# CHAPTER II

# LITERATURE REVIEW

## 2.1. Lactic acid bacteria (LAB)

The LAB, frequently termed "the Lactics'' consisting of a number of diverse genera, were originally classified as either homofermenters or heterofermenters by Orla-Jensen (1919) based on the end product of their fermentation. The homofermenters produce lactic acid as the major product of fermentation of glucose (Fig. 2.1a). The heterofermenters produce a number of products beside lactic acid including carbon dioxide, acetic acid, and ethanol from the fermentation of glucose (Fig. 2.1b). The acidification and enzymatic processes accompanying the growth of LAB impart the key flavor, texture, and preservative qualities to a variety of fermented foods. The type and amount of lactic acid formed during fermentation is critical in food manufacture and plays a prominent role in taxonomic classification of the LAB (Frank *et al.*, 2002).Lactic acid may be extracted from the fermentation product and a determination is made by ability to optically rotate light. If the rotation is to the right, it is termed Dextrorotary (D); if to the left, it is termed Levorotary (L), or, if there is a mixture of both D and L, it is termed racemic (DL). The classical approach to bacterial classification is based on morphological and physiological features. LAB are Gram-positive, non-sporeforming cocci, coccobacilli, or rods with a DNA base composition of less than 50 mol% G + C (Stiles & Holzapfel, 1997). LAB associated with food are generally restricted to the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc, Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Table 2.1 shows differential characteristics of LAB at the genus level (Axelsson, 1998).

The current taxonomic classification of prokaryotes is based on polyphasic taxonomy (Vandamme *et al.*, 1996). This approach combines the genomic and phenotypic characteristics of a strain. The minimum amount of genomic information required for the description of a novel bacterial species must include its phylogenetic classification, DNA–DNA relatedness and the mol% G+C content of DNA. Sequence data of ribosomal RNA (rRNA) still provide a solid basis for microbial taxonomy and identification. All species description should include an almost complete 16S rDNA

sequence (>1300 nts, <0.5% ambiguity) (Stackebrandt *et al.*, 2002). It has been suggested that microorganisms showing more than 10 mol% difference in DNA G+C contents might not belong to the same genus and that 5 mol% is the common range found within a species (Goodfellow *et al.*, 1997). Several molecular biology-based techniques, such as multilocus sequence analysis (MLSA), randomly amplified polymorphic DNA, (RAPD) analysis, 16S rRNA gene sequencing, amplified fragment length polymorphism (AFLP) analysis, pulsed-field gel electrophoresis (PFGE), and intergenic ribosomal PCR, have been used to identify LAB to the species and the strain levels (Naser *et al.*, 2005)

**Table 2.1.** Differential characteristics of lactic acid bacteria at the genus level, based on morphology and physiology.

| | Rods | | Cocci | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Characteristic | *Carnobacterium* | *Lactobacillus* | *Enterococcus* | *Lactococcus, Vagococcus* | *Leuconostoc, Oenococcus* | *Pediococcus* | *Streptococcus* | *Tetragenococcus* | *Weissella* [a] |
| Tetrad formation | − | − | − | − | − | + | − | + | − |
| $CO_2$ from glucose [b] | −[c] | ± | − | − | + | − | − | − | + |
| Growth: | | | | | | | | | |
| at 10°C | + | ± | + | + | + | ± | − | + | + |
| at 45 °C | − | ± | + | − | − | ± | ± | − | − |
| in 6.5% NaCl | ND[d] | ± | + | − | ± | ± | − | + | ± |
| in 18% NaCl | − | − | − | − | − | − | − | + | − |
| at pH 4.4 | ND | ± | + | ± | ± | + | − | − | ± |
| at pH 9.6 | − | − | + | − | − | − | − | + | − |
| Lactic acid [e] | L | D,L, DL | L | L | D | L, DL | L | L | D, DL |

+, positive; −, negative; ±, response varies between species; ND, not determined.
[a]may also be rods.
[b]test for homo- or heterofermentation of glucose;
+, homofermentation; −, heterofermentation. [c]small amounts of $CO_2$ can be produced.
[d]no growth in 8% NaCl has been reported.
[e]configuration of lactic acid produced from glucose.
Reproduced from M.P. Doyle and J. Meng, 2006.

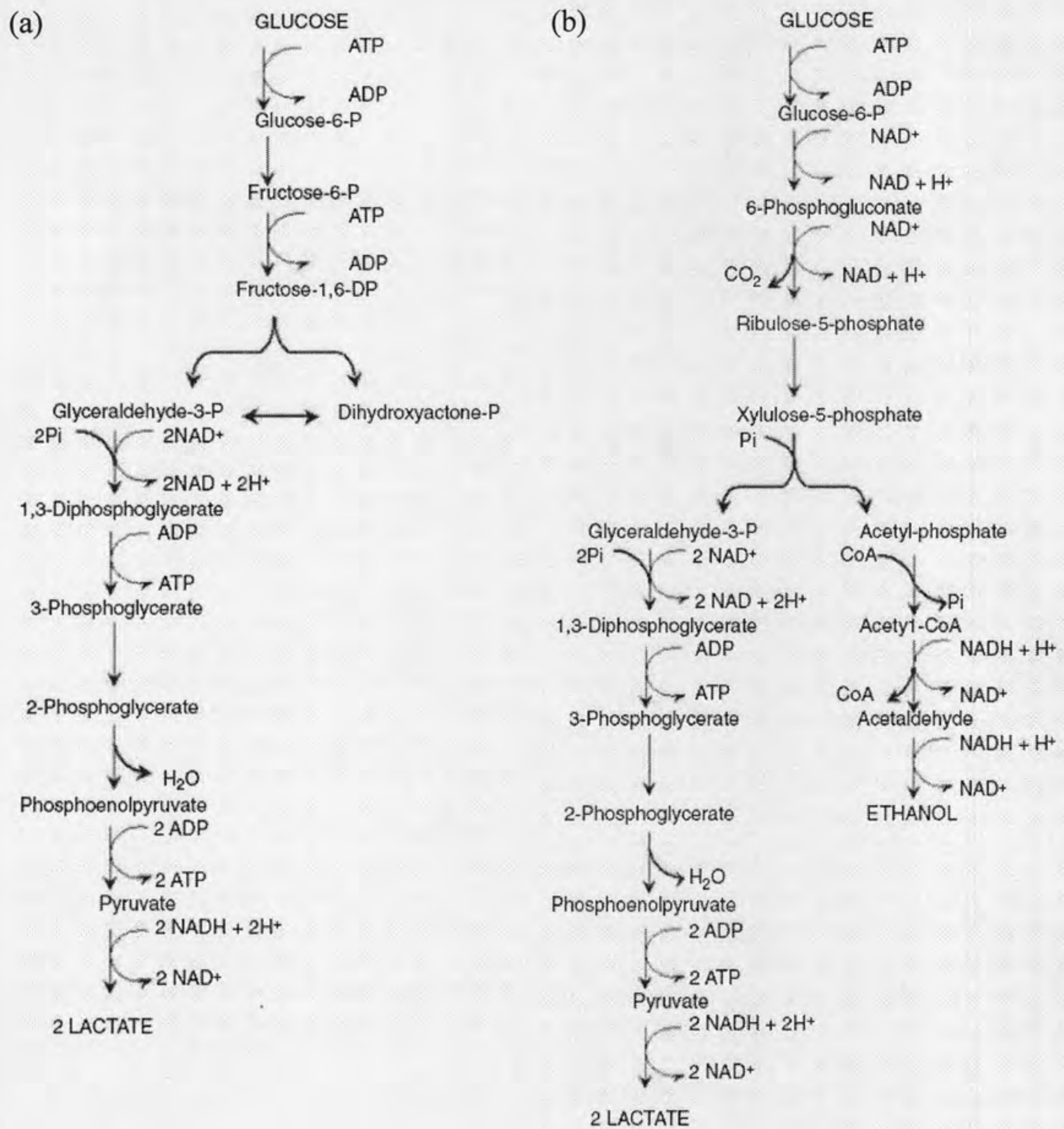



**Fig. 2.1.** Metabolism of glucose by LAB. (a) Homolactic fermentation pathway of glucose (glycosis, Embden-Meyerhof pathway) and (b) Heterolactic fermentation pathway of glucose (6-phosphogluconate/phosphoketolase pathway) from Doyle & Meng, 2006.

More recently, LAB are being used in the production of industrial chemical and biological products including biopolymers (*Leuconostoc* spp.), bulk enzymes (*Lactobacillus brevis*), ethanol, and lactic acid (*Lactobacillus casei, lactis, delbrueckii, brevis*) (Gold *et al.*, 1996; Hofvendahl & Hahn-Hagerdal 2000). The LAB are also strong candidates for development as oral delivery vehicles for digestive enzymes and vaccine antigens (Wells *et al.*, 1996; Pouwels *et al.*, 1998; Steidler *et al.*,

2000). Their innate acid tolerance, ability to survive in gastric passage, and safety record during human consumption, are key features that can be exploited to effectively deliver biologics to targeted locations and tissues.

Realizing their practical significance in fermentation, bioprocessing, agriculture, food, and more recently, medicine, the LAB have been the subject of considerable research and commercial development over the past decade. Contributing to this explosion have been the recent efforts to determine the genome sequences of a representative collection of LAB species and strains (Klaenhammer *et al.*, 2002). The first complete genome of the LAB group was published on *Lactococcus lactis* subsp. *lactis* IL1403 by Bolotin *et al.* (2001). The genome was 2.4 Mb in size and revealed a number of unexpected findings: biosynthetic pathways for all 20 amino acids, albeit not all are functional, a complete set of late competence genes, five complete prophages, and partial components for aerobic metabolism. The newly released genome of LAB, *Lactobacillus plantarum* WCFS1 (3.3 Mb) revealed that the genome encodes more than three thousand genes (Kleerebezem *et al.*, 2003). This data is being systematically 'mined' for genes of potential economic significance using state of the bioinformatics analysis. Genome sequencing of selected LAB are listed in Table 2.2. Looking to the future, the knowledge of the complete genetic potential of numerous LAB and bifidobacteria will now allow integration of genomics, transcriptomics, proteomics and metabolomics data, aimed at developing metabolic models and understanding gene regulation (Francke *et al.*, 2005; Siezen *et al.*, 2004; Liu *et al.*, 2005).

The availability of genome sequences has spawned the new scientific disciplines of comparative genomics, which allows the comparison of genome sequence data between strains, species, genera and even kingdoms. Such studies will provide important taxonomic insights and will have far-reaching implications for the study of evolution (Wren, 2006). Bioinformatics is essentially the evolution of computer-based technology dedicated to the analysis of genome sequences. It is a cross disciplinary activity, including aspects of computer science, software engineering, molecular biology, and mathematics. The past few years have seen vast improvements in the algorithms used to analyze sequence data, and an increasing range of bioinformatics software has been developed and released into the public domain by way of the internet.

**Table 2.2.** List of genome sequencing of selected LAB

| Species | Strain | Size (Mb) | GC (mol%) | Origin/use | Institution[a] | Status[b] |
|---|---|---|---|---|---|---|
| ***Lactococcus*** | | | | | | |
| *lactis* subsp. *lactis* | IL1403 | 2.37 | 35.5 | Cheese, model strain | INRA and Genoscope, France | P |
| *lactis* subsp. *cremoris* | MG1363 | 2.53 | 35.8 | Cheese, model strain | Univ. of Groningen, The Netherlands; Univ. College Cork, Republic of Ireland; IFR, UK;Univ. of Bielefeld, Germany | C |
| ***Lactobacillus*** | | | | | | |
| *acidophilus* | NCFM | 1.99 | 34.7 | Probiotic | NC State Univ., California Poly. State Univ., USA | P |
| *brevis* | ATCC 367 | 2.29 | 46.2 | Beer, sourdough | UC San Diego, USA; JGI | C |
| *casei* | Shirota | 3.04 | 46.3 | Probiotics | Yakult, Japan | NP |
| *delbrueckii* subsp. *bulgaricus* | ATCC 11842 | 1.86 | 49.7 | Yoghurt | INRA, Genoscope, France | C |
| *gasseri* | ATCC 33323 | 1.95 | 35 | Human, probiotic | NC State Univ., USA; JGI | C |
| *helveticus* | CM4 | 2.03 | 37.1 | Functional foods | Calpis, Kitasato Univ., Japan | NP |
| *johnsonii* | NCC533 | 1.99 | 34.6 | Probiotic | Nestle´, Switzerland | P |
| *plantarum* | WCFS1 | 3.31 | 44.5 | Human | WCFS, The Netherlands | P |
| *rhamnosus* | HN001 | ~2.4 | 46.4 | Cheese | Fonterra Research Centre, New Zealand | NP |
| *sakei* | 23K | ~1.9 | 41.2 | Meat starter | INRA, France | C |
| *salivarius* subsp. *salivarius* | UCC118 | 1.83 | 32.9 | Human, probiotic | Univ. College Cork, Republic of Ireland | C |
| ***Enterococcus*** | | | | | | |
| *faecalis* | V583 | 3.21 | 37.5 | Clinical isolate | The Institute for Genomic Research, USA | C |
| *faecium* | DO | 2.84 | 37.9 | Opportunistic pathogen | JGI | NC |

[a] JGI, Joint Genome Institute, US Department of Energy. [b] P, Public; C, complete (to be published); NP, not public; NC, not completed.

The most common use of bioinformatics is the search for sequence similarity with homologous genes/gene products deposited in the numerous nucleotide and protein databases worldwide. The basic local alignment search tool (BLAST) is the most widely used program for such analysis (Altschul, 1997). The molecular database collection 2007 updated includes 968 databases (Galperin, 2006). Selected useful bioinformatic resources are listed in Table 2.3.

**Table 2.3.** Bioinformatic resources

| **Databases** | | |
|---|---|---|
| General | DDBJ (Japan) | http://www.ddbj.nig.ac.jp/ |
| | EMBL (EU) | http://www.ebi.ac.uk/Databases/ |
| | GenBank (USA) | http://www.ncbi.nlm.nih.gov/ |
| Genomes | TIGR | http://www.tigr.org/tdb/mdb/mdbcomplete.html |
| | JGI | http://www.jgi.doe.gov/JGI_microbial/html/index.html |
| | Sanger | http://www.sanger.ac.uk/Projects/Microbes/ |
| | NCBI | http://ncbi.nlm.nih.gov/Genomes/index.html |
| Lists of genomes in progress | | http://wit.integratedgenomics.com/GOLD/<br>http://www.tigr.org/tdb/mdb/mdbinprogress.html |
| **Data acquisition (search engines)** | | |
| Keyword | Entrez | http://www.ncbi.nlm.nih.gov/Entrez/ |
| Similarity | BLAST | http://www.ncbi.nlm.nih.gov/BLAST/ |

Lactic acid bacteria have been isolated form various food sources in Thailand such as fermented fissh (pla-ra, pla-paengdaeng, pla-chao, pla-chom, pla-som, etc.) and shrimp (kung-chom), fermented pork (nham), fermented vegetables (phak-gard-dong, phak-koom-dong, thuangok-dong, hom-dong, etc.), tea leaves (miang), sweetened rice (khaomak), etc. (Tanasupawat & Komagata,1995). These fermented foods are used for main- and side dishes, condiments, desserts and so on. The lactic acid bacteria are responsible for the souring and ripening of these foods. Some of these foods contain a high concentration of salt (pla-ra and pla-chom).*Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Staphylococcus* strains have been isolated from the above-mentioned foods so far (Tanasupawat & Komagata, 1995; Tanasupawat *et al.*, 1998).

### 2.1.1 The genus Lactobacillus

The genus Lactobacillus, consisting of a large number of diverse species, are Gram-positive, nonsporeforming rods, catalase-negative when growing without a heme source (e.g., blood), usually nonmotile, occasionally nitrate reducers and heterogenous with 32-53 mol% G+C contents in the DNA. They utilize glucose fermentatively and may be either homofermentative, producing more than 85% lactic acid from glucose, or heterofermentative, producing lactic acid, CO2, ethanol, and/or acetic acid in equimolar amounts. The type species is *Lactobacillus delbrueckii* Leichmann 1896 (Beijerinck, 1901). The classical division of the lactobacilli was based on their fermentative characteristics: (1) obligately homofermentative; (2) facultatively heterofermentative; and (3) obligately heterofermentative. Key characteristics of the 3 groups were listed in Table 2.4-2.6 (Hammes & Hertel. 2006). This division suited the interest of food microbiologists. Several lactobacilli of group 1 ans 2 and some of the heterofermentative group 3 latobacilli are either used in fermented foods, but group 3 are also commonly associated with food spoilage. On the basis of comparative analysis of 16S rRNA sequences, the phylogenetic relatedness of lactobacilli was investigated by using the 16S rRNA sequences of all species validly described (Hammes & Hertel. 2006) was shown in Fig. 2.2 The genus *Lactobacillus* constitutes together with the genus *Pediococcus* the family Lactobacillaceae and presently comprises 80 recognized species and 15 subspecies.

Media for the isolation of lactobacilli must take into account the aciduric or acidophilic nature of these organisms and their complex nutritional requirements. In some cases, species have adapted to extreme environmental conditions and can only grow on media that simulate their natural habitat. This includes in some cases even strictly anaerobic growth conditions. All media must contain adequate growth factors, usually with yeast extract as a source of vitamins, as well as peptone, manganese, acetate, and the stimulatory Tween 80. A low pH, ranging between 4.5 and 6.2 favors growth. In some habitats, particular spoilage situations, lactobacilli may constitute the only organisms present; more often, they occur together with other organisms, which may include other LAB and yeasts. MRS broth or agar can be used as a general culture medium for all-purpose because of its ability to support a variety of LAB and also used as a basal medium for performing tolerance tests such as temperature, pH, alcohol and salt. The MRS name originates from the formula of deMan, Rogosa, and Sharpe (1960). The glucose normally found in MRS broth may be omitted or replaced

by the others carbon sources for detection of gas/acid from the fermentation of carbohydrates.

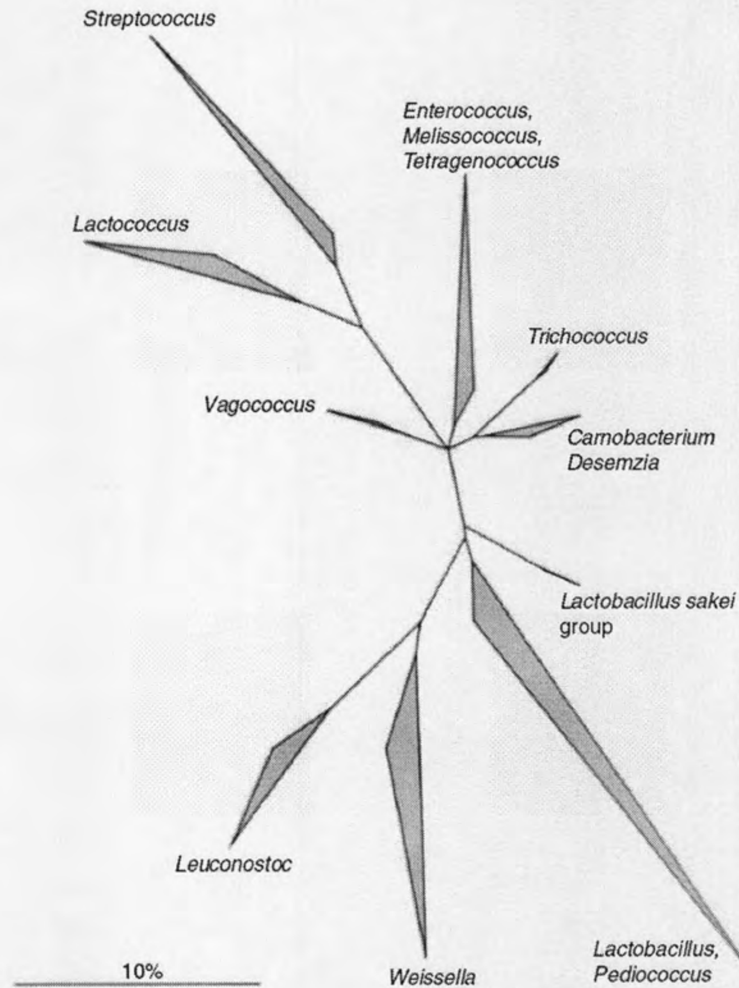



**Fig. 2.2.** Phylogenetic tree depicting groups of the family Lactobacillaceae. The consensus tree is based on maximum parsimony analyses of all available, at least 90% complete 16S rRNA sequences of the depicted genera and/or species. The topology was evaluated and corrected according to the results of distance matrix and maximum likelihood analyses with various data sets. Alignment positions that share identical residues in at least 50% of all sequences of the depicted genera were considered. The bar indicates 10% estimated sequence divergence. (from Hammes & Hertel., 2006)

**Table 2.4**. Key characteristics of the group 1 Lactobacilli: obligately homofermentatives.

| Species | Peptidoglycan type | G+C content (mol%) | Lactic acid isomer(s) | Growth (°C) 15/45 | $NH_3$ from arginine | Carbohydrates fermented: Amygdalin | Cellobiose | Galactose | Lactose | Maltose | Mannitol | Mannose | Melibiose | Raffinose | Salicin | Sucrose | Trehalose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L. acidophilus* | Lys-DAsp | 34–37 | DL | –/+ | – | + | + | + | + | + | – | + | d | d | + | + | d |
| *L. amylolyticus* | Lys-DAsp | 39 | DL | –/+ | – | d | – | + | – | + | – | + | d | d | d | + | – |
| *L. amylophilus* | Lys-DAsp | 44–46 | L | +/– | ND | – | – | + | – | + | – | + | – | – | – | – | – |
| *L. amylovorus* | Lys-DAsp | 40–41 | DL | –/+ | ND | + | + | + | – | + | – | + | – | – | + | + | + |
| *L. animalis* | Lys-DAsp | 41–44 | L | –/+ | – | d | + | ND | d | + | – | ND | + | d | + | ND | d |
| *L. aviarius* subsp. *aviarius* | Lys-DAsp | 39–43 | DL | /ND | ND | d | ı | d | d | ı | | ı | d | ı | ı | ı | ı |
| *L. aviarius* subsp. *araffinosus* | Lys-DAsp | 39–43 | L(D) | –/ND | ND | d | d | – | – | + | – | + | – | – | d | + | + |
| *L. crispatus* | Lys-DAsp | 35–38 | DL | –/+ | – | + | + | + | + | + | – | + | – | – | + | + | – |
| *L. delbrueckii* subsp. *delbrueckii* | Lys-DAsp | 49–51 | D | –/+ | d | – | – | – | – | d | – | + | – | – | – | + | d |
| *L. delbrueckii* subsp. *bulgaricus* | Lys-DAsp | 49–51 | D | –/+ | – | – | d | – | + | – | – | – | – | – | – | – | – |
| *L. delbrueckii* subsp. *lactis* | Lys-DAsp | 49–51 | D | –/+ | d | + | d | d | + | + | – | + | – | – | + | + | + |
| *L. equi* | ND | 38–40 | DL | –/+ | ND | – | – | + | + | + | + | d | + | + | – | + | – |
| *L. farciminis* | Lys-DAsp | 34–36 | L(D) | +/– | + | + | + | + | + | + | – | + | – | – | + | + | + |
| *L. gallinarum* | Lys-DAsp | 36–37 | DL | +/+ | ND | + | + | + | d | + | – | + | + | + | + | + | – |
| *L. gasseri* | Lys-DAsp | 33 35 | DL | –/+ | – | + | + | + | d | d | – | + | d | d | + | + | d |
| *L. helveticus* | Lys-DAsp | 38–40 | DL | –/+ | – | – | – | + | + | d | – | d | – | – | – | – | d |
| *L. iners* | Lys-DAsp | 34.4 | L | –/ND | – | ND | ND | ND | – | d | – | ND | – | – | ND | – | – |
| *L. johnsonii* | Lys-DAsp | 33–35 | DL | +/+ | ND | + | + | + | d | + | – | + | d | d | + | + | d |
| *L. kefiranofaciens* | ND | 34–35 | D(L) | –/– | ND | – | – | + | + | + | – | ND | + | + | – | + | – |
| *L. kefirgranum* | Lys-DAsp | 34–39 | D(L) | w/– | – | – | d | + | + | + | – | + | + | + | d | d | d |
| *L. mali* | mDpm | 32–34 | L | +/– | – | + | d | d | – | – | – | + | – | – | + | + | + |
| *L. manihotivorans* | Lys-DAsp | 48.4 | L | + | + | + | + | + | + | + | – | + | + | + | + | + | + |
| *L. nagelii* | mDpm[b] | ND | DL | +/+ | – | + | + | + | – | + | + | + | – | – | + | + | + |
| *L. pantheris* | mDpm[b] | 52.7 | D | +/– | – | – | + | + | + | + | – | + | – | – | + | – | + |
| *L. psittaci* | ND | ND | ND | ND | – | ND | ND | ND | – | ND | – | ND | – | + | ND | + | ND |
| *L. ruminis* | mDpm | 44–47 | L | /d | | ı | ı | ı | d | ı | | ı | ı | ı | ı | ı | |
| *L. salivarius* subsp. *salivarius* | Lys-DAsp | 34–36 | L | –/+ | – | – | – | + | + | + | + | – | + | + | – | + | + |
| *L. salivarius* subsp. *salicinius* | Lys-DAsp | 34–36 | L | –/+ | – | – | – | + | + | + | + | – | + | + | + | + | + |
| *L. sharpeae* | mDpm | 53 | L | +/– | – | + | + | + | + | + | – | + | – | – | + | – | – |

Symbols and abbreviations: +, 90% or more of strains are positive; –, 90% or more are negative; d, 11–89% of strains are positive; w, weak positive reaction; ND, no data available; (), isomers in parenthesis indicate <15% of total lactic acid; and mDpm, *meso*-diaminopimelic acid.
The symbols and abbreviations are used in Table 5 and 6. Copied from Hammes & Hertel., 2006.

**Table 2.5.** Key characteristics of the group 2 Lactobacilli: facultatively heterofermentatives.

| Species | Peptidoglycan type | G+C content (mol%) | Lactic acid isomer(s) | Growth (°C) 15/45 | Carbohydrates fermented: Amygdalin | Arabinose | Cellobiose | Esculin | Gluconate | Mannitol | Melezitose | Melibiose | Raffinose | Ribose | Sorbitol | Sucrose | Xylose |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L. acetotolerans* | Lys-DAsp | 35–36.5 | DL | –/– | – | – | d | + | – | d | – | – | – | d | – | – | – |
| *L. acidipiscis* | Lys-DAsp | 39–42 | L(+) | –/– | d | d | – | – | – | d | – | – | – | + | – | d | – |
| *L. agilis* | mDpm | 43–44 | L | –/+ | + | – | + | + | – | + | + | + | + | + | d | + | – |
| *L. algidus* | mDpm | 36.8 ± 3 | L+ | +/– | d | + | d | + | – | – | – | d | d | + | – | d | – |
| *L. alimentarius* | Lys-DAsp | 36–37 | L(D) | +/– | ND | d | + | + | + | – | – | – | – | + | – | + | – |
| *L. arizonensis* | mDpm[b] | 48 | DL | +/+ | ND | d | + | ND | ND | + | ND | + | + | d | + | + | – |
| *L. bifermentans* | Lys-DAsp | 45 | DL | +/– | – | – | – | – | – | + | – | – | – | + | + | – | – |
| *L. casei* | Lys-DAsp | 45–47 | L | +/– | + | – | + | + | + | + | + | – | – | + | + | + | – |
| *L. coryniformis* subsp. *coryniformis* | Lys-DAsp | 45 | D(L) | +/– | – | – | – | d | + | + | – | d | d | – | d | + | – |
| subsp. *torquens* | Lys-DAsp | 45 | D | +/– | – | – | – | – | + | + | – | – | – | – | – | + | – |
| *L. curvatus* subsp. *curvatus* | Lys-DAsp | 42–44 | DL | +/– | d | – | + | + | – | – | – | – | – | + | – | d | – |
| subsp. *melibiosus* | Lys-DAsp | 42–44 | DL/L(D) | +/– | – | – | d | + | + | – | – | + | – | + | – | + | – |
| *L. cypricasei* | ND | ND | ND | –/– | ND | d | + | + | ND | – | – | – | – | d | – | d | – |
| *L. fornicalis* | ND | 37 | DL | –/– | + | – | + | + | ND | + | + | – | – | + | + | + | – |
| *L. fuchuensis* | mDpm | 41–41.7 | L(D) | +/ND | + | – | + | + | + | – | – | – | – | + | – | – | – |
| *L. graminis* | Lys-DAsp | 41–43 | DL | +/– | + | – | + | + | – | – | – | – | – | – | – | + | + |
| *L. hamsteri* | Lys-DAsp | 33–35 | DL | –/ND | ND | + | + | + | + | + | – | + | + | + | + | + | d |
| *L. homohiochii* | Lys-DAsp | 35–38 | DL | +/– | – | – | d | ND | – | d | – | – | – | d | – | – | – |
| *L. intestinalis* | Lys-DAsp | 33–35 | DL | –/+ | – | – | d | – | ND | + | – | d | d | d | – | + | – |
| *L. jensenii* | Lys-DAsp | 35–37 | D | –/+ | + | – | + | + | – | d | – | – | – | +[d] | – | + | – |
| *L. kimchii* | Lys-DAsp[b] | 35 | DL | +/– | + | + | + | + | + | – | + | – | – | + | – | + | + |
| *L. murinus* | Lys-DAsp | 43–44 | L | –/+ | d | + | + | + | – | d | – | + | + | + | – | + | – |
| *L. paracasei* subsp. *paracasei* | Lys-DAsp | 45–47 | L[c] | +/d | + | – | + | + | + | + | + | – | – | + | d | + | – |
| *L. paracasei* subsp. *tolerans* | Lys-DAsp | 45–47 | L | +/– | – | – | – | – | w | – | – | – | – | – | – | – | – |
| *L. paralimentarius* | Lys-DAsp[b] | 37–38 | ND | +/+ | + | – | + | + | – | – | – | – | – | + | – | + | – |
| *L. paraplantarum* | mDpm | 44–45 | DL | +/– | + | d | + | + | + | + | + | + | d | + | d | + | – |
| *L. pentosus* | mDpm | 46–47 | DL | +/– | + | + | + | ND | + | + | d | + | + | + | + | + | + |
| *L. perolens* | Lys-DAsp | 49–53 | L | +/– | + | d | + | + | + | – | + | + | + | – | d | + | d |
| *L. plantarum* | mDpm | 44–46 | DL | +/– | + | d | + | + | + | + | + | + | + | + | + | + | d |
| *L. rhamnosus* | Lys-DAsp | 45–47 | L | +/+ | + | d | + | + | + | + | + | – | – | + | + | + | – |
| *L. sakei* subsp. *sakei* | Lys-DAsp | 42–44 | DL/L(D) | +/– | – | d | d | + | + | – | – | + | – | + | – | + | – |
| *L. sakei* subsp. *carnosus* | Lys-DAsp | 42–44 | DL/L(D) | +/– | – | d | d | + | + | – | – | + | – | + | – | + | – |
| *L. zeae* | Lys-DAsp | 48–49 | L(D) | +/+ | + | – | + | + | + | + | + | – | – | + | – | + | – |

Symbols and abbreviations, refer to footnote in Table 4. Copied from Hammes & Hertel., 2006.

**Table 2.6.** Key characteristics of the group 3 Lactobacilli: obligately heterofermentatives.

| Species | Peptidoglycan type | G+C content (mol%) | Growth (°C) 15/45 | $NH_3$ from arginine | Carbohydrates fermented | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Arabinose | Cellobiose | Esculin | Galactose | Maltose | Mannose | Melezitose | Melibiose | Raffinose | Ribose | Sucrose | Trehalose | Xylose |
| *L. brevis* | Lys-DAsp | 44–47 | +/– | + | + | – | d | d | + | – | – | + | d | + | d | – | d |
| *L. buchneri* | Lys-DAsp | 44–46 | +/– | + | + | – | d | d | + | – | + | + | d | + | d | – | d |
| *L. coleohominis*[b] | mDpm | ND | –/+ | – | – | – | – | – | d | – | – | – | – | + | – | – | – |
| *L. collinoides* | Lys-DAsp | 46 | +/– | + | + | – | + | + | + | – | + | + | – | + | – | – | + |
| *L. diolivorans* | ND | 40 | +/– | ND | + | – | – | + | + | – | – | + | – | + | – | – | + |
| *L. durianis* | ND | 43.3 | +/– | – | + | – | d | d | – | – | – | – | – | + | – | – | + |
| *L. ferintoshensis* | mDpm[c] | ND | +/– | + | + | d | + | + | + | + | d | d | d | + | + | + | + |
| *L. fermentum* | Orn-DAsp | 52–54 | –/+ | + | d | d | – | + | + | w | – | + | + | + | + | d | d |
| *L. fructivorans* | Lys-DAsp | 38–41 | +/– | + | – | – | – | – | d | – | – | – | – | w | d | – | – |
| *L. frumenti* | Lys-DAsp | 43–45 | –/+ | +? | d | + | + | + | + | + | d | + | + | + | + | + | – |
| *L. hilgardii* | Lys-DAsp | 39–41 | +/– | + | – | – | – | d | + | – | d | – | – | + | d | – | + |
| *L. kefiri* | Lys-DAsp | 41–42 | +/– | + | d | – | – | – | + | – | – | + | – | + | – | – | – |
| *L. kunkeei* | Lys-DAsp | ND | +/– | – | ND | – | – | – | – | – | – | – | w | – | + | – | – |
| *L. lindneri* | Lys-DAsp | 35 | +/– | – | – | – | – | – | + | – | – | – | – | – | – | – | – |
| *L. malefermentans* | Lys-DAsp | 41–42 | +/– | + | – | – | – | – | + | – | – | – | – | + | – | – | – |
| *L. mucosae* | Lys-DAsp | 46–49 | –/+ | + | d | – | + | d | + | – | – | d | d | + | + | – | d |
| *L. oris* | Lys-DAsp | 49–51 | –/d | – | + | d | d | + | + | d | – | + | + | + | + | d | + |
| *L. panis* | Lys-DAsp | 48 | –/+ | – | + | – | + | + | + | + | – | + | + | + | + | – | + |
| *L. parabuchneri* | Lys-DAsp | 44 | +/ND | + | + | – | – | + | + | ND | + | + | + | + | + | – | – |
| *L. parakefiri* | Lys-DAsp | 41–42 | +/+ | + | + | – | – | + | + | – | d | d | – | + | – | – | – |
| *L. pontis* | Orn-DAsp | 53–55 | +/+ | + | – | – | – | d | + | – | – | d | d | + | + | ND | – |
| *L. reuteri* | Lys-DAsp | 40–42 | –/+ | + | + | – | ND | + | + | – | – | + | + | + | + | – | – |
| *L. sanfranciscensis* | Lys-Ala | 36–38 | +/– | – | – | – | ND | d | + | – | – | – | – | d | d | – | – |
| *L. suebicus* | mDpm | 40 | +/d | ND | + | d | – | + | + | ND | – | d | – | + | d | – | + |
| *L. vaccinostercus* | mDpm | 36 | –/– | – | + | w | – | w | + | – | – | – | – | + | – | – | + |
| *L. vaginalis* | Orn-DAsp | 38–41 | –/+ | ND | – | – | d | + | + | + | – | + | + | d | + | – | – |

Symbols and abbreviations: +?, not determined; and for other symbols and abbreviations, refer to footnote in Table 4. Copied from Hammes & Hertel., 2006.

Sequence analysis of the phenylalanyl-tRNA synthase alphasubunit (*phe*S) housekeeping gene has been proved to be a robust approach for the identification of enterococci (Naser *et al.*, 2005). *Lactobacillus brevis* strain LMG 11494 (=NCFB 1058) isolated from farmhouse red Cheshire cheese and strain LMG 11984 (=ATCC 53295) isolated from wheat were reclassified as *Lactobacillus parabrevis* sp. nov. by investigating of the *phe*S sequence analysis and confirmed by fluorescent amplified fragment length polymorphism, DNA–DNA hybridization and DNA G+C content (Vancannyt *et al.*, 2006). The neighbour-joining tree depicted in Fig. 2.3 (a) (based upon comparison of partial sequences of 309 bp) revealed the relatedness between thee strains LMG 11494 and LMG 11984 and type strains of related taxa and showed that the two strains under study constituted a distinct cluster with a gene sequence similarity of 97 %. Nearest neighbours were the type strains of *L. acidifarinae, L. hammesii, L. spicheri* and *L. zymae,* with sequence similarities in a significantly lower range of 85–87 %. *L. brevis* and other taxa of the *L. buchneri* species group were more distantly related, with sequence similarities below 82 % while the phylogenetic position of strains LMG 11494 and LMG 11984 determined by complete 16S rRNA gene sequence analysis compared with deposited sequences available in the EMBL database in Fig. 2.3 (b) classified strains LMG 11494 and LMG 11984 as part of the *L. buchneri* group with the nearest neighbours (>97% sequence similarity) *L. hammesii* and *L. brevis*, showing sequence similarities of 99.2 and 98.1%, respectively. The data clearly showed that *phe*S gene sequences are much more discriminatory than 16S rRNA.

*Lactobacillus acidipiscis* sp. nov. FS60-1$^{T}$(a.ci.di'pis.cis. L. adj. *acidus* sour; L. n. *piscis* fish; L. adj. *acidipiscis* pertaining to sour fish, an isolation source of strains of this species) isolated from fermented fish in Thailand using MRSH medium (a half-strength version of MRS medium) by Tanasupawat *et al.* (2000). Cells are Gram-positive rods, non-motile, non-sporing and occur singly, in pairs and in chains. Colonies on MRSH agar plate are circular, slightly convex with an entire margin and unpigmented. Microaerophilic. Utilizes D-glucose homofermentatively and does not produce gas from glucose. Produces L-lactic acid from glucose (>91%).Negative for catalase activity, nitrate reduction, hydrolysis of arginine, aesculin, gelatin and starch and for the formation of slime from sucrose. Most strains show no reaction in litmus milk. Grows at 25-37 °C but not at 15 or 42 °C. Does not grow at pH 4.0 or pH 8.5. Grows in 10% NaCl. Some strains grow in 12% NaCl. Produces acid from D-fructose,

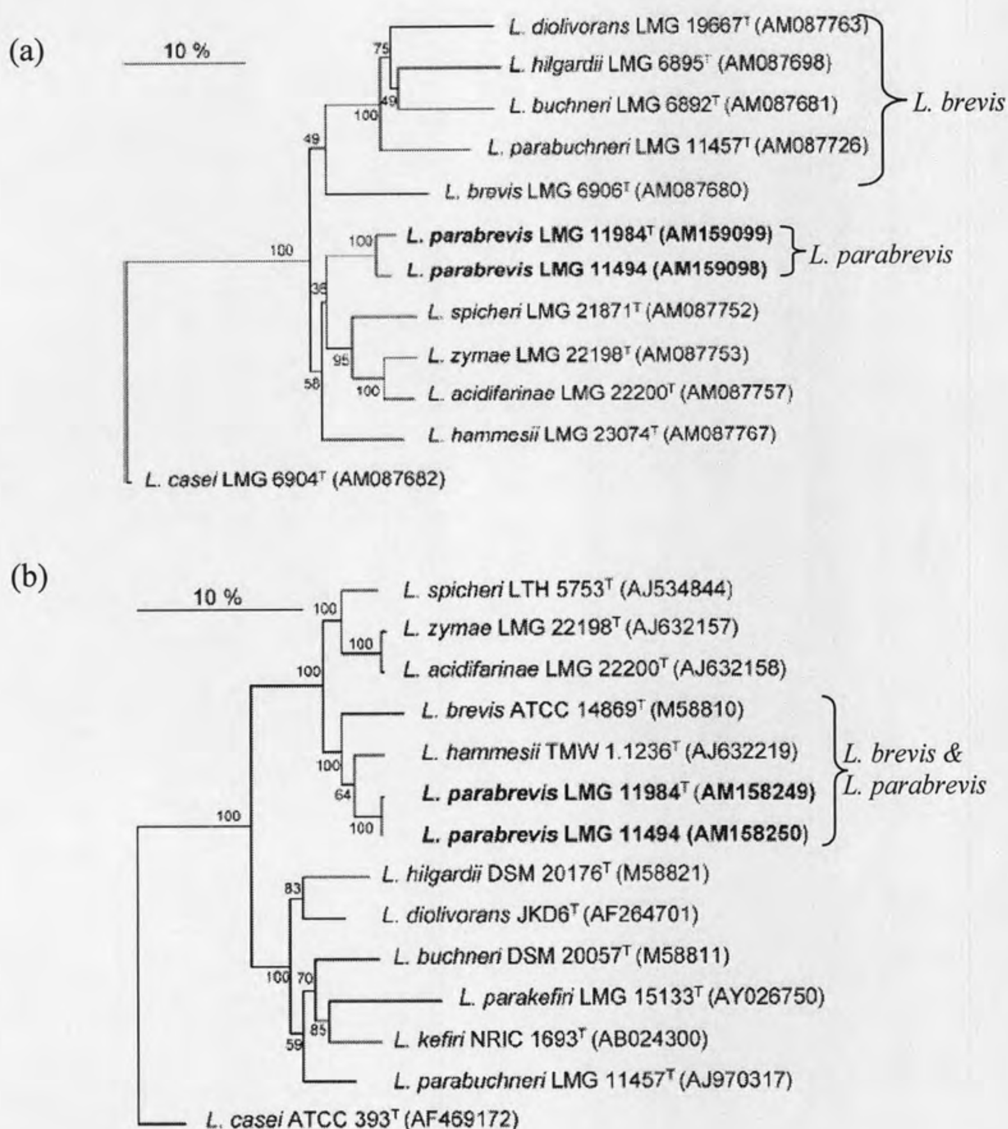



**Fig. 2.3**. Neighbour-joining tree based on the partial *phe*S gene. (a) and 16s rRNA gene sequences of *L. parabrevis* and other reference species. Bootstrap percentages after 500 simulations are shown. *Lactobacillus casei* LMG 6904T was included as an outgroup. Bar, 10% difference in nucleotide sequence. (Modified from Vancannyt *et al.*, 2006)

D-galactose, D-glucose and D-mannose but does not produce acid from cellobiose, gluconate, D-melibiose, D-melezitose, methyl $\alpha$-D-glucoside, raffinose, D-sorbitol and dxylose. Some strains produce acid weakly from lactose and salicin. Variable reactions for D-amygdalin, L-arabinose, glycerol, maltose, D-mannitol, L-rhamnose, sucrose and D-trehalose. Requires niacin and calcium pantothenate for growth. Major cellular fatty acids are straight-chained $C_{16:0}$ and $C_{18:1}$. L-Lys-D-Asp in the cell wall. The G-C content of the DNA ranges from 38.6 to 41.5 mol%. *Lactobacillus cypricasei* Lawson *et al.* 2001 was reclassified as a later heterotypic synonym of *Lactobacillus acidipiscis* Tanasupawat *et al.* 2000 by multilocus sequences analysis (MLSA)-based identification of three housekeeping genes that code for phenylalanyl-tRNA synthase $\alpha$-subunit (*pheS*), RNA polymerase $\alpha$-subunit (*rpoA* ) and $\alpha$-subunit of ATP synthase (*atpA*) (Naser *et al.*, 2006). The MLSA data revealed close relatedness between *Lactobacillus cypricasei* and *Lactobacillus acidipiscis*, with 99.8-100% *pheS, rpoA* and *atpA*.

### 2.1.2 The genus Enterococcus

Originally classified in the 1930s as Group D *Streptococci, Enterococci* were officially given genus status in 1984 after hybridization studies showed a more distant relationship to *Streptococci*. *Enterococci* are gram-positive, spherical or ovoid bacteria, which arranged in single cells, pairs, or chains, non-spore-forming, oxidase- and catalase-negative. They are naturally found as part of the digestive tract flora in many organisms including humans. However, enterococci are ubiquitous and can be found free-living in soil, on plants, or in dairy products. They are robust microbes able to tolerate relatively high salt and acid concentrations.

The genus *Enterococcus* comprises 33 validly published species (www.bacterio.cict.fr/e/enterococcus.html). Phenotypic characterization of this genus has been discussed. It is generally agreed that the genus *Enterococcus* comprises gram-positive cocci that are catalase negative, usually facultative, anaerobic bacteria that grow in 6.5% NaCl, 40% bile salts, and 0.1% methylene blue milk and at pH 9.6. They grow at 10 and 45°C and can resist 30 min at 60°C. There is clear evidence of the genotypic identity of *Enterococcus*, based on molecular studies. Four species groups; *Enterococcus feacium, Enterococcus avium, Enterococcus gallinarum,* and *Enterococcus cecorum* and other miscellaneous species group, e.g. *Enterococcus*

*faecalis, Enterococcus saccharolyticus, Enterococcus sulfureus*, and *Enterococcus dispar* were revealed by 16S rDNA comparative sequence analysis (Hardies & Whiley, 1997). There are two species of *Enterococci* which opportunistic cause complicated abdominal infections, skin and skin structure infections, urinary tract infections and infections of the blood stream. In a study conducted between 1995 and 1997 data were collected from over 15,000 *Enterococcus* isolates. Of those, less than 2% of *E. faecalis* were found to be resistant to ampicillin and vancomycin, whereas 83% of the *E. faecium* isolates were resistant to ampicillin and 52% were resistant to vancomycin. *E. faecium* is known to have a resistance to several types of antibiotics including quinolones and aminoglycosides. Resistance to penicillin was first observed in *E. faecium* in 1983 and in 1988 the first cases of resistance to the "antibiotic of last resort", vancomycin, were detected in Europe. Vancomycin resistant strains of *E. faecium* were reported in the US in 1989.

16S rRNA gene sequencing, DNA–DNA hybridization and SDS-PAGE of whole-cell proteins are among the most common techniques currently used for Enterococcus species identification. On the basis of 16S rRNA gene sequences, a phylogenetic tree was constructed from which species groups can be distinguished. Homology values within the genus *Enterococcus* ranged from 93.7% to 99.8% for a 1,452-nucleotide region limited discriminating power for several closely related enterococcal species, e.g. the *E. faecium* species group. Because this is a costly and time-consuming method, several other tools have been studied for the ability of identification. Recently, the gene that encodes α- subunit of ATP synthase (*atp*A), the a-subunit of bacterial RNA polymerase (rpoA) and phenylalanyl-tRNA synthase a-subunit (*phe*S) have been used as an alternative identification tool for all enterococcal species (Naser *et al.*, 2005). The data clearly show that rpoA and pheS gene sequences are much more discriminatory than 16S rRNA. The phylogenetic tree of all validly described *Entercoccus* species based on *rpo*A sequence was shown in Fig. 2.4 (Naser *et al.*, 2005). Reclassification of *Enterococcus flavescens* Pompei et al. 1992 as a later synonym of *Enterococcus casseliflavus* (ex Vaughan et al. 1979) Collins et al. 1984 and *Enterococcus saccharominimus* Vancanneyt et al. 2004 as a later synonym of *Enterococcus italicus* Fortina et al. 2004 were proposed by the used of MLSA techniques ( Naser *et al.*, 2006).

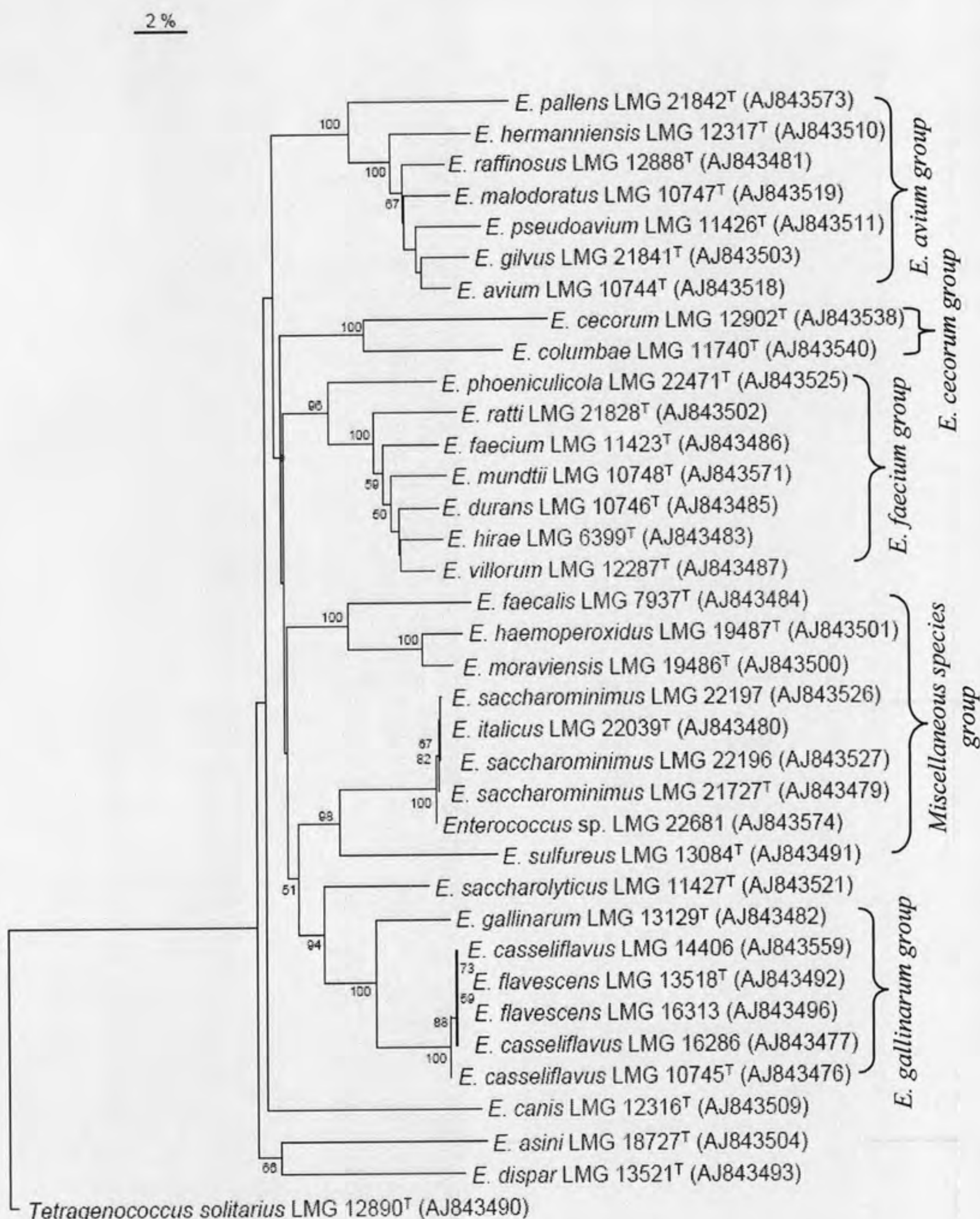



**Fig. 2.4.** Neighbour-joining tree based on *rpoA* gene sequences of enterococcal strains (Naser *et al.*, 2006). *Tetragenococcus solitarius* LMG 12890T was included as an outgroup. Bootstrap percentages ($\geq$ 50) after 500 simulations are shown. Bar, 2 % sequence divergence. Four species group and a miscellaneous species group are showed in bracket.

## 2.2 GABA (γ-aminobutyric acid) producing LAB

Since, GABA (γ-aminobutyric acid, 4-aminobutanoic acid, $C_4H_9NO_2$) discovered in 1950 by Awapara *et al.*, that it exists in the vertebrate central nervous system which is also usually an inhibitory neurotransmitter. GABA acts like a brake to the excitatory neurotransmitters that lead to anxiety. People with too little GABA tend to suffer from anxiety disorders, so GABA and their agonists have been used for medicine and also added in some functional foods commercially for the propose of brain metabolic function and improvement of hypertension (de Wardener, 2001; Hayakawa *et al*, 2004). GABA production is known to occur in cheese production. It was suggested that GABA has a direct or indirect impact on cheese flavor and correlated increased production of $CO_2$ and GABA in cheese with an increase in the number of eyes (Doyle & Meng, 2006). Lactic acid bacteria (LAB), especially lactobacilli were found to have ability to produce GABA from glutamic acid by catalytic activity of glutamic acid decarboxylase (L-glutamate 1-carboxy-lyase, Gad; EC 4.1.1.15). GABA producing LAB comes to being interested in research and development both in scientific and industrial as GABA containing fermented food processing by *Lactobacillus brevis* IFO3960 was patented in 2002 (US Patent Pub. No. US2002/0106424 A1). The chemical structure of GABA and enzymatic reaction were shown in Fig. 2.5.

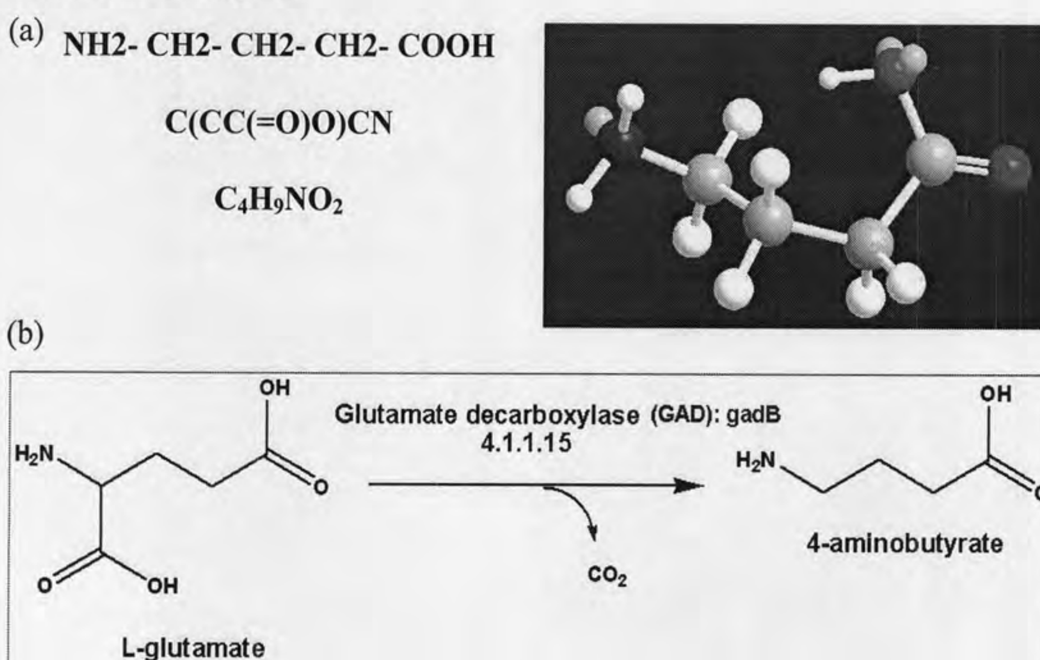



**Fig. 2.5.** Chemical structure of GABA (a) and the enzymatic reaction catalyzed by glutamate decarboxylase (b).Te ball and stick model created with ChemDraw.

Gad is a pyridoxal 5'-phosphate (PLP) –dependent enzyme, which catalyzes the irreversible α-decarboxylation of L-glutamate to γ-aminobutyrate (GABA) as shown in Fig. 2.5 (b). Gad has a crucial role in the vertebrate central nervous system where it is responsible for the synthesis of GABA, the major inhibitory neurotransmitter. Gad occurs in two isoforms, GAD65 and GAD67 in the majority of vertebrates, both active at neutral pH (Soghomonian and Martin, 1998). A unique feature of plant and yeast Gad is the presence of a calmodulin (CaM)-binding domain in the C-terminal region. Gad expression is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae* (Coleman *et al.*, 2001) while it is thought to be a stress-adapter chaperonin sensing Ca2+ signals in plant. The plant enzyme has a pH optimum of 5.8, but its activity is also significant at pH 7.3 in the presence of the Ca2+/CaM complex (Zik et al., 1998), which is ~500 kDa in size (Baum *et al.*, 1996).

Bacterial Gad has some features similar to those of the plant enzyme: it exhibits an acidic pH optimum (3.8-4.6), forms a hexamer and is expressed in response to environmental stresses (Blankenhorn et al., 1999; De Biase et al., 1999). GAD isoforms (GadA and GadB) have also been reported in some bacterial species, including the Gram-negative bacterium *Escherichia coli* (Smith *et al.*, 1992) and the Gram-positive bacterium *Listeria monocytogenes* (Cotter *et al.*, 2001). Separate expression of the two isoforms demonstrated that they are biochemically indistinguishable (De Biase *et al.*, 1996). In *E.coli* and in other enteric bacteria-both commensal and pathogenic-GadA and GadB have been identified as structural constituents of the *gad* system (De Biase *et al.*,1999; Cotter et al., 2001). Of the three acid resistance systems known in *E.coli* (Lin *et al.*, 1995, 1996), the gad system is by far the most potent and is involved in conferring acid resistance to the bacteria in stationary phase, giving them survival capacity for at least 2 h in a strongly acidic environment (pH < 2.5).

The genes encoding the two Gad isoforms, *gadA* and *gadB*, are 2100 kb apart in the *E.coli* chromosome with 99.8% identity (Blattner *et al.*, 1997).In Escherichia coli, expression of glutamate decarboxylase (GadB), a 330 kDa hexamer, is induced to maintain the physiological pH under acidic conditions, like those of the passage through the stomach en route to the intestine. GadB, together with the antiporter GadC, constitutes the gad acid resistance system, which confers the ability for bacterial survival for at least 2 h in a strongly acidic environment. GadB undergoes a pH-dependent conformational change and exhibits an activity optimum at low pH.

The crystal structures of GadB at acidic and neutral pH were determined (Capitani *et al.*, 2003). The revealed molecular details of the conformational change and the structural basis for the acidic pH optimum demonstrated that the enzyme is localized exclusively in the cytoplasm at neutral pH, but is recruited to the membrane when the pH falls. However, only one *gadB* were found in *Lactococcus lactis* subsp. *lactis* (Nomura *et al.*, 1999). The lactococcus GadB was 466 amino acid residues with 53.9 kDa. Frameshift mutation were found in *gadB* of *Lactococcus lactis* subsp *cremoris* which have no GAD activity (Nomura *et al.*, 2000)

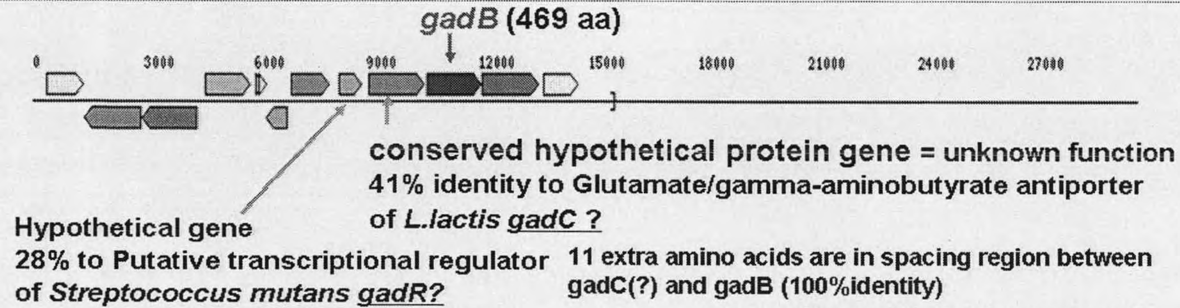
The homology of subunit primary sequence of 40 glutamate decarboxylases (Gad) of different origin was analyzed by multiple alignment. (Sukhareva & Mamaeva, 2002). Only one LAB *gadB* of *Lactobacillus lactis* was included in the analysis. A phylogenetic tree was designed on the basis of the resulting data. The following groups are distinguished in the consensus tree: archeans, bacteria, plant eukaryotes, and animal eukaryotes. The results of multiple alignments during structural analysis of the 40 Gad confirmed and extended data on conserved residues that arrange the position of the coenzyme (PLP) in the enzyme active center. The length of PLP domains in the studied proteins varies to a significantly lower extent: they contain 254 residues in animals, 244 in higher plants, 242-246 in bacteria, and 221-228 in archeans. In this case the yeast enzyme also is an exception, its PLP domains consisting of 262 amino acids. The following residues should be noted: lysine forming a Schiff base with the PLP aldehyde group, an adjacent histidine, and aspartic acid that establishes a link with nitrogen of the PLP pyridine ring. The homology of the primary sequence fragments was also found in the residues in contact with the PLP phosphate group. Comparison of the Gad amino acid sequence with that of another PLP enzyme, aspartate aminotransferase, revealed a binding site for carboxylic group of the substrate--glutamic acid.

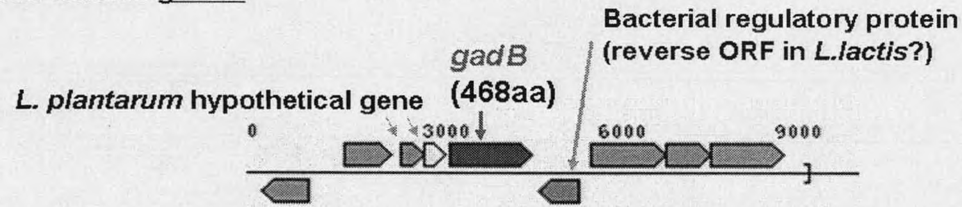
Gad enzyme of *Lactobacillus brevis* IFO12005 was purified and characterized by Ueno *et al.*(1997). The purified enzyme was dimer that shown 60 kD on SDS-PAGE and 120 kD by gel filtration. The N-terminal amino acid sequence of the purified enzyme was NH2-Met-Asn-Lys-Asn-Asp-Gln-Glu-Gln-Thr-. Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase were reported . In order to produce large amount of GABA, Gad gene cloning and further study in molecular level are important and useful for production of functional foods with beneficial effect for health.

The genome sequence data of LAB inspire and persuade the way to study on Gad activity of LAB since predicted glutamate decarboxylase genes (*gadB*) were found in released genome sequence analysis of *Lactobacillus brevis* ATCC367 and *Lactobacillus plantarum* WCSF by comparison of the known functional genes of the others microorganism. (JGT; http://www.jgi.doe.gov/) However, the predicted *gadB* genes of have not been proved for their functional properties to date so the data are valuable treasures that can be used for predict and optimize the molecular components and pathways leading to the production of interest such as flavours, health-promoting and other functional ingredients. The predicted *gadB* encoding gene of *L. brevis* ATCC 367 were summarized with annotation in compared with the predicted *gadB* of *L. plantarum* WCSF1, the known *Lactococcus lactis gadB* and that of *E. coli* (Fig. 2.6). There are 3 predicted *gadB* located separately on *L. brevis* ATCC 367 genome. The genes and their deduced amino acid are 1440, 1407 and 1881 bp which can be deduced to 479, 468 and 626 amino acid respectively. The genome sequence of *Lactobacillus plantarum* WCSF1 consists of only one predicted *gadB* (1410 bp, 469 amino acid residues). All retrieved sequences of predicted *gadB* from NCBI are in Appendix III. The predicted 479 bp genes of *L. brevis* show 99.5% identity to *gadB* of *L. brevis* IFO12005 (Ueno *et al.*, 2007; unpublished data).

**Draft genome sequence annotation of *L. brevis* ATCC367**

1. *L. brevis* ATCC367: lbre_Scaffold18 : 99.5% identity to KIT L.brevis IFO12005




2. *L. brevis* ATCC367: lbre_Scaffold49: 80.3% identity to *L.plantarum*
82.7% identity to LSF8-13

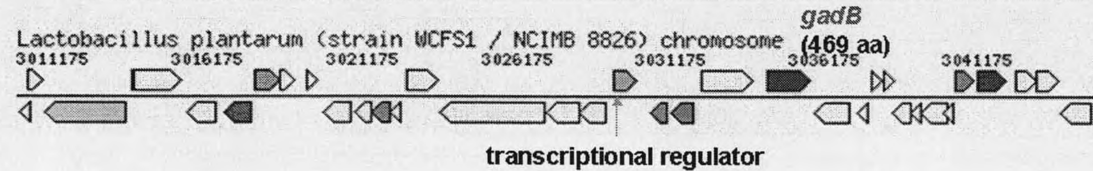



**Fig. 2.6.** Summary of the predicted *gadB* encoding gene on the draft genome sequence annotation of *Lactobacillus brevis* ATCC 367 in compared with the others predicted *gadB* and the known genes.

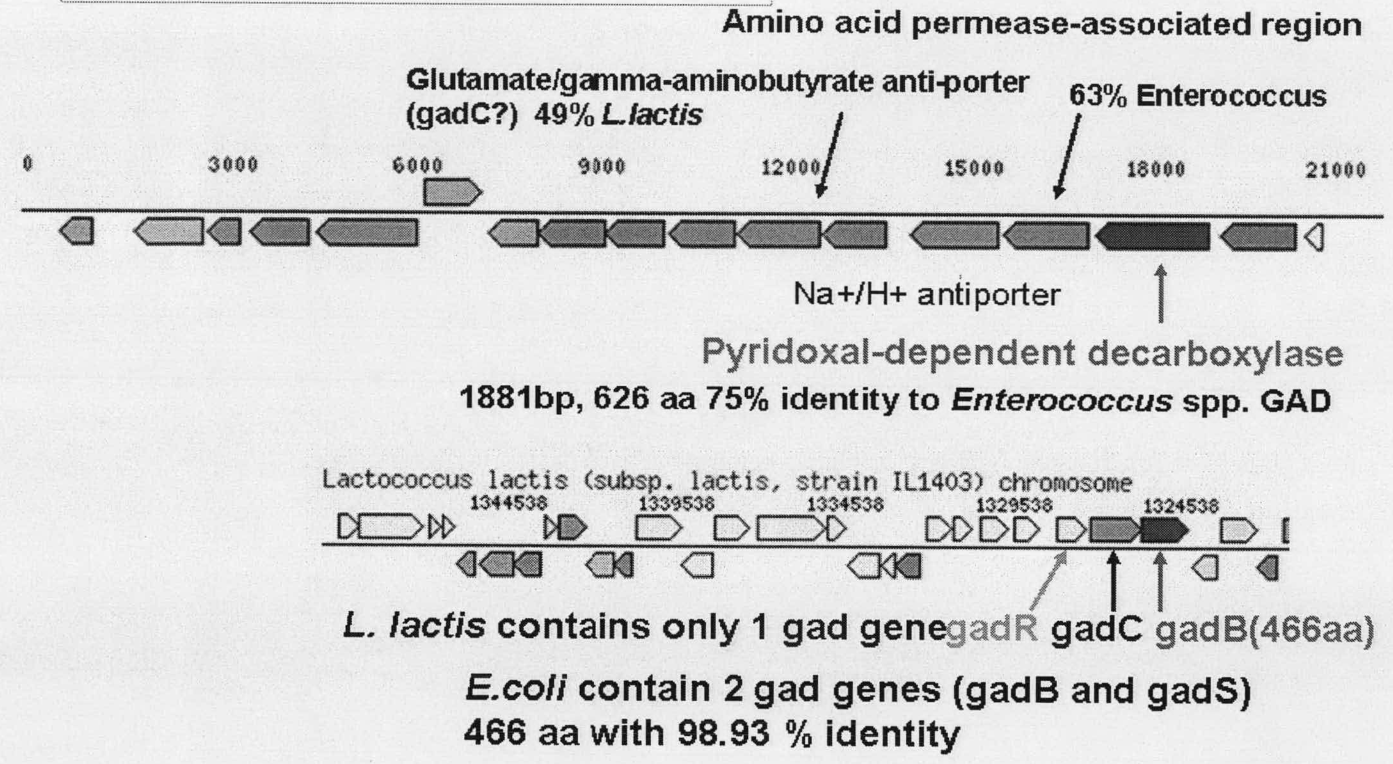



**Fig. 2.6.** Summary of the predicted *gadB* encoding gene on the draft genome sequence annotation of *Lactobacillus brevis* ATCC 367 in compared with the others predicted *gadB* and the known genes (continued).

**2.3. Starch hydrolyzing LAB**

Interestingly, LAB are able to hydrolyze starch by catalytic activities of amylase. α-amylase (1,4-α-D-glucan glucanohydrolase, E.C.3.1.2.1) is the name given to glycoside hydrolase enzymes that break down starch into glucose molecules. Raw-starch-degrading amylases are commercially important enzymes in the beverage, food, and textile industries. Starch is commonly used as a renewable raw material for the industrial production of lactic acid. The amylolytic lactic acid bacteria are attractive alternatives for this type of process, since they can directly produce lactic acid from starch. Direct fermentation of starch to L-(+)-lactic acid using *Lactobacillus amylophilus* was reported by Yumoto & Ikeda (1955). The strain can produce 30 g of L-(+)-lactic acid from 50 g of soluble starch when the pH of the culture was ranging from pH 5 to pH 6.8 at 28°C. α-amylase producing LAB in fish silage were isolated. The isolated strain was belonging to the genus *Leuconostoc* (Lindgren & Refai, 1984). An amylolytic of *Lactobacillus plantarum* was isolated from cassava roots (*Manihot esculenta* var. Ngansa) during retting (Giraud *et al.*, 1991). Investigated properties of the *L. plantarum* enzyme indicated its extracellular and acidotolerant characteristics. Nearly 70% of the enzyme activity remains at pH 4.0 after the 3rd day of retting. The α-amylase genes of *L. plantarum* A6 and *L. amylovorus* were cloned and characterized (Giraud *et* al., 1997). Various *Lactobacillus* strains exhibit amylase activity: *L. cellobiosus*, *L. amylovorus*, *L. amylophilus*, *L. plantarum*, *L. manihotivorans*, and *L. amylolyticus* (Rodiguez-Sanoja *et al.*, 2000). An extracellular α-amylase of a new LAB: *L. manihotivorans* LMG $18010^T$ was purified and characterized (Aguilar *et al.*, 2000). The molecular weight was found to be 135 kDa. The temperature and pH optimum were 55°C and 5.5, respectively. The α-amylase had good stability at pH range 5 to 6 and the enzyme was sensitive to temperature, losing activity within 1 h of incubation at 55 °C. Amylase genes from *L. amylovorus* (2,861 bp), *L. plantarum* (2,738 bp), and *L. manihotivorans* (2,705 bp) were comparative analyzed (Rodiguez-Sanoja *et al.*, 2005). The catalytic domains were highly conserved among the gene while the starch binding domains are different. Starch-binding domains (SBDs) are present in about 10% of amylases and their related enzymes. They are found in bacterial and fungal α-amylases, bacterial cyclodextrin glucanotransferases, bacterial β-amylases and fungal glucoamylases. All

of these enzymes have different catalytic properties and structures, however, all are multidomain proteins that show significant SBD sequence homology. This domain is usually localized at the C-terminal end of enzymes.

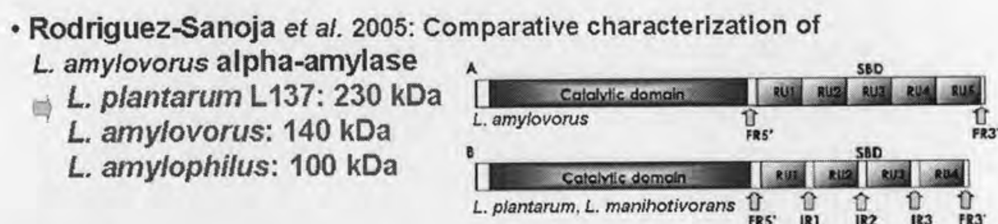
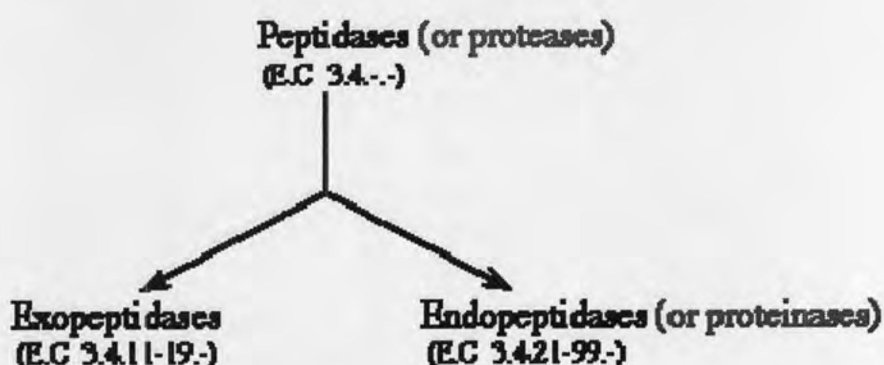



**Fig. 2.7.** Comparison of amylase gene structure *Lactobacillus amylovorus, Lactobacillus plantarum*, and *Lactobacillus manihotivorans*.

A new starch-binding domain (SBD) was recently described in α-amylases from three lactobacilli (*Lactobacillus amylovorus*, *Lactobacillus plantarum*, and *Lactobacillus manihotivorans*) as showed in Fig. 2.7. Usually, the SBD is formed by 100 amino acids, but the SBD sequences of the mentioned lactobacillus α-amylases consist of almost 500 amino acids that are organized in tandem repeats. The three lactobacillus amylase genes share more than 98% sequence identity. In spite of this identity, the SBD structures seem to be quite different. To investigate whether the observed differences in the SBDs have an effect on the hydrolytic capability of the enzymes, a kinetic study of *L. amylovorus* and *L. plantarum* amylases was developed, with both enzymes acting on several starch sources in granular and gelatinized forms. Results showed that the amylolytic capacities of these enzymes are quite different; the *L. amylovorus* α-amylase is, on average, 10 times more efficient than the *L. plantarum* enzyme in hydrolyzing all the tested polymeric starches, with only a minor difference in the adsorption capacities. *Enterococcus* strains isolated from Thai fermented foods show starch hydrolyzing activity (unpublished data). α-amylase from *Enterococci* have not been studied in detail. Analyzed genome sequence of *Enterococci* consist predicted non-experimental evidence amylase gene by its catalytic domain. The retrieved sequence of the three amylase of lactobacilli and predicted genes of *Enterococci* were in Appendix III.

**2.4. Proteinase producing LAB**

The term peptidase is used for the subset of peptide bond hydrolases (Subclass E.C 3.4.). The widely used term protease is synonymous with peptidase. Peptidases comprise two groups of enzymes: the endopeptidases and the exopeptidases, which cleave peptide bonds at points within the protein and remove amino acids sequentially from either N or C-terminus respectively. The term proteinase is also used as a synonym word for endopeptidase and four mechanistic classes of proteinases are recognized by the IUBMB. The modern scheme of nomenclature is thus:




**Scheme 2.1.** Nomenclature of proteinase (http://delphi.phys.univ-tours.fr/Prolysis/introprotease.html)

LAB have a very limited capacity to synthesize amino acids using inorganic nitrogen sources. Some strains are prototrophic for most amino acids, whereas others may require 13–15 amino acids. The quantities of free amino acids present in food often are not sufficient to support the growth of bacteria to a high cell density; therefore, they require a proteolytic system capable of utilizing the peptides and proteins present in food that hydrolyzes proteins proteins to obtain essential amino acids. All dairy lactococci used for acidification of milk (e.g., in cheese manufacture) have proteolytic activity. The lactococcal proteolytic system consists of enzymes outside the cytoplasmic membrane, transport systems, and intracellular enzymes. The transport systems on the cell membrane include oligopeptide transport, di/tripeptide transport, and amino acid transport systems (Fig. 2.8; Doyle & Meng, 2006). The transport of free amino acids contributes very little to the total growth of lactococci in milk. Two general classes of proteolytic enzymes of LAB are proteinases and

peptidases. An extracellular, membrane-anchored serine proteinase (PrtP) has been identified as being essential for this activity. Once inside the cell, peptides are hydrolyzed by peptidases.

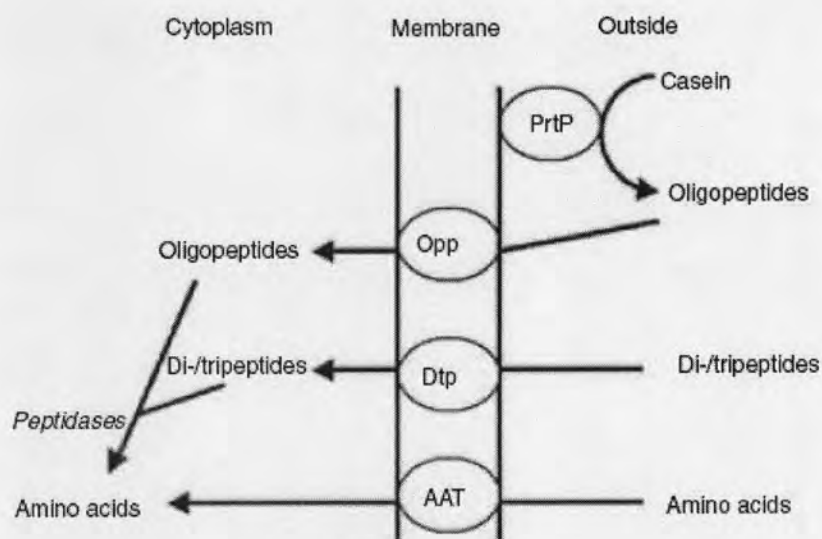



**Fig. 2.8**. Model of the lactococcal proteolytic system. Included also is transport of di- and tripeptides and free amino acids, but they contribute very little to the total growth of lactococci. PrtP, membrane-anchored proteinase; Opp, oligopeptide transport system; Dtp, di-/tripeptide transport system; AAT, amino acid transport system. (Doyle & Meng, 2006)

The conversion of peptides to free amino acids and the subsequent utilization of these amino acids is a central metabolic activity in LAB. The peptidase system is involved in the hydrolysis of exogenous peptides to obtain essential amino acids for growth and hydrolysis of peptides formed by housekeeping proteinases. The amino acids formed by this system can be utilized for processes such as protein synthesis, generation of metabolic energy, and recycling of reduced cofactors.

At least 16 peptidases from LAB have been characterized biochemically and/or genetically. Among LAB, the peptidase systems of *Lactobacillus helveticus* and *Lactococcus lactis* have been examined in greatest detail. While there are homologous enzymes common to both systems, significant differences exist in the peptidase complement of these organisms. The characterization of single and multiple peptidase mutants indicate that these strains generally exhibit reduced specific growth

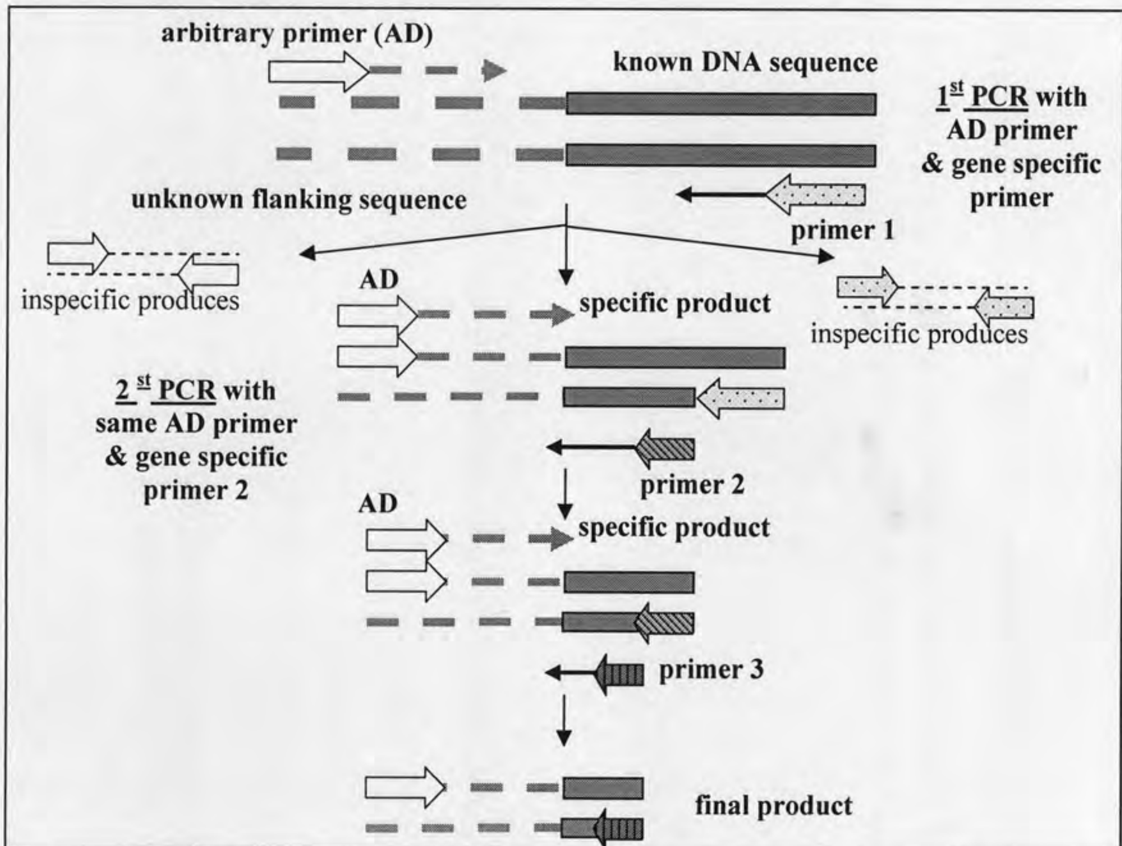
rates in milk compared to the parental strains. LAB can also catabolize amino acids produced by peptide hydrolysis. While the catabolism of amino acids such as Arg, Thr, and His is well understood, few other amino acid catabolic pathways from lactic acid bacteria have been characterized in significant detail. Increasing research attention is being directed toward elucidating these pathways as well as characterizing their physiological and industrial significance. The PrtP hydrolysis and Opp transport of peptides derived from purified casein by *Lactococcus lactis* has been investigated by subtractive evaluation of the remaining media peptides and accumulation of intracellular peptides and amino acids. The results indicate that most of the essential amino acids can be obtained from transported peptides derived from the C-terminal end (residues 161–191) of purified casein. The exception was His, which was previously shown to be a required supplement for growth on purified casein. Opp was shown to transport from ten to fourteen of the casein derived peptides ranging in size from five to ten residues. However, subsequent kinetic analysis of Opp indicates that peptides from four to at least eighteen residues can be transported with little specificity for particular side chains. These studies begin to define the potential peptidase substrates available to *Lc. lactis* during growth in milk (Chriatensen *et al*, 1999).

Recently, a draft-quality genome sequence for *L. helveticus* CNRZ32 was assembled and screened for genes encoding additional proteolytic enzymes. New endopeptidase genes were identified from the genomic sequence and their properties in hydrolysis of model bitter peptides were revealed (Sridhar *et al.*, 2005). Three endopeptidases, designated PepE2, PepF, and PepO3, appeared to be paralogs or orthologs of known endopeptidases.

**2.5 Thermal Asymmetric Interlaced (TAIL) PCR**

Liu and Whittier (1995) developed a thermal asymmetric interlaced (TAIL)–PCR strategy to isolate gene flanking regions from short known sequence genomic DNA. This strategy uses nested sequence-specific primers together with 16-bp degenerate primers (AD, arbitrary degenerate primer) in a multistep thermal cycling program. The procedure consists of the alternation of low-stringency and high-stringency cycling to allow amplification of gene-specific flanking regions. This strategy is based on thermal asymmetric PCR (Mazars *et al.*, 1991), which uses 2 primers of different length and annealing stability. PCR cycles at higher temperatures

favor the annealing of the longer gene-specific primers, while lower temperature incubation allows annealing of both specific and random primers. A typical thermal cycling program consists of 12-15 TAIL cycles containing 1 low-stringency and 2 high-stringency cycles. This technique is amenable for amplifying fragments from P1 and YAC clones (Liu and Whittier, 1995) or for mapping *Arabidopsis thaliana* T-DNA insert junctions (Liu *et al.*, 1995). Most amplified fragments range from 300-700 bp, but it can also be used for larger fragments of about 2 kb. Specific PCR-amplification products are selected by using agarose gel electrophoresis. Diagram of TAIL-PCR showed in scheme 2.2




**Scheme 2.2.** TAIL-PCR strategy to isolate unknown regions sequence from short known sequence DNA.

Specific products were not always seen in the primary reactions due to their low concentration. Size of specific product will decrease gradually. However, these specific products becomes visible after the subsequent secondary reaction. Because of high specificity, unpurified TAIL-PCR products can be directly sequenced by specific primer on the known sequence. This method is useful for identification of new genes from their conserved regions or known sequence.