDO 2764
3	NCDO 2762
4	DSM 20019
5	DSM 20017
6	DSM 20184
7	DSM 20054
8	ATCC 8014
9	UP 1092
10	UP 20409
11	UP 20160
12	60
13	83
14	LV6
15	LV60
16	LV14
17	M2
18	L90
19	N3
20	N61

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7.8 SIMILARITY COEFFICIENTS CALCULATED FOR PHYSIOLOGICAL, MORPHOLOGICAL AND BIOCHEMICAL DATA OF BACTERIA ISOLATED ON DETA-MRS

Cluster number	1
Number of isolates	2
Average similarity	0,9583
Isolates	F2 F5
Cluster number	2
Number of isolates	2
Average similarity	0,9375
Isolates	F9 F32
Cluster number	3
Number of isolates	2
Average similarity	0,9375
Isolates	F33 F37
Cluster number	4
Number of isolates	3
Average similarity	0,9271
Isolates	F2 F5 F8
Cluster number	5
Number of isolates	3
Average similarity	0,9271
Isolates	F3 F33 F37
Cluster	6
Number of isolates	2
Average similarity	0,9236
Isolates	F7 F40
Cluster	7
Number of isolates	5
Average similarity	0,9108
Isolates	F3 F33 F37 F7 F40

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Cluster number	8
Number of isolates	2
Average similarity	0,9167
Isolates	F31 F41

Cluster number	9
Number of isolates	2
Average similarity	0,9167
Isolates	F44 F49

Cluster number	10
Number of isolates	4
Average similarity	0,9103
Isolates	F3 F41 F44 F49

Cluster number	11
Number of isolates	2
Average similarity	0,9103
Isolates	F46 20017

Cluster number	12
Number of isolates	5
Average similarity	0,9100
Isolates	F2 F5 F8 F9 F32

Cluster number	13
Number of isolates	6
Average similarity	0,9099
Isolates	F2 F5 F8 F9 F32 F38

Cluster number	14
Number of isolates	6
Average similarity	0,9089
Isolates	F31 F41 F44 F49 F46 20017

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Cluster number	15
Number of isolates	6
Average similarity	0,9017
Isolates	F3 F33 F37 F7 F40 F36

Cluster number	16
Number of isolates	7
Average similarity	0,8970
Isolates	F3 F33 F37 F7 F40 F36 F35

Cluster number	17
Number of isolates	7
Average similarity	0,8935
Isolates	F30 F31 F41 F44 F49 F46 20017

Cluster number	18
Number of isolates	7
Average similarity	0,8895
Isolates	F2 F5 F8 F9 F32 F38 F42

Cluster number	19
Number of isolates	14
Average similarity	0,8856
Isolates	F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017

Cluster number	20
Number of isolates	15
Average similarity	0,8805
Isolates	F3 F33 F37 F7 F40 F36 F35 F30 F31 F44 F49 F46 20017 F28

Cluster number	21
Number of isolates	16
Average similarity	0,8787
Isolates	F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47

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Cluster number	22
Number of isolates	17
Average similarity	0,8689
Isolates	F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47 F39

Cluster number	23
Number of isolates	8
Average similarity	0,8682
Isolates	F2 F5 F8 F9 F32 F38 F42 F29

Cluster number	24
Number of isolates	18
Average similarity	0,8569
Isolates	F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47 F39 F22

Cluster number	25
Number of isolates	2
Average similarity	0,8542
Isolates	F11 F12

Cluster number	26
Number of isolates	10
Average similarity	0,8696
Isolates	F2 F5 F8 F9 F32 F38 F42 F29 F11 F12

Cluster number	27
Number of isolates	2
Average similarity	0,8340
Isolates	20623 2762

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Cluster number 28
Number of isolates 2
Average similarity 0,8336
Isolates 20409 20160

Cluster number 29
Number of isolates 20
Average similarity 0,8333
Isolates F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47 F39 F22 20623 2762

Cluster number 30
Number of isolates 30
Average similarity 0,8330
Isolates F2 F5 F8 F9 F32 F38 F42 F29 F11 F12 F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47 F39 F22 20623 2762

Cluster number 31
Number of isolates 31
Average similarity 0,8206
Isolates F2 F5 F8 F9 F3 F38 F42 F29 F11 F12 F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47 F39 F22 20623 2762 F34

Cluster number 32
Number of isolates 32
Average similarity 0,8199
Isolates F2 F5 F8 F9 F32 F38 F42 F29 F11 F12 F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47 F39 F22 20623 2762 F34 F43

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Cluster number	33
Number of isolates	34
Average similarity	0,8114
Isolates	F2 F5 F8 F9 F32 F38 F42 F29 F11 F12 F3 F33 F37 F7 F40 F36 F35 F30 F31 F41 F44 F49 F46 20017 F28 F47 F39 F22 20623 2762 F34 F43 20409 20160

7.9 BECKMAN DU-8 SPECTROPHOTOMETER SETTINGS

Absorbtion
Wavelenth 500nm
Average reading 1
Slit width 0,2nm
Lower scale 0,00
Upper scale 2,00
Chart speed 30 cm/min
Gel speed 10 cm/min
Gel slit width 5nm
Gel start 0mm
Gel end 140mm
Sensitivity 1

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