LITERATURE REVIEW

1. Nham

Nham is a traditional Thai fermented pork sausage popularly consumed in various parts of Thailand. It made from minced pork, shredded cooked pork rinds, garlic, cooked rice, salt, chili, sugar, and sodium nitrite. Nham is considered a local authentic due to its flavor and texture varied in accordance which the region in which it is produced (Valyasevi and Rolle, 2002). Currently, the scale of production of Nham has been increasing from household to small industrial scale with an estimated production value of 20 million USD annually (Visessanguan *et al.*, 2004).

1.1 Production of Nham

Nham is prepared by combining of minced pork, salt and sodium nitrite in the first step. The mixture is, then thoroughly mixed with sucrose, monosodium glutamate, erythrobate, phosphate and minced cooked rice and garlic. After, thoroughly mixed the mixture was added with shredded cooked pork rinds and chili. Finally, the mixture is stuffed in casings and fermented for 3-5 day at room temperature (~30^{O}C). During fermentation, complex changes in microbiological, physico-chemical and biochemical characteristics take place that result in a significant change of Nham characteristics (Visessanguan *et al.*, 2006a,b). Mechanical processes for the production of Nham are well developed but the fermentation process is remained rely on contamination of desire microorganisms. Acid production contributes to both intrinsic quality and safety in the production of Nham, including sour taste, typical fermented aroma, firmness of texture and as well as prevent the growth of acid sensitive pathogens (Valyasevi and Rolle, 2002 and Visessanguan *et al.*, 2004). At the end of fermentation, Nham usually has a pH of 4.4–4.8 with titratable acidity values of 0.77–1.60% (Phithakpol *et al.*, 1995). Methodologies for the production of Nham are summarized in Figure 1.

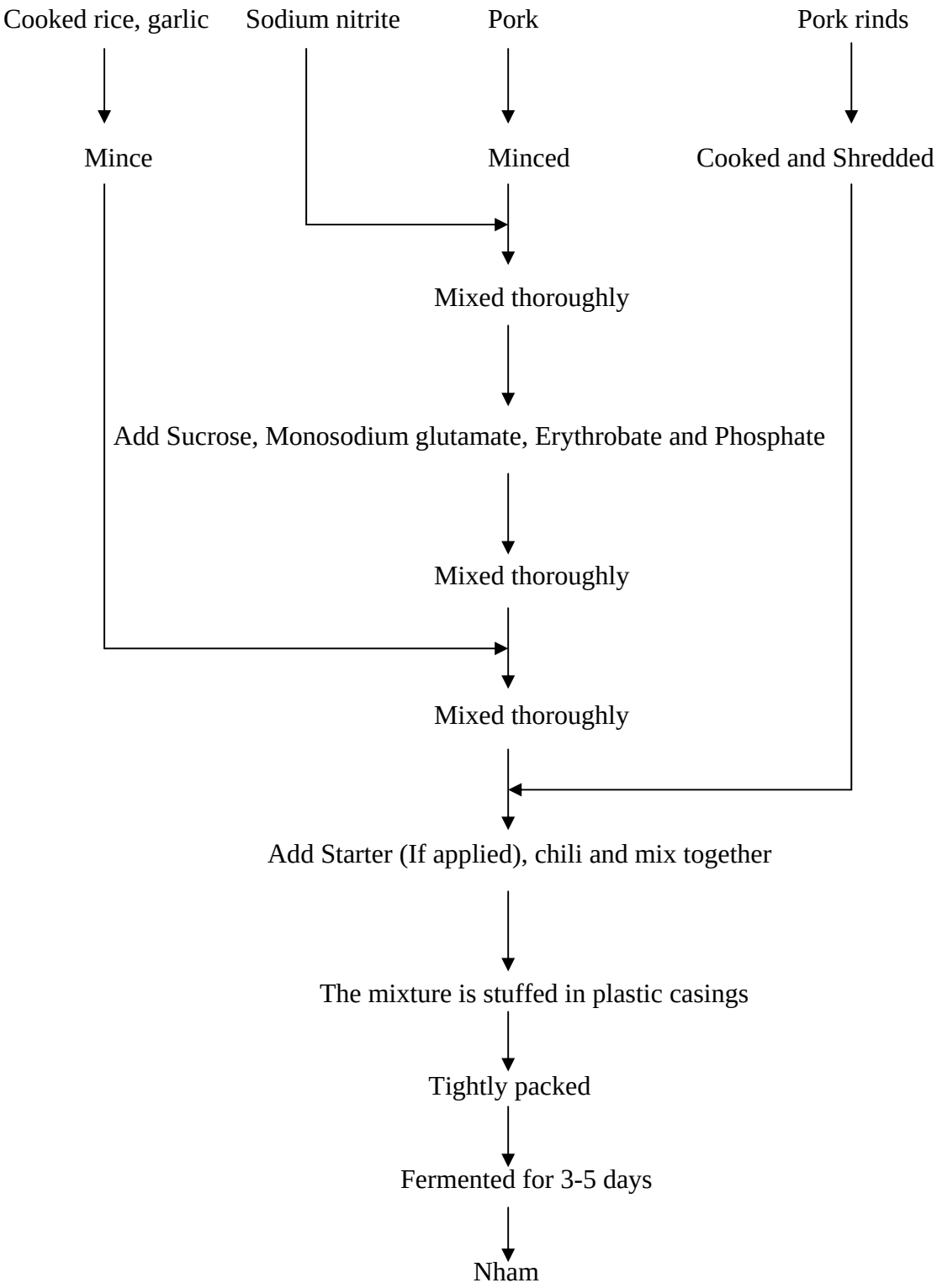


Figure 1 The flow chart of the manufacturing process of Nham

Source: Modified from Phoonsawat (2005)

1.2 Ingredients of Nham

Ingredients of Nham, according to Product Development Division, Department of Livestock are listed below;

Formula for 1 kg of Nham

Ingredients	Weight (g)
Minced pork	600
Pork rind	400
Garlic	50
Cooked rice	50
Whole bird chili	20
Nitrite salt	22
• NaCl (99.4%) 21.9 g	
• Nitrite (0.6%) 0.1 g	
Sucrose	4
Monosodium glutamate	2
Erythrobate	2
Phosphate	2

1.3 Functional properties of ingredients during Nham fermentation

The success in production of Nham is influenced by several factors, including natural microflora, quantity and properties of raw ingredients (e.g. meat, salt, sucrose, erythrobate, phosphate, nitrate and nitrite). The varying proportion of these ingredients is thought to play important role in chemical, physical and sensory properties of Nham.

1.3.1 Pork and Pork rind

Minced pork and cooked pork rind are two major typical ingredients used in Nham, comprised over 90% of the ingredients. Pork meat should be fresh in order to minimized bacterial contamination (Kunawasen, 2000). Proteins, derived from raw meat and cooked pork rind, exhibit a wide range of functional properties. They are able to form networks structures, interact with others ingredients, and thus play an important role in the textural, sensory, and nutritional quality of foods. Visessanguan *et al.* (2005) has found that the proportion of minced pork and rind in the formulation could affect texture, color, water binding capacity, and sensory quality but not the fermentation characteristic of Nham ($p>0.05$). However, the ratio of minced pork to rind at 5:5 was the most appropriate to minimizing the cost of production for the Nham formulation.

1.3.2 Cooked rice

Cooked rice can be used as the carbon sources by LAB. Wiriyacharee *et al*. (1994) found that Nham formulation consisted of 3% cooked rice and 1% cooked glutinous rice, exhibited low pH (pH 4.3) and high total acid (1.1%) within 48 hour at 30^{O}C. Cooked rice is the suitable source for Nham production which using starter cultures (*Lb. plantarum, P. acidilactici* and *M. varians*) (Opaswatcharanon, 2004).

1.3.3 Garlic

Garlic has an impact on flavor and it may also have antioxidant and antimicrobial effects (Työppönen *et al.*, 2003). Swetwiwathana *et al.* (1999) found that garlic enhanced the growth of three commercial meat starter cultures (*Lb. curvatus, Lb. sake* and *P. acidilactici*) for increasing the lactic acid production and rapid decreasing of pH value, which leaded to shorten Nham fermentation time.

1.3.4 Nitrate and nitrite compounds

Nitrate and nitrite compounds are curing reagents which played an important role in the color development as well as can inhibit pathogen growth. Nitrate is reduced to nitrite by temperature, bacteria or acid condition. In the first step of color development in meat product, nitrite is reduced to nitric oxide in acidic condition. The occurring nitric oxide reacts with myoglobin to form nitrosomyglobin which exhibit red color (Opaswatcharanon 2004). The mechanism of color development is shown in Figure 2. Nitrite in the form of undissociated nitrous acid (HNO_2) is able to pass the ion barrier of bacterial cell wall and disturb the function of bacterial enzyme and, therefore, bacterial growth (Työppönen *et al.,* 2003). Wiriyacharee *et al*. (1995a) reported that *Kocuria varians*, formerly known as *Micrococcus varians* is important in converting nitrate to nitroso-haemoglobin (NOMb), thus, imparting a pink color to Nham. The maximum allowable level of nitrite residue is 125 ppm (Codex, 1994). Since the excess of residual nitrite can react with secondary amines in certain meat products to form nitrosamine which are proved carcinogen (Kunawasen, 2000).

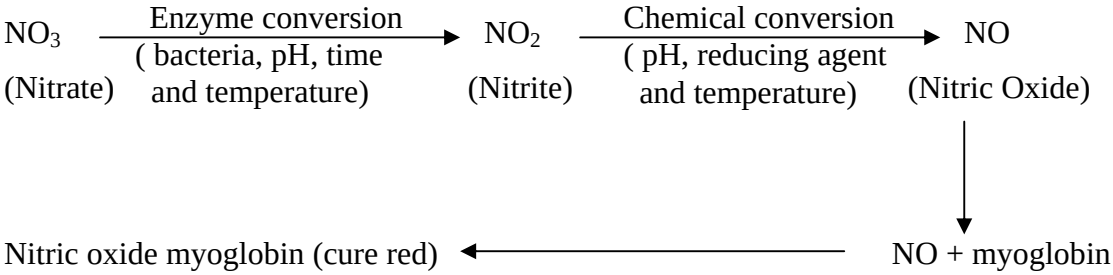


Figure 2 Simplified mechanism of curing agent for color development in sausage.
Source: Modified from Opaswatcharanon (2004)

1.3.5 Sucrose

Sucrose or glucose is added as fermentable substrates for growth of LAB, and production of acid (Työppönen *et al.,* 2003). Wiriyacharee *et al.* (1995b) found that Nham formulation consisted of 5% glucose, exhibited low pH (pH 4.2) and high total acid (1.0%) within 48 hour at 30^{O}C. The products obtained have good sensory quality.

1.3.6 Salt

Salt is usually included as an ingredient to enhance the organoleptic properties of Nham and to inhibit the growth of undesirable microorganism (Kunawasen, 2000). The amount of salt in Nham inhibits the growth of spoilage bacteria contaminated in pork but it doesn't inhibit growth of fermentative bacteria. It also induces the solubilisation and diffusion of myofibrillar proteins from muscle forming gel between meat and fat particles of the raw sausage material (Työppönen *et al.*, 2003). Generally, the amount of salt in fermented sausage is in the range of 2.5-3.5% (Opaswatcharanon, 2004). Techapinyawat (1975) found that Nham with 3% salt obtained the highest score from sensory analysis. Moreover, salt concentration lower than or equal to 2% can cause fermentation failure while salt concentration higher than 4% can increase fermentation time.

1.3.7 Erythrobate and ascorbic acid compounds

Erythrobate and ascorbic acid compounds are used in meat product to improve color of product by enhancing the color formation. They are catalyst in development and maintenance of pink-red color in meat. There were several reports which showed the effect of using the combination of ascorbic acid and nitrite compound to improve color formation in pork product (Thungtakul, 1988 and Dandamrongrak, 1991). The color, flavor and taste of pork product were improved when used both ascorbic acid and nitrite compound (Opaswatcharanon, 2004).

1.3.8 Phosphate compounds

Phosphate compound are widely used in meat products. These compounds improve the chemical and physical properties of the product such as texture, color, stability and water absorption (Rotsachakul, 2002 and Munikanonth, 2004). In addition, phosphate compounds also have buffering capacity which can prevent an increase in pH during early period of meat fermentation, and can prolong the time before pH dropping (Opaswatcharanon, 2004).

2. Microbiological changes during Nham fermentation

In spontaneous fermentation, the raw materials are converted by natural microflora to products that have acceptable food qualities. Therefore, the quality of the product is depends on the occurrence of desirable microorganisms in the ingredients used. Over the past decade, the scientific community has paid special attention to the correct identification of microorganisms involved in Nham fermentation as well as the role of microorganisms during fermentation to find potential starter for ensuring safety and stability of Nham products. The dominant microorganisms have already been isolated from Nham by traditional methods. Several reports have focus on the isolation and identification of microflora from commercial Nham samples during fermentation. In previous studies, pediococci (*Pediococcus* spp., *P. cerevisiae* (reclassified as *P. pentosaceus*) and heterofermentative lactobacilli was found to be dominated at the early stage of fermentation and Lactobacilli (*Lactobacillus* spp., *Lb. plantarum* and *Lb. brevis*) grew at the later stage and became dominant at the end of fermentation (Techapinyawat, 1975). Several studies on Nham microflora have shown lactobacilli (*Lb. plantarum*, *Lb. pentosus* and *Lb. sakei)* and pediococci (*P. acidilactici* and *P. pentosaceus*) are the dominant microorganisms in Nham fermentation (Tanasupawat and Daengsubha, 1983; Tanasupawat *et al.*, 1992). Other microorganisms identified at early phases of the fermentation include *Micrococcus varians*, yeasts and molds. Khieokhachee *et al.* (1997) reported a total non-lactic acid bacterial count in the order of 10^6 cfu/g, and a yeast count in the order of 10^3 cfu/g during the initial 16 h of Nham fermentation. Recently, Kunawasen (2000) have focused on the identification of LAB in Nham from different commercial brands. The study was used both phenotypic and Randomly Amplified Polymorphism DNA (RAPD) to confirm the identification of lactic acid bacteria strains during Nham fermentation. The results from this study have shown that the dominant genetic groups are members of lactobacilli including *Lb. acidophilus, Lb. cellubiose, Lb. graminis, Lb. plantarum, Lb. pentosus, Lb. curvatus, Lb. sakei, Lb. delbruckeii, Lb. paracasei* and *Lb. brevis*, while *Leuconostoc mesenteroides* and *P. pentosaceus* were found in a much lower proportion.

Nham fermentation is usually resulting in product of inconsistent quality due to difficulty in control and/ or prediction of the process as well as increasing risk of pathogenic bacterial growth. Somathiti (1982) examined Coliform counts, *Salmonella* spp and *Shigella* spp. in commercial Nham samples from 4 provinces of Thailand, namely Bangkok, Chiang Mai, Chiang Rai and Ubonratchathani. This study has found that the average of Coliform counts were 1.1-6.1 x 10^4 CFU/g, 2.5 x 10^5 CFU/g, 2.5 x 10^5 CFU/g and 30 CFU/g, in Nham products obtained from Bangkok, Chiang Mai, Chiang Rai and Ubonratchathani, respectively. In addition, *Salmonella* spp. was detected at 12%, 25%, 42% and 11% in Nham obtained from Bangkok, Chiang Mai, Chiang Rai and Ubonratchathani, respectively. However, none of these samples were found to have contaminated with *Shigella* spp. Paukatong *et al*. (2000) reported that food-borne pathogenic bacteria such as *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* can be found in Nham with pH higher than 4.6 at 16%, 15% and 12%, of commercial Nham samples, respectively. This renders the product to be unsafe to consumers. Therefore, the safety of Nham fermentation relies on the rapid growth of LAB and a rapid decrease of pH value to minimize the risk of the growth of food-borne pathogens.

The complexity and variability of microbial populations associated with spontaneous fermentation can partially reduce in back-slopping practice, i.e. inoculation of the raw material with a small quantity of a previously performed successful fermentation (Holzapfel, 2002, Leroy and De Vuyst, 2004). Techapinyawat (1975) found that Nham fermentation which was operated by back-slopping, exhibited a higher rate of fermentation than naturally fermented Nham as indicated by greater rate of pH drop and acidity production. Based on pH and total acidity, the fermentation completed within 96 and 36 h for control and back-slopping, respectively. This might indicated the potential of using starter culture in Nham fermentation. Currently the use of starters as functional flora is gaining importance. Starter cultures are widely used for producing various kinds of fermented meat products including Nham to shorten the fermentation time, ensure proper acid production and the color development, enhance the flavor and improve the product safety, drip loss improvement, and inhibition of undesirable microorganisms.

3. Functional starter cultures for improved sausage fermentation

A meat starter cultures can be described as viable microorganisms added directly to meat in order to improve the storage quality, safety and/or enhance consumer acceptability of meat product (Kunawasen, 2000). A wide variety of microorganisms, mainly lactic acid bacteria (LAB), *Staphylococcus* and *Kocuria* spp., have already been isolated from sausage fermentations. These microorganisms have been selected for using in meat ecosystems, to improve the quality and safety of the final product (Baruzzi *et al.*, 2006). Starter cultures can be implemented for varieties of purpose as described below

3.1 Starter cultures for a more tasty product

Basic flavor results from the interaction of taste (mainly determined by lactic acid production and the pattern of peptides and free amino acids resulting from tissue-generated proteolysis) and aroma (mainly determined by volatile components derived from bacterial metabolism and lipid autoxidation) (Leroy *et al*., 2006). Lactic and acetic acids are often suggested to be major contributors to the acid aroma and taste and the development of the Nham's texture (Visessanguan, *et al*., 2004). The autooxidative phenomenon, especially lipid oxidation in Nham, was likely to be intense and might partially contribute to the Nham characteristics (Visessanguan *et al.*, 2006a). The LAB neither possessed high proteolytic nor lipolytic capabilities. Since microbial lipases generally are very sensitive to pH, lipolysis at conditions relevant to Nham fermentation was likely mediated by lipases present in lysosomes of the muscle tissues (Visessanguan *et al.*, 2006a). Various *Staphylococcus* spp. are often used as starter cultures in production of dry fermented sausages due to their nitrate reductase activity and flavour enhancing capacity, Additionally, *Staphylococcus* often possesses catalase activity which indirectly could alter flavour formation by preventing chemical oxidation of fatty acids (Talon *et al*., 1999, Tjener *et al*., 2003). Staphylococci, in particular *Staphylococcus xylosus* and *S. carnosus*, modulate the aroma through the conversion of amino acids (particularly the branched-chain amino acids leucine, isoleucine, and valine) and free fatty acids. In addition,

additives such as nitrate, nitrite, or ascorbate, pre-cultivation parameters, and environmental factors clearly influence the generation of aroma compounds (Leroy *et al*., 2006).

3.2 Starter cultures for a safer product

Bacteriocins produced by LAB are antibacterial peptides or proteins that kill or inhibit the growth of other Gram-positive bacteria. These peptides are most active towards closely related bacteria likely to occur in the same ecological niches (Leroy *et al*., 2006). Many bacteriocin producing strains have been isolated and these increasing interesting in using theses isolates as starter or protective culture in traditional fermented food including Nham. Lactobacilli that being isolated from sausage frequently produce bacteriocins or bacteriocin-like compounds, as has been shown for *Lb. sakei*, *Lb. curvatus*, *Lb. plantarum*, *Lb. brevis* and *Lb. casei* (Leroy *et al.*, 2006). Ngeamduang (2002) have focus on the isolation of LAB and screening for bacteriocin production from 43 samples of a Thai traditional fermented food. *Lb. helviticus* NL 13 exhibited high potential antagonistic effects. Bacteriocin-producing lactococci have also been isolated from fermented sausage. *Lc. lactis* strain WNC 20 was able to produce the bactericocin nisin-Z originally isolated from Nham (Noonpakdee *et al*., 2003). Bacteriocin-producing *Lc. lactis* strains indicated a potential use as protective culture to improve the food safety of the fermented product. However, the fact that bacteriocin-producing *Lc. lactis* strain are not particularly adapted to sausage technology, e.g. displaying sensitivity to nitrite (Leroy *et al.*, 2006). In contrast, Chiwprasertphol, 2002 showed that *Lc. lactis* BCC 11499 could compete with microflora in Nham at the early stage of fermentation. Moreover, low concentration of sodium chloride and nitrite as using in Nham does not effect on growth and bacteriocin production of *Lc. lactis* strain BCC 11499. Therefore, the application of *Lc. lactis* strain BCC 11499 as starter culture in Nham fermentation has been developed. These observations suggest that BCC 11499 strain may be used as a co-cultures that are effective during the first stages of the fermentation process.

3.3 Starter cultures with a technological advantage

The use of functional starter cultures may be useful to reduce levels of nitrite and nitrate, which received a lot of attention because of their contribution to the formation of health affecting nitrosamines (Leroy *et al.*, 2006). The primary species of bacteria successfully used as starter cultures are *Lactobacillus* sp., *Pediococcus* sp., and *Micrococcus*/*Staphylococus* sp. Lactic acid is produced from carbohydrates by genera of *Lactobacillus* sp., *Pediococcus* sp., which leads to a drop in pH and inhibits the growth of undesirable microorganisms in the products. Moreover, contributes to the development of flavor. *Micrococcus* sp. and *Staphylococus* sp. reduce nitrate and ensure the color development (Swetwiwathana *et al*., 1999).

3.4 Starter cultures for a healthier product

Reduction of undesirable compounds which is important during strain selection that no undesirable compounds such as toxins, biogenic amines, or D (-)-lactic acid, that could adversely affect health, are formed. The use of decarboxylase-negative starter cultures that are highly competitive and fast acidifiers prevents the growth of biogenic amine producers and leads to end-products nearly free of biogenic amines. In addition, the introduction of starter strains that possess amine oxidase activity might be a way to further decreasing the amount of biogenic amines produced in Nham (Limsuwan, 2004)**.**

During the last 20 years, much attention has been focused on the use of starter cultures to guarantee safety and standardize product propertied, including consistent flavor and color and reduce fermentation time (Baruzzi *et al*., 2006). The first stage in designing starter culture for fermented meat is to characterize the microorganisms strains isolated from the meat fermented products (Ammor and Mayo, 2007). The most promising bacteria for starter cultures are those which are isolated from the indigenous microflora of traditional products. These microorganisms are well adapted in the meat environment and are capable of dominating the microflora of products (Drosinos *et al*., 2005).

4. Development of starter microorganisms for Nham fermentation

The use of starter cultures for improving the quality of Nham has received a lot of attention. The development of starter culture involves intense research on the roles of microorganisms during Nham fermentation. The biochemical and physiological roles of these microorganisms in the development of flavor and aroma of the Nham product must be elucidated. This information can be used as the criteria in the selection of starter microorganism for used in the fermentation. Finally, the starter culture selected would be the one giving a satisfactory performance in the process and also giving an acceptable organoleptic evaluation of the Nham products.

Svetvivadhana (1990) had reported on the advantage of using mixed culture strains of *Pediococcus* spp. and *Lactobacillus* spp., which gave the highest antagonistic effect on the inoculated salmonellae (*S. anatum, S. derby* and *S. newport*) in Nham. Moreover this study achieved shorter fermentation time and more safety in getting "Salmonellae free products" when using starter cultures in contrast to naturally fermented products. Based on the organoleptic tests carried out by both consumer test panelists and producers, Nham prepared with starter cultures were favorably preferred more than the naturally fermented products.

Wiriyacharee *et al*. (1990) proposed that *Lb. plantarum and P. cerevisiae* are important for acid production and *M. varians* (reclassified as *K. varians*) produces nitrite reductase which is important in converting nitrate to nitroso-haemoglobin giving pink color to the product. Krairojananan *et al*. (1997) found that most of the lactic acid bacteria and other aerobic bacteria isolated from Nham produce hydrogen peroxide, a strong oxidizing agent causing discoloration and rancid off-odor. Hence the ability to produce catalase is a desirable trait for bacterial strains used in Nham fermentation. The first starter formulated for Nham fermentation was developed by Wiriyacharee *et al*. (1990) at Chiang Mai University, Thailand. The formulae which consists of lactobacilli, pediococci and micrococci along with fixed amounts of various ingredients, has been successfully utilized in the industrial production of

Nham by the Wanasanun Co.Ltd, Thailand which have exclusively use of the culture. This is the first commercial production of Nham that used starter technology.

Furthermore, developments of starter formula for Nham have been carried out by Valyasevi *et al.* (2001) to improve control of microbial processes. Selected isolates from the dominant genetic groups has been selected for using as starter. Nham products were evaluated by their sensory quality and the ability of the starter culture bacteria to ferment based on different quality factors of the final product such as final pH, total acidity, color and texture. Both *Lb. curvatus* and *Lb. plantarum* were found to give product with higher scores of overall acceptability based on 9 points hedonic scale sensory analysis than natural fermented Nham. Nham fermented with *P. pentosaceus* and *P. acidilactici* had pH of less than 4.6 within 72 h and have acceptable texture and color values. Nham fermented using *P. acidilactici* gave a significantly higher overall acceptability than the product fermented by *P. pentosaceus*.

Combinations of lactobacilli and pediococci did not give products of higher sensory scores than single strain fermentation using either *Lb. curvatus* or *Lb. plantarum* (Smitinont *et al.*, 1999). Nham fermented with combined cultures of *Lb. curvatus* and yeast gives products of higher aroma than single strain fermentation. The product also does not have a strong sour taste and higher overall acceptability scores (Valyasevi *et al.*, 2001).

Petchsing and Woodburn (1990) found that Nham inoculated with 1.5% of starter culture can be used to inhibit the growth of *S. aureus* and *E.coli* within 36 h and 96 h, respectively. Rakphoa, (1996) found that the application of three strains, *Lb. plantarum*, *P. cerevisiae* and *M. varians* as starter cultures could inhibit growth of *S. aureus* in Nham fermentation. However, inoculation with a single strain of *Lb. plantarum* gave a higher potential of inhibition of 10^2 and 10^3 cfu/g at 32 h and 36 h, respectively.

Twichatwitayakul (1996) reported the effect of using mixed starter cultures of *L. plantarum*, *P. cerevisiae* and *M. varians* on the growth of Salmonella (*S.* Typhimurium and *S. anatum*) at 10^3 cfu/g during Nham fermentation. The mixed starter cultures can be used to reduce the growth of Salmonella after 5 days of fermentation at pH 4.37 and 1.39% of total lactic acid

Kwanmuang (2003) studied on the mixed culture strains of *Lactobacillus johnsonii* and *P. pentosaceus*, which were previously isolated from commercial Nham samples. Based on the organoleptic tests carried out by consumer test panelists, Nham prepared with starter cultures were favorably preferred more than the naturally fermented products ($p<0.05$).

Visessanguan *et al.* (2006b) studied on the effect of inoculum of *Lb. curvatus* during Nham fermentation. Nham inoculated with *Lb. curvatus* at 10^4 and 10^6 cfu/g exhibited a higher rate of fermentation than natural Nham fermentation based on pH and lactic acid production. In terms of acceptability, Nham inoculated with *Lb. curvatus* at 10^4 cfu/g received the highest score on flavor, sourness, saltiness, and texture. However, unusual smell was detected in Nham inoculated with *Lb. curvatus* at 10^6 cfu/g. Therefore, inoculation of *Lb. curvatus* at high level may cause off-flavor in the product

Chiwprasertphol *et al.* (2002) have focused on the isolation of lactic acid bacteria and screening for bacteriocin production. *Lactococcus lactis* strain BCC 11499 produced a bacteriocin that not only inhibited closely related LAB, but also *L. monocytogenes* and *S. aureus*. Application of *Lc. lactis* strain BCC 11499 as starter culture in Nham showed that *Lc. lactis* BCC 11499 predominated from 12-24 h resulting in a decrease of the total number of bacteria and lactic acid bacteria at 24 h. Moreover, there was no significant difference in pH, texture, color, total acidity and sensory evaluation at 72 h in spontaneous and starter culture inoculated Nham. Thus, *Lc. lactis* strain BCC 11499 may be useful in improving the food safety of the fermented product.

5. Lactic Acid Bacteria

The two categories of bacteria which play a significant role and commonly found in fermented sausages are lactic acid bacteria and Gram-positive, catalase positive cocci (Coppola *et al.*, 1998). These microorganisms have been used as starter cultures for promoting meat fermentation (Papamanoli *et al.*, 2003). Lactic acid bacteria improve safety and stability of the product, whereas Gram-Positive, catalase-positive cocci enhance colour stability, prevent rancidity and release various aromatic substances (Coppola *et al.*, 1998 and Papamanoli *et al.*, 2003).

5.1 Taxonomy and physiology

Lactic acid bacteria have been used for centuries in the preparation and processing of foods and beverages as well as in numerous fermentation processes. They are used in the manufacture of fermented dairy products, in the production and preservation of sausages and meat, in the fermentation of olives and vegetables, and in baking. Their long history of safe use commonly referred to as the GRAS (Generally Recognized As Safe) status, combined with a variety of interesting metabolic characteristics have led to a wide range of industrial applications (Holzapfel *et al.*, 2001).

LABs are a diverse group of genera which can be characterized as Gram-positive, catalase negative, non-sporulating, non-pigmented mesophiles (Brookes and Buckle, 1992). The tolerated temperature range is generally between 5^{o}C and 50^{o}C, with the optimum for most strains being around 30^{o}C (McDonald *et al.*, 1990). Shape is variable, from cocci to elongated rods. This group of organism has strictly fermentative metabolism from which lactic acid is the key metabolite (Temmerman *et al.*, 2004).

LAB species can be subdivided on metabolic grounds into three groups according to Kandler and Weiss (1986). These subdivisions are based on the principal of saccharolytic pathway employed by the species

5.1.1 Group I, obligate homofermentative (Figure 3), these convert hexose into lactic acid via the Embden-Meyerhof Parnas pathway, but they are unable to ferment pentoses or gluconate

5.1.2 Group II, facultative heterofermentative, usually ferment hexoses homo-fermentative into lactic acid but, in some strains and under some conditions, hetero-fermentative metabolism (Figure 4) can convert hexose into lactic acid, carbon dioxide and ethanol (or acetic acid). Acetic acid production occurs under conditions where NAD^+ can be regenerated without the formation of ethanol, for example through the reduction of fructose or molecular oxygen. Pentoses are fermented into lactic and acetic acid via a phosphoketolase pathway.

5.1.3 Group III, obligate heterofermentative (Figure 4), hexoses are fermented to lactic acid, carbon dioxide and ethanol (or acetic acid in the presence of an alternative electron acceptor). Pentoses are converted to lactic and acetic acids.

The largest group of LAB belongs to the genus of Lactobacillus that comprises of more than 50 different species (Stiles and Holzapfel, 1997; Tannock, 2004). In many cases, these lactobacilli are also used as starter cultures in industrial and artisanal food fermentation since they contribute to the conservation, flavor, and texture of the fermented foods. Moreover, the fermentative conversion of other metabolites is other important properties (Ross *et al.*, 2002, Tamime, 2002).

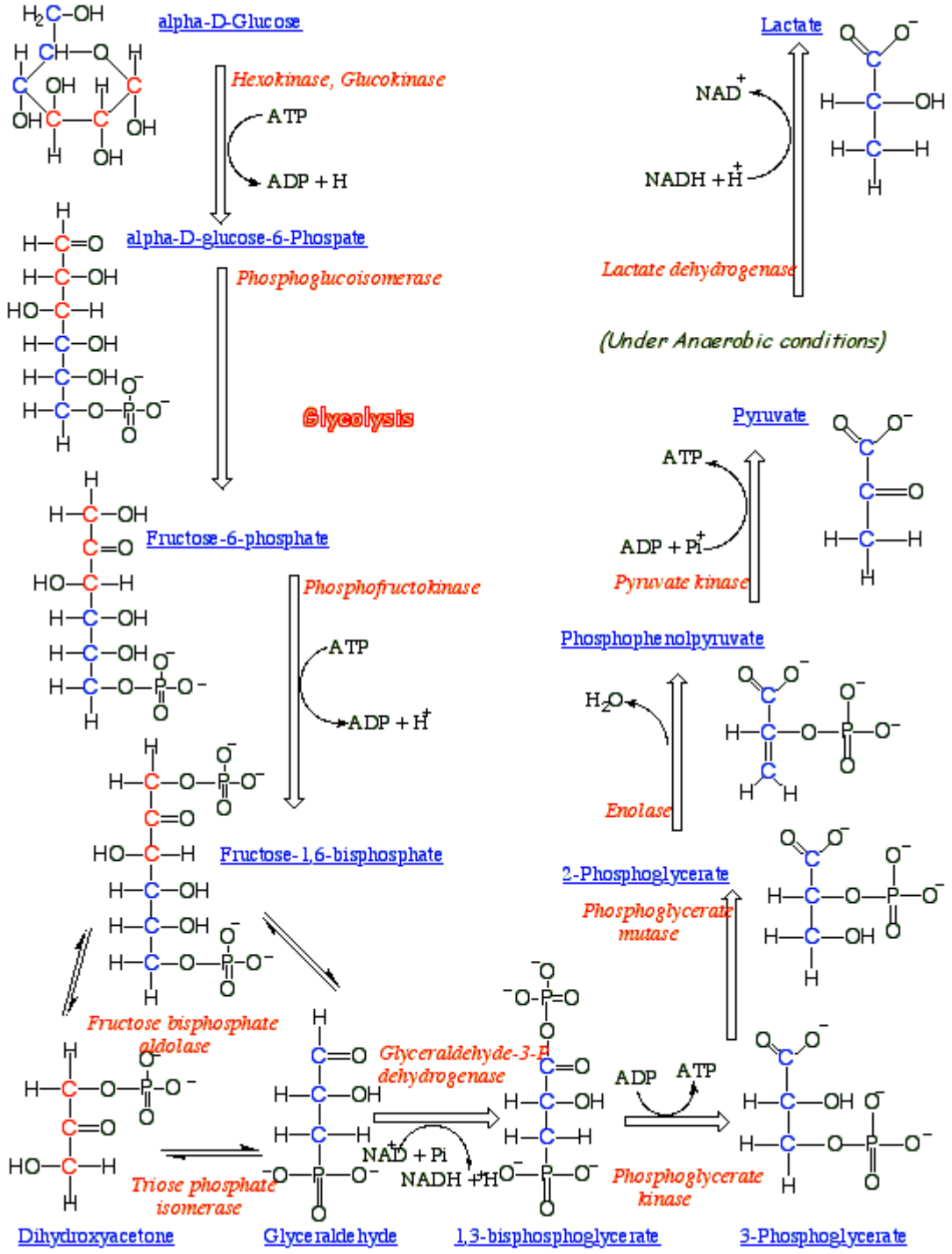


Figure 3 The homofermentative pathway of lactic acid bacteria.

Source: Brook (2005)

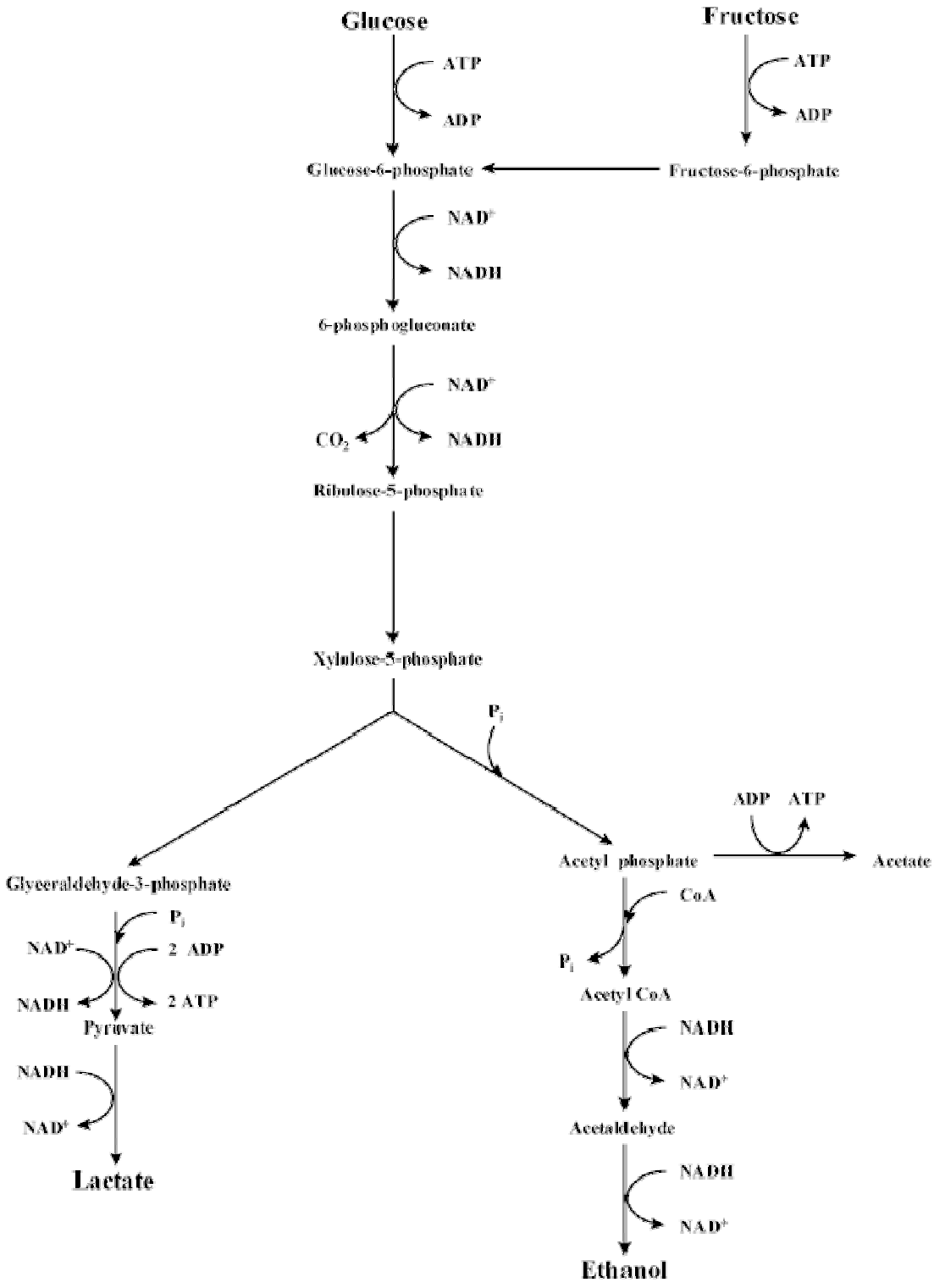


Figure 4. The heterofermentative pathway of lactic acid bacteria.

Source: Lee (2005)

6. Identification of Lactic Acid Bacteria

Over the past decade, the scientific community has paid special attention to the correct identification of LAB used for food fermentation (Temmerman *et al.*, 2004). Several methods have been developed and applied for identifying LAB. These methods can be used for the screening and selection of LAB, assessing their roles during fermentation and to find potential starter for ensuring safety and stability of products. A broad range of identification techniques are available, all displaying difference in discriminatory power, reproducibility and work load. An overview of phenotypic and genotypic methods that are currently used for identification of LAB as described below.

6.1 Phenotypic methods

Traditionally, LABs have been identified on the basis of phenotypic methods. These methods include morphological and physiological characterization, and carbohydrate fermentation patterns (Gonzalez *et al.*, 2000). Phenotypic tests are still being used on a routine basis for the identification of food-associated LAB. The popularity of these methods is mainly due to the fact that no specialized equipment is required to carry out most tests and because of the availability of an identification database (Temmerman *et al.*, 2004). Additionally, phenotypic methods are less expensive and require less training for lab personnel compared to genetic methods.

6.1.1 Morphological and cultural characteristics

In general pediococci, streptococci, enterococcci and lactococci can be differentiated morphologically, in contrast to lactobacilli and carnobacteria. However, all of them have similar colonial appearance (Kunawasen, 2000). Drosinos *et al.*, (2005) identified 288 LAB isolates from a traditional Greek fermented sausages using cell morphology and physiology characteristic followed by further identification using the commercial API 50 CHL system (BioMérieux, France). These studies

demonstrated that cell morphology gave several doubtful and low discrimination profiles.

6.1.2 Physiological characteristics

The ability to grow at various conditions (presence or absence of oxygen, pH, temperature, etc.) can discriminate among genus of lactic acid bacteria. For example, discrimination between Lactobacillus and Carnobacterium can readily be achieved because the latter can not grow at pH 4.5 or on acetate agar but can grow at pH 9.0 (Kunawasen, 2000). Gonzalez *et al*. (2000) identified 249 LAB isolates from freshwater fish and their environment using 44 morphological and physiological tests. A high percentage (90%) of the isolates could only be identified at the genus level, demonstrating the low taxonomic resolution of this labor-intensive approach

6.1.3 Carbohydrate fermentation patterns

The determination of the carbohydrate fermentation pattern using conventional test tube techniques is subject to immense variation, depending on the procedures used (Kunawasen, 2000). API (BioMérieux) and BIOLOG system are the most popular commercially available miniaturized identification systems (Temmerman *et al.,* 2004). The determination of carbohydrate fermentation is not very convenient and may be misleading (Shaw and Harding, 1984). Furthermore, it is unsatisfactory to consider only carbohydrate fermentation patterns because variable fermentations often occur (Champomier *at el*., 1987). Andrigetto *et al.* (1998) found that some strains were falsely assigned to species or subspecies, on the basis of sugar fermentation profiles obtained by API 50 CHL.

Although the application of phenotypic techniques has proven to be useful for certain LAB, these identification methods still have their limitations. Because of relatively poor reproducibility and low taxonomic resolution that often only allows differentiation at the genus level (Temmerman *et al.,* 2004). The shortcomings of

phenotypically based typing methods have led to the development of typing methods based on the microbial genotype or DNA sequence (Olive and Bean, 1999).

6.2 Genotypic methods

Molecular techniques have been successfully applied for LAB identification which permitted accurate and fast identification (Ammor *et al.*, 2005). Genotypic methods exhibit various levels of discriminatory power, from species level to strains level. In recent years, several molecular methods have been used for differentiating LAB such as restriction fragment length polymorphism (RFLP) (Temmerman *et al.*, 2004), pulsed-field gel electrophoresis (PFGE) (Drosinos *et al*., 2005, ribotyping and DNA-DNA hybridization (do Toit *et al.*, 1998). Furthermore, many genotypic methods are based on the principle of polymerase chain reaction (PCR) including amplified fragment length polymorphism (AFLP) (Temmerman *et al.*, 2004), random amplified polymorphic DNA (RAPD) (Ammor *et al*., 2005), 16S rDNA sequence, 16S-23S rDNA Internal Transcribed Spacer (ITS)-PCR (Song *et al*., 2000), and rep-PCR (Gevers *et al.*, 2001). Each technique is described in more detail as followed.

6.2.1 Restriction fragment length polymorphism (RFLP)

This technique involved the digestion of genomic DNA with restriction enzymes, followed by electrophoresis, southern transfer, radioactive or non-radioactive probing and exposure of the filters to film. This restriction endonuclease analysis based on sequence data of those fragments was used for differentiation among species (Kunawasen, 2000). In additional, the discriminatory power of these methods is very high (i.e. strain level) making them very useful for typing LAB starter cultures, of which the strain-specific properties are crucial to the production process (Temmerman *et al.*, 2004).

6.2.2 Ribotyping

Ribotyping combines an enzymatic restriction digest with the detection of the resulting fragments by means of rDNA probes. Either fluorescent or radioactively labeled probes can be used to hybridize with specific DNA sequences (Temmerman *et al.,* 2004). Ribotyping has been applied with success for the identification of LAB from vacuum-packaged 'gravad' rainbow trout (Lyhs *et al*., 2002) and morcilla de Burgos (Santos *et al*., 2005). However, ribotyping provides high discriminatory power at the species and subspecies level rather than on the strain level (Ammor *et al*., 2005).

6.2.3 Pulsed Field Gel Electrophoresis (PFGE)

PEGE employs an alternating field of electrophoresis to allow separation of the large DNA fragment obtained from restriction digest with rare-cutting enzymes (Temmerman *et al.,* 2004). PFGE is the most discriminatory power and reproducible method for differentiating lactobacilli at the strain level (Plengvidhya *et al*., 2004 and Drosinos *et al*., 2005). PFGE is often considered the "gold standard" of molecular typing methods (Olive and Bean, 1999). However, it is a laborious and expensive method, with only a limited number of samples can be analyzed at the same time. Moreover, performing PFGE is very time-consuming and not suited for a routine use in many laboratories.

6.2.4 DNA-DNA hybridization

DNA-DNA hybridization has higher resolution than 16S rDNA sequencing (Temmerman *et al.,* 2004). Various approaches exist such as the nitrocellulose filter methods, free-solution methods, and more recently the use of microarray technology (Cho and Tiedje, 2001). DNA-DNA hybridization was used to select probiotic species out of 297 lactobacillus isolates (do Toit *et al.,* 1998). Although the DNA-DNA hybridization technique has improved knowledge on taxonomic relationships between *Lactobacillus* species, but this technique is still time-consuming and labor-intensive (Kwon *et al*., 2004)

6.2.5 Amplified fragment length polymorphism (AFLP)

AFLP is a genome fingerprinting technique that combines PCR amplification with double enzyme restriction digestion. Total genomic DNA is digested using two restriction enzymes and double-stranded adapters are usually ligated to the DNA fragments serving as primer binding sites for PCR amplification. The AFLP procedure is more labor-intensive than Rep-PCR, but results are obtained more rapidly than with PFGE (Olive and Bean, 1999). AFLP has been found to be a very useful fingerprinting technique for bacteria, allowing both species resolution and strain differentiation (Temmerman *et al.,* 2004).

6.2.6 Random amplified polymorphic DNA (RAPD)

RAPD are based on the use of arbitrary primers, 9 to 10 base in length which hybridized with sufficient affinity on chromosomal DNA sequences at low annealing temperatures that they are able to bind under low stringency to a number of partially complementary sequences of unknown location in the genome of an organism. The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis a pattern of bands, which in theory is characteristic of the particular bacterial strain results (Olive and Bean, 1999). RAPD has been widely reported as a rapid, sensitive, and inexpensive method for genetic typing of different strains of LAB and bifidobacteria (Ammor *et al*., 2005). However, primer with a high discriminatory power and a broad applicability within a large group of LAB species level is not available. Moreover, the resulting band patterns are often exhibit a poor reproducibility because RAPD primers are not directed against a particular genetic locus, thus many of priming events are the result of imperfect hybridization between primer and the target site (Olive and Bean, 1999; Gevers *et al.,* 2001). RAPD was more discriminating than RFLP analysis of 16S rRNA gene or the 16S-23S rRNA spacer region but less discriminating than Rep-PCR (Olive and Bean, 1999).

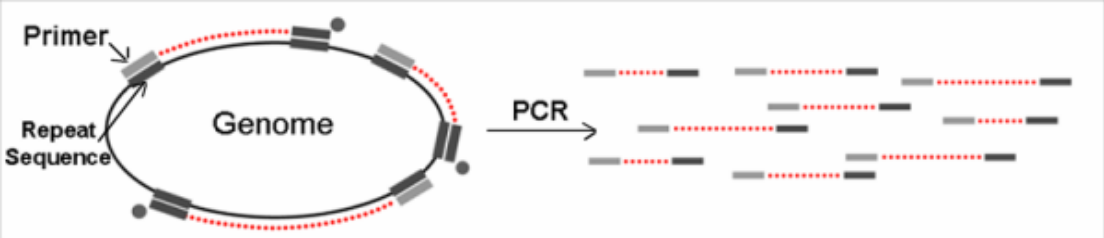
6.2.7 Repetitive DNA element -based PCR (Rep-PCR)

Rep-PCR genomic fingerprinting developed by Versalovic *et al.* (1991) is currently used to estimate relative degrees of similarity between different isolates and to generate species-specific fingerprint. Depending on the type of primer such as REP, ERIC, BOX or $(GTG)_5$ used and on the conditions of the reaction (Fani *et al.*, 1993, Versalovic *et al.*, 1994 and Olive and Bean, 1999), this technique is easy to perform and can be applied to a large number of isolates. In addition, rep-PCR has a high discriminatory power, low cost to perform making this technique suitable for a high-throughput of strains, and considerable to be a reliable tool for classifying and typing a wide range of Gram negative and several Gram-positive bacteria (Gevers *et al.,* 2001). Rep-PCR shows broader species applicability and better discriminatory power than either plasmid profiling or RAPD. Rep-PCR has considerably better discriminatory power than restriction analysis of the 16S rRNA gene or the 16S-23S spacer region (Temmerman *et al.,* 2004). Furthermore, studies which have compared Rep-PCR to other typing methods such as multilocus enzyme electrophoresis, biochemical characterizations, or ribotyping have shown Rep PCR to be superior to these methods (Ammor *et al*., 2005). Finally studies have shown Rep PCR to have good correlation with PFGE but, in general, with slightly less discriminatory power (Olive and Bean, 1999).

Repeated sequences are present in the genomes of all organisms. The first described repeated sequences in prokaryotes and may be the most extensively studied is the repetitive extragenic palindrome (REP) or palindromic unit (PU) sequence initially identified in *Salmonella* Typhimurium and *Escherichia coli*. The repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria (Lupski and Weinstock 1992). Three families of repetitive sequences have been identified, including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element (Versalovic *et al.* 1994). These sequences appear to be located in distinct, intergenic positions around the genome. Oligonucleotide primers have been designed to prime

DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX, in the polymerase chain reaction (PCR) (Versalovic *et al.* 1994). The use of these primer(s) and PCR leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprint. Rep-PCR genomic fingerprinting was found to be extremely reliable, reproducible, rapid and highly discriminatory (Versalovic *et al.*, 1994). The process known as repetitive sequence-based PCR (rep-PCR), allows for the amplification of many different sized fragments (amplicons) representing the DNA within the non-coding, repetitive sequences in the genome (Figure 5).

Step 1 rep-PCR primers bind to many specific repetitive sequences interspersed throughout the genome. Multiple fragments of various lengths are amplified.



Step 2 Fragments can be separated by size and charge. A unique rep-PCR fingerprint profile is created containing multiple bands of varying sizes and intensities.

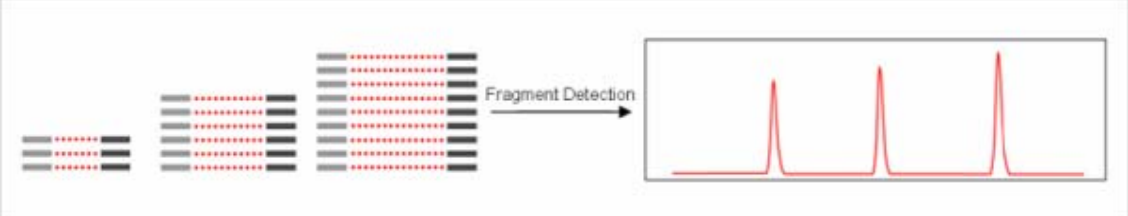


Figure 5 Repetitive sequence-based PCR (Rep-PCR) steps.

Source: Versalovic *et al.*, 1994

6.2.8 16S rDNA sequencing

DNA sequencing generally begins with PCR amplification of a sample DNA directed at genetic regions of interest, followed by sequencing reactions with the PCR products. The obtain sequence is to be compared with DNA sequences stored in online databases of previously sequence DNA, of which the most popular ones are the EMBL (http://www.ebi.ac.uk/embl) and Genbank (http://www.ncbi.nlm.nih.gov/Genebank/) databases. The 16S rRNA-targeted hybridization probes and polymerase reaction (PCR) primers have been successfully used to identify and detect of some *Lactobacillus* species. However, among closely related species, 16S rRNA gene probes or primer have limited success when using in identification of little variation of the 16S rRNA sequence (Song *et al*., 2000). In general, DNA sequencing is expensive and requires a high degree of technical competency to perform (Olive and Bean, 1999). Although a very powerful tool, the reliability and the taxonomic coverage the technique are rely on the availability of the database. Furthermore, the degree of strain and inter-operon sequence variation may in some cases yield confusing identification results (Temmerman *et al.*, 2004).

6.2.9 16S-23S rDNA Internal Transcribed Spacer (ITS)-PCR

The sequence of the 16S-23S rRNA intergenic spacer region exhibits greater variations than that of 16S rRNA structural gene. The amplification of the 16S-23S rRNA intergenic spacer region prove to be a useful alternative to 16S rDNA sequencing for designing specific primers or probes to identify closely related species such as *Lb. curvatus* and *Lb. casei* or *Lb. acidophilus* and *Lb. helveticus* (Nour, 1998 and Song *et al*., 2000). Recently, length, sequence variation and RFLP analysis of the 16S-23S rDNA spacer regions have been used to provide adequate discriminatory of species in which 16S rDNA sequence are similar (Laganowska and Kaznowski, 2004).

The spacer regions of ribosomal operon are transcribed, and for this reason they are called intergenic transcribed spacer or internal transcribed spacer

(ITS) regions (Përez-luz *et al*., 2002). De Vries *et al.* (2006) reported the ITS between the 16S and 23 S genes contain between zero and two tRNA genes, usually encoding a tRNA-Ile, tRNA-Ala or tRNA-Glu (Figure 6). The amplification of the 16S-23S rDNA spacer region and the size of polymorphism of the resulting PCR products have been successfully used for the species-level differentiation and identification of a broad range of bacteria (Gürtler and Stanisich, 1996; Mendoza *et al*., 1998; Riffard *et al*., 1998; Christensen *et al*., 1999) including of *Lactobacillus* species (Tannock *et al*., 1999; Song *et al*., 2000).

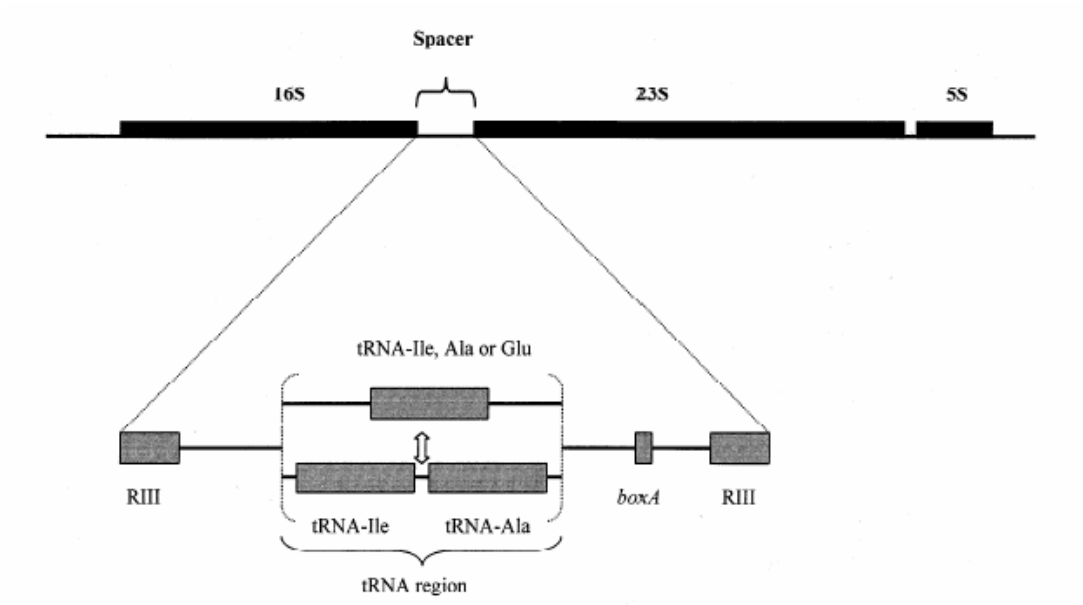


Figure 6 Schematic representation of a 16S–23S spacer and organization of its functional regions (shadowed boxes). As indicated by brackets, the presence of tRNA genes is not universal and their number and type may vary among species

Source: García-Martínez *et al*. (1999)