

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Lactobacillus is ubiquitous bacteria in nature and has a long history of safety in food, beverage and healthcare (Klaenhammer, 1993; Holzapfel *et al.*, 1998) because of their contribution to flavour and aroma development and to spoilage retardation (Holzapfel *et al.*, 1998). In human the lactobacilli are found in the mouth, lower intestine, and vagina. Over the past decades, an increased drive has existed for the applications of lactobacilli as ingesting live microorganisms for therapy (probiotic) (Holzapfel *et al.*, 1998) and antimicrobial agents for prevention of certain infectious disease (bacteriocin) (Huttunen *et al.*, 1987; Klaenhammer, 1995; Kabuki *et al.*, 1997; Holzapfel *et al.*, 1998; Suma *et al.*, 1998; Kaewnopparat, 1999; Onda *et al.*, 2003; Kaewsrichan *et al.*, 2004), although the mechanisms are not entirely understood (Montville *et al.*, 1995).

Bacteriocins are gene-coded, ribosomally synthesized antimicrobial peptides produced by bacteria (Hancock, 2001; Zasloff, 2002). The bacteriocin of the same structure can be produced by different species from the same environment, probably due to conjugal transfer in ecological nich (Kawai, 2001). Some bacteriocins show enhanced activity upon complementation with other components. They are not necessarily linked to one species or restricted to organisms

sharing a specific environment (Papagianni, 2003; Fimland *et al.*, 2006). The evidences have shown an inhibitory activity towards both Gram positive and negative bacteria but still restrict to the closed species (Suma *et al.*, 1998). New bacteriocins are still being discovered and regularly reviewed. Several bacteriocins have been characterised with respect to their protein sequence, molecular mass, biochemical properties and antimicrobial activity spectrum (Klaenhammer, 1993; Kaewnopparat, 1999; Huttunen *et al.*, 1987; Kabuki *et al.*, 1997; Onda *et al.*, 2003). Bacteriocins and synthetic antimicrobial peptides that contain all D-amino acids usually have antibacterial activity but exhibit more resistance to proteolytic enzymes and are less cytotoxic compared with other kinds of antimicrobial peptides that have all L-amino acids (Boman, 1991; Zasloff, 2002; Ryadnov *et al.*, 2002). Their antimicrobial activity strongly indicates that they act without interacting with stereo-specific receptors. Amino acid sequenced analysis assist in understanding the activity of bacteriocin, however, only few of them have been completely characterized. Comparison of the bacteriocin specific activities described in the literature remains difficult due to the fact that the methods and the target strains used are heterogeneous.

Mechanisms that have been proposed to account for activity of bacteriocins include the possible formation of a barrel/stave poration complex, the destabilization of the bacterial membrane as a consequence of pore formation, and the depletion of the proton-motive force, which can be a primary or secondary event (Montville *et al.*, 1995; Hancock, 2001; Duche, 2002). The composite amino acid residues of general bacteriocin confer a cationic amphiphilic structure, and such a characteristic is likely to contribute to the mechanisms of action (Hancock,

2001; Duche, 2002; Zasloff, 2002). Some have combination structures of protein, lipid, and/or carbohydrate moieties (Klaenhammer, 1993). Although it is generally accepted that bacteriocins act by targeting bacterial cytoplasmic membranes (Klaenhammer, 1993; Montville *et al.*, 1995; Hancock, 2001; Duche, 2002; Zasloff, 2002), few studies have been reported with the mechanism of their action.

In oral cavity, bacteriocins from lactobacilli are one of feasible survival strategies for the microbial population when lactobacilli are approximately 1% of the cultivable oral microflora. Such bacteriocins may play an important role in subgingival microflora control (Boman, 1991; Grenier, 1996; Zasloff, 2002). For example, the oral lactobacilli are related to oral health and some of these species have the ability to inhibit the growth of both periodontal and caries-related pathogens *in vitro* (Testa *et al.*, 2003; Köll-Klais *et al.*, 2005). Furthermore, when periodontal health status is obtained after therapy, periodontal pocket are recolonized by Gram-positive bacteria, among them lactobacilli have been isolated (Testa *et al.*, 2003). Other evidence, such that antagonistic relationship between *Lactobacillus* spp. and *Porphyromonas gingivalis* may occur as the number of *Lactobacillus* spp. on supragingival plaque increases, the number of *P. gingivalis* decreases or it is absent (Almstahl *et al.*, 2001). *Lactobacillus paracasei* is one of most common species found within the human oral cavity (Sookkhee *et al.*, 2001; Köll-Klais *et al.*, 2005). Even *L. paracasei* HL32 was isolated from the feces in healthy volunteers, it showed the reduction of growth of food-borne and spoilage pathogens (Kaewnopparat, 1999). However, in earlier studies, the antimicrobial effects from organic acids were not eliminated and

the antimicrobial effects to periodontal pathogen have never been investigated (Kaewnopparat, 1999; Sookkhee *et al.*, 2001; Testa *et al.*, 2003; Köll-Klais *et al.*, 2005).

P. gingivalis is of interest because it not only produces localized inflammation of the periodontium (periodontal ligament, gingival, cementum and alveolar bone) (Kinane, 2001; Southard and Gowdowski, 1998), but also cardiovascular disease and preterm low birth weight (Southard and Gowdowski, 1998; Collins *et al.*, 1994; Kang *et al.*, 2002). The cell wall of *P. gingivalis* contains lipopolysaccharides that provoke a host immune response, which results in the release of a variety of cytokines by connective macrophages, such as tumour necrosis factor alpha and interleukin 1 beta (Collins *et al.*, 1994; Kang *et al.*, 2002). Obviously, the pathway of cytokine induction occurs not only at periodontal infection sites, but also in vascular tissues and in the fetus (Collins *et al.*, 1994; Kang *et al.*, 2002; Lamont and Yilmaz, 2002). *P. gingivalis*, a dominant pathogen for adult periodontitis (Kinane, 2001; Masuda *et al.*, 2001; Bhatti *et al.*, 2001), is an obligatively anaerobic Gram-negative rod bacterium that produces extracellular proteases and toxins, and sheds outer membrane vesicles (Kinane, 2001; Southard and Gowdowski, 1998; Collins *et al.*, 1994; Kang *et al.*, 2002). These products can enhance the survival of the bacterium, suppress host defence mechanisms and destroy periodontal tissues (Kinane, 2001; Southard and Gowdowski, 1998). *P. gingivalis* can attach to and penetrate periodontal tissues using fimbriae, while maintaining deeper inflammation by increased depth of periodontal pockets, which is the sign of periodontitis (Kinane, 2001; Southard and Gowdowski, 1998; Lamont and Yilmaz, 2002; Slot, 2002). It also causes alveolar bone destruction, leading to

tooth loss, and this is the most significant symptom of periodontitis (Kinane, 2001; Southard and Gowdowski, 1998; Slots, 2002). In Thailand, the community periodontal index of treatment needs survey was conducted in subjects from 12 to 74 years of age. 76.9% of the 12 yr-old children had signs of gingivitis, 86.5% in the 17-19 yr-olds had signs of periodontal diseases. 37.3% of subjects (35-44 and 60-74 yr-olds) had periodontitis. Subjects with 6 mm or deeper pockets were observed among the 17-19-yr-old age group (Dental Health Division, 2001).

Current treatment for periodontitis involves mechanical therapy (scaling and root planning), followed by either systemic or local delivery of antibiotics. Tetracycline and metronidazole are the drugs of choice in periodontal treatment (Southard and Gowdowski, 1998; Slots, 2002). Tetracycline is a broad-spectrum antimicrobial agent, whereas metronidazole has the ability to kill anaerobic bacteria, including periodontal pathogens (Edward, 2001). Metronidazole is effective against obligative anaerobes, but *Streptococcus mutans* and microaerophilic bacteria increase after drug treatment (Loesche *et al.*, 1991). In spite of successful treatment with these antibiotics, the development of resistant bacterial strains has led to increasing problems for periodontal therapy (Southard and Gowdowski, 1998; Chan and Chan, 2003; Sanai *et al.*, 2002; Walker, 1996). As the prevalence of antibiotic-resistant strains of periodontal pathogens has increased, there is a greater requirement to develop a selective means of killing microorganisms. In recent years, bacteriocins have been proposed as a potential solution to the widespread appearance of resistance to classical antibiotics in clinically relevant bacteria. Such expectations are based on the fact that these peptides exert their antimicrobial

action through simple mechanisms of membrane disruption (Andersson *et al.*, 1988; Ennahar *et al.*, 1999) which are unlikely to promote the appearance of resistance.

The activity of bacteriocins has been found to be selective killing toward closely related species to that of the producer (Daeschel *et al.*, 1990; Suma *et al.*, 1998c; Teanpaisan *et al.*, 1998), and their secretion has been proposed to occur as part of a feasible survival strategy for organisms in the oral microbial population (Fujimura and Nakamura, 1979; Nakamura *et al.*, 1981; Teanpaisan *et al.*, 1998). The properties of bacteriocins, i.e. resistance to proteolytic enzymes, a narrower spectrum of antimicrobial activity, and the antagonism phenomena, could make bacteriocins as promising chemotherapeutic agents for the treatment of periodontal infections, particularly to solve the problems of *P. gingivalis* re-infection (Kaewsrichan *et al.*, 2004; Zasloff, 2002; Ryadnov *et al.*, 2002, Grenier, 1996). However, lactobacilli multiplication in periodontal pocket may become a disadvantage since lactobacilli may induce a secretion of inflammatory cytokines such as TNF- α and IFN- γ (Maragkoudakis *et al.*, 2006). So far, organic acids production from lactobacilli itself enhance root caries and deep caries in oral cavity. Therefore living lactobacilli may not be feasible answer to the periodontal treatment, even though they are characteristically members of indigenous microbiota.

Objectives

1. To purify antimicrobial protein(s) from *L. paracasei* HL32
2. To study antibacterial activity of bacteriocin from *L. paracasei* HL32 against *P. gingivalis*, and

also compared to effects of tetracycline and metronidazole

3. To characterize and identify the bacteriocin obtained from *L. paracasei* HL32
4. To evaluate the pH, temperature, and biological fluids on bacteriocin activity
5. To evaluate cytotoxicity of the purified bacteriocin

1.2 *Lactobacillus*

1.2.1 Background of *Lactobacillus*

Lactobacillus is ubiquitous in nature since it can be found either in humans, plants, soil, water, sewage, manure, cereal products, silage, mucous of mammals, milk, dairy food, meat, or food fermentation (Sandine *et al.*, 1972; Klaenhammer, 1993; Leal *et al.*, 1998; De Martinis and Franco, 1998; Holzapfel *et al.*, 1998; Caridi, 2003; Ayad *et al.*, 2004). Obviously, 21 strains of lactobacilli were isolated from newborn infant faeces including *L. paracasei* ssp. *paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, *Lactobacillus buchneri*, *Lactobacillus brevis*, and *Lactobacillus curvatus* (Arici *et al.*, 2004). The relationships are represented in Diagram 1-1. For instance, we receive lactobacilli from foods and milk and selected strains are isolated. Then the isolated ones are used in manufactory as a starter culture for the fermentation of vegetables and sausage products. The organisms from human can be passed to the soil and back to plants. Pregnancy woman transfers the microorganisms to the newborn in the breast feeding and during birth (Sandine *et al.*, 1972). Noticeably, dairy foods mostly contain *Lactobacillus acidophilus* and *Lactobacillus casei*. When those strains were identified on the basis of DNA-homology analysis, most of them was *L. acidophilus* and *L. paracasei* whereas the rest was *Lactobacillus johnsonii*, *Lactobacillus crispatus*, *L. rhamnosus* and *L. casei* (Holzapfel *et al.*, 1998).

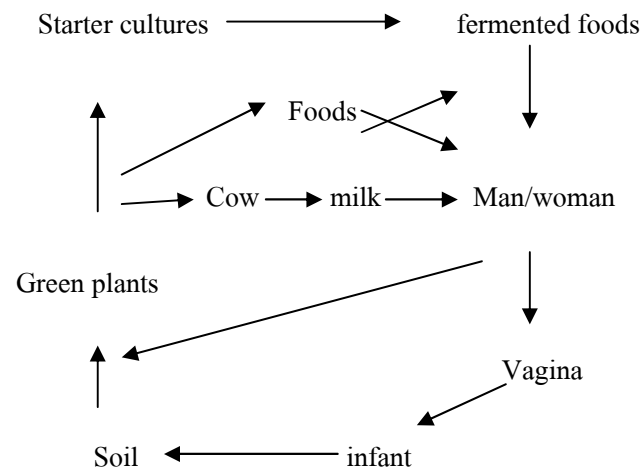


Diagram 1-1 Transfer pathway of organisms in environment

Lactobacilli are strictly fermentative Gram-positive bacteria, producing pH 4.0 in nutrition containing carbohydrate. Some strains can grow up to a maximum pH of 7.2 depending on substrate. They are member of lactic acid bacteria (LAB), since it has similarity in production of lactic acid as a major end-product of carbohydrate catabolism (Holzapfel *et al.*, 1998), see in section 1.2.2. Generally, lactic acid bacteria are generally recognized as safe (GRS) status except *Leuconostocs* and *L. rhamnosus* which are prohibited in European countries (Schillinger & Lucke, 1989). Initially lactobacilli are the magnificent interest for food fermentation and food preservation because they are able to inhibit the growth of undesirable microorganisms (e.g. *Clostridium botulinum*, *Bacillus* spp., *Enterococcus faecalis*, *Listeria monocytogenes* and *Staphylococcus aureus*) that cause food spoilage and food-borne diseases, resulting in less use of chemical preservatives. Subsequently, there has been an increase in potential health purposes as a live microorganism ingestion for promoting or supporting a balance

of indigenous microflora as a probiotic, such as probiotic yogurts or yogurt-like products as well as providing a potential source of antimicrobial agents, such as bacteriocins, for the prevention of certain infectious diseases (Holzapfel *et al.*, 1998; Kaewsrichan *et al.*, 2004). Recently, probiotic has been used for greater than ever: reducing carcinogenesis in preventative therapy for intestinal tumors; restoring the imbalance in the gut microflora, reducing blood level of urine toxin in treatment of chronic kidney failure, degradation of oxalate to prevent the subsequent evolution of kidney stones, reducing blood levels of cholesterol, and enhancing immunity by stimulating the activity of splenic NK cells and increasing gut IgA (Prakash and Jones, 2005). However, the mechanisms of such protective effects are not entirely understood (Montville *et al.*, 1995; Holzapfel *et al.*, 1998; Hancock, 2001; Duche, 2002).

As a food additive, the bacteriocin is incorporated into the product as a dried concentrated powder prepared from a skim-milk derived fermentate, or use of live cultures which produced bacteriocins in situ in the food. Even though there has been a dramatic increase in the number of novel bacteriocin discovered, the potential applications are limited by incomplete information such as spectrum of inhibition, physical and chemical properties, and stability. In food matrices, the bacteriocin activity may be affected by (1) changes in solubility and charge of the bacteriocins, (2) binding of the bacteriocins to food components, (3) inactivation by proteases, and (4) changes in the cell envelope of the target organisms as a response to environmental factors. Salt and Ca^{2+} can affect bacteriocin adsorption, because of ion binding competition on the bacterial target cell surface, or the blockage of the binding sites of the

bacteriocin (Atrih *et al.*, 2001). In addition, lipoteichoic acid also inhibited adsorption of bacteriocin to the cells, since that lipoteichoic acid might represent the nonspecific receptor.

1.2.2 Classification of lactobacilli

The lactobacilli can be divided into three groups based on carbohydrate fermentation process: (1) obligatively homofermentative; (2) facultatively heterofermentative; and (3) obligatively heterofermentative. The homofermentative groups use the Embden-Meyerhof-Parnas pathway to convert 1 mol of glucose into 2 mol of either D-, L- or DL-lactate and do not produce gas (CO₂) from glucose fermentation (Fig.1-1). In contrast, heterofermentative bacteria produce equimolar amounts of DL-lactate, CO₂ and ethanol from glucose fermentation using the hexose monophosphate or pentose pathway, and as such an only half amount of energy is generated as compared to homofermentative group (Fig.1-2). Obligatively homofermentative and obligatively heterofermentative do not ferment the pentose sugars (xylose, ribose or arbinose), whereas facultatively heterofermentative ferment at least one of the 5-carbon sugars mentioned above. The obligately heterofermentative strains are not good acid producers as strains from the obligately homo- and facultatively heterofermentative group (mostly *Lactobacillus plantarum*).

The genera of *Lactobacillus* widely distributes in nature: *L. bavaricus*, *L. caucancus*, *L. cellobiosis*, *L. hilgardi*, *L. fructivorans*, *L. pentosus*, *L. bulgaricus*, *L. xylosus* (Schillinger and Lucke, 1989; De Martinis and Franco, 1998; Holzapfel *et al.*, 2001; Ayad *et al.*, 2004).

<u>Obligately homofermentative strains</u>	<u>Heterofermentative strains</u>	
	Facultative group	Obligate group
<i>L. salivarius</i>	<i>L. plantarum</i>	<i>L. fermentum</i>
<i>L. acidophilus</i>	<i>L. rhamnosus</i>	<i>L. oris</i>
<i>L. delbrueckii</i>	<i>L. casei</i>	<i>L. brevis</i>
<i>L. gasseri</i>	<i>L. paracasei</i>	<i>L. buchneri</i>
-----	<i>L. agilis</i>	-----
<i>L. helveticus</i>	-----	<i>L. vaginalis</i>
<i>L. crispatus</i>	<i>L. pentosus</i>	<i>L. reuteri</i>
<i>L. johnsonii</i>	<i>L. intestinalis</i>	<i>L. viridescens</i>
<i>L. amylovorus</i>	<i>L. sake</i>	<i>L. sanfrancisco</i>
<i>L. aviaries</i>	<i>L. curvatus</i>	
<i>L. farciminis</i>	<i>L. paraplantarum</i>	
	<i>L. arizonensis</i>	
	<i>L. acetolerans</i>	
	<i>L. alimentarius</i>	
	<i>L. coryniformis</i>	

Lactobacillus spp. above drawing line (-----) refer to most common species in oral cavity

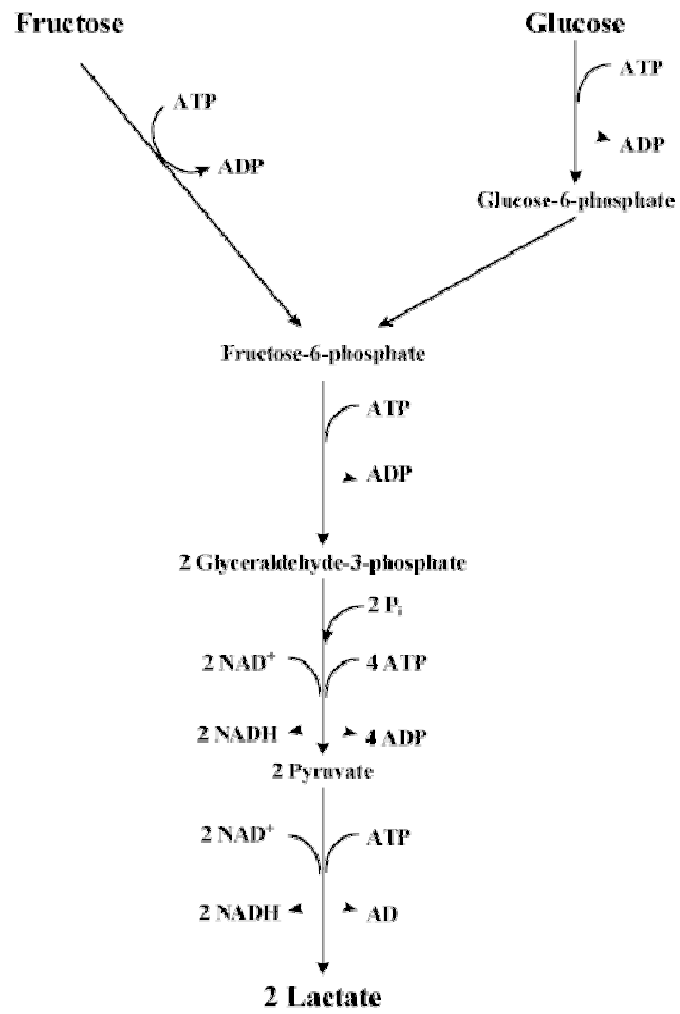


Figure 1-1 The homofermentative pathway of lactic acid bacteria

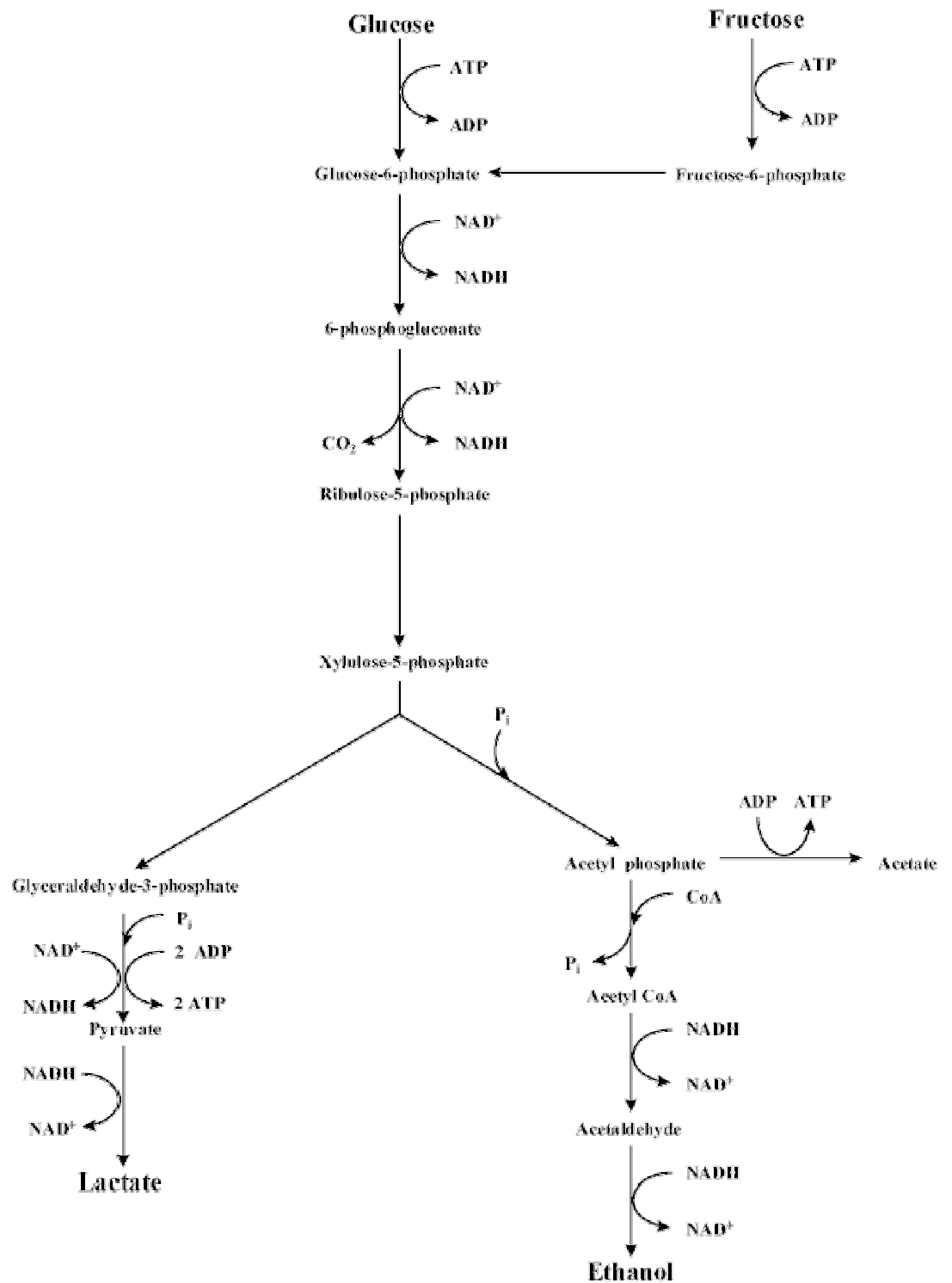


Figure 1-2 The heterofermentative pathway of lactic acid bacteria

1.2.3 Role of *Lactobacillus* in oral cavity

In humans, lactobacilli are found in the mouth, lower intestine and vagina (Holzapfel *et al.*, 1998). They are approximately 1% of the cultivable oral microflora (Mager *et al.*, 2003). The most common species are *L. plantarum*, *L. rhamnosus*, *L. salivarius*, *L. casei*, *L. fermentum*, *L. acidophilus*, *L. oris*, *L. paracasei*, *L. brevis*, *L. buchneri*, *L. delbrueckii*, *Lactobacillus jensenii*, *L. gasseri*, and *L. agilis* as shown above (Sookkhee *et al.*, 2001; Koll-Klais *et al.*, 2005). *L. gasseri* and *L. fermentum* are the most frequently found in saliva of healthy individuals, whereas *L. casei*, *L. rhamnosus*, *L. salivarius*, and *L. plantarum* in the saliva of periodontitis patients. *L. plantarum* and *L. rhamnosus* occupy on the tongue mucosa of healthy gum. Although, lactobacilli are not a common habitat in the subgingival region, *L. gasseri*, *L. oris*, *L. salivarius*, *L. crispatus*, and *L. paracasei* have much more chance to discover in healthy gingival crevice than in periodontal pocket (Mager *et al.*, 2003; Koll-Klais *et al.*, 2005).

There is a relationship between number of lactobacilli and oral lesions. Lactobacilli in the oral cavity are recognized to be cariogenic bacteria in deep lesions, in retentive sites, and in secondary carious lesions. Individuals with high lactobacilli counts usually have high carbohydrate contents in their diet and high frequency intake (Testa *et al.*, 2003). *L. paracasei*, *L. rhamnosus*, *L. fermentum* have been identified in both caries lesions and caries free, but *L. fermentum* is significantly found in caries free (Botha *et al.*, 1998). Lactate, acetate, propionate, and butyrate were detected in most carious dentine, and limited amounts of isobutyrate, valerate, isovalerate, caproate, and isocaproate were occasionally detected. In carious dentine under

fillings or restorations had little lactate, but a high percentage of acetate plus propionate. The differences in acid profiles of carious dentine may reflect differences in the microbial ecology, and a stage of progress of dentinal caries or a type of dentinal caries (Hojo *et al.*, 1991). The lactobacilli isolated from saliva and subgingival sites of chronic periodontitis patients were able to inhibit *Streptococcus mutans* which was partly explained an important role of lactobacilli in contributing to the balance of oral microflora (Koll-Klais *et al.*, 2005).

There are several reports on the relationship between lactobacilli and periodontal microbiota in human mouth. A high proportion of *Lactobacillus* spp. might decrease the number of *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*; Nørskov-Lauritsen and Kilian, 2006) or even disappear in plaque specimens taken from subgingival sites (Almstahl *et al.*, 2001). None of the species of lactobacilli isolated from saliva derived from periodontal patient inhibited the growth of *Prevotella intermedia* or *Fusobacterium nucleatum* (Testa *et al.*, 2003). Lactobacilli has an important role in the maintenance of indigenous microflora in periodontitis and has antagonistic properties against periodontal pathogen (Sookkhee *et al.*, 2001; Koll-Klais *et al.*, 2005).

1.2.4 Bioactive components produced by *Lactobacillus*

Competition among *Lactobacillus* in the oral cavity can be achieved, since it is capable of producing inhibitory substances: (1) bacteriocins; (2) by-products of primary metabolic pathways such as lactic acid and formic acid, as well as other organic acids (acetic acid), hydrogen peroxide, diacetyl, and ethanol; (3) bacteriophages; (4) secondary metabolites such as hypothiocyanite and other inhibitory substances (Annuk *et al.*, 2003; Jack *et al.*, 1995). Isolated bacteriocin activity has to be excluded from the inhibitory activities of the by-products, bacteriophages and the secondary metabolites.

1.2.4.1 Bacteriocins

The word “bacteriocins” was appeared firstly in 1953 (Jacob *et al.*, 1953). However, in 1925 Gratia A. first demonstrated an antibacterial substance produced from *Escherichia coli*, which was later on named as a colicin (Reeves, 1972). According to Klaenhammer, 99% of all bacteria may produce at least one bacteriocin. Only a few have been isolated. Bacteriocins play an important role in mediating microbial population or community interactions and may also play a defensive role and act to prohibit the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells, see also in section 1.3. Typical bacteriocins have a narrow antibacterial activity, but a few have a broad spectrum (Suma *et al.*, 1998; Zamfir *et al.*, 1999). Most of bacteriocins produced by *Lactobacillus* spp. has activity against Gram-positive strains and has rarely reported of activity against Gram-negative strains. Gram-negative bacteria have an

additional outer membrane barrier to cross with highly negative charge carried by the anionic lipopolysaccharide, when a few bacteriocin isolated from Gram-positive bacteria has self-promoted uptake across such a membrane.

The bacteriocin of the same structure can be produced by different lactobacilli species from the same environment (Kawai *et al.*, 2001), probably due to conjugal transfer in ecological niches. Some bacteriocins show enhanced activity upon complementation with others components, for example lactacin F containing LafA and LafX (Muriana and Klaenhammer, 1991). Bacteriocins can be found in different forms such as conjugated form, oxidized forms or fragmented forms.

There are many techniques for detecting bacteriocin production. Most are based on the diffusion of bacteriocins through solid or semisolid culture media to inhibit growth of a sensitive organism. Deferred antagonism or indirect methods include the flip streak and the spot-on-the-lawn assays. A more direct assay is the well-diffusion assay. Several different indicator organisms should be used in bacteriocin-screening protocol. The flip-streak deferred-antagonism method was cumbersome and of limited value. The well diffusion method gave a large number of false-negative results compared to the spot-on-the-lawn method due to aggregation, nondiffusable bacteriocins, and protease inactivation and concentration effects. However, to allow time for the bacteriocin diffusing into the agar prior to incubation or increase the amount of samples will increase sensitivity of well-diffusion assay (Lewus and Montville, 1991). Well diffusion assay is

routinely used to assess the ability of bacteriocin to inhibit microorganisms. The microorganisms commonly selected to determine the activity are summarized in Table 1-1.

Table 1-1 Summary of microorganism commonly use in susceptibility tests of bacteriocin

Indicator species	Reference strains
Gram-positive bacteria	
Competitor	
<i>Lactobacillus</i> spp.	
<i>Lactococcus lactis</i>	
<i>Leuconostoc mesenteroides</i>	NRRL B-512; ATCC 8293, FFL
<i>Leuconostoc paramesenteroides</i>	NRRL B-3471; DSM 20288
<i>Micrococcus luteus</i>	ATCC 9341
<i>Pediococcus</i> sp.	
<i>Streptococcus thermophilus</i>	DSM 20617T
Spoilage or pathogenic	
<i>Bacillus cereus</i>	FMC 19; LMG 13569; ATCC 9634; ATCC 1778
<i>Bacillus subtilis</i>	ATCC 6633; ATCC 6051
<i>Clostridium perfringens</i>	ATCC 13124
<i>Enterococcus faecalis</i>	ATCC 29218
<i>Listeria monocytogenes</i>	NCTC 0488, 05105, 05214, 1052,10888, 10890

Table 1-1 (cont.)

Indicator species	Reference strains
Others	
<i>Staphylococcus aureus</i>	ATCC 25923, 2392, 28213, 6538
<i>Listeria innocua</i>	ATCC 33090; DSM 20649
<i>Staphylococcus epidermidis</i>	ATCC 12228
Gram-negative bacteria	
<i>Alcaligenes sp.</i>	
<i>Escherichia coli</i>	ATCC 25922, 13762; LTH 1600, 4346
<i>Klebsiella pneumoniae</i>	ATCC 13883
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Serratia marcescens</i>	ATCC 8100
<i>Shigella flexneri</i>	ATCC 14028
<i>Salmonella typhimurium</i>	ATCC 14028
Yeast	
<i>Candida albicans</i>	ATCC 13803

1.2.4.2 Metabolic by-products

- Organic acids

During the fermentation of dairy products, lactic acid bacteria metabolize lactose into lactic acid by-product, whereas the fermentation of carbohydrate sources metabolize lactose into lactate by-product. In fermented food, short chain acids (acetic, lactic, or propionic acid) have been found. In general, propionic acid has broader inhibition than acetic and lactic acids. Acid production lowers the pH and creates an environment that is unfavorable for the growth of pathogens and spoilage organisms. In addition, the low pH of fermented foods potentiates the antimicrobial effects of organic acids. Furthermore, those acids can dissolve and diffuse across cell membrane into the cell. At higher internal pH of the cell, the acid dissociates and acidifies. The net effect is disruption of the pH gradient across the membrane interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and cessation of related metabolic function that are essential to cellular survival. Organic acids have a very broad spectrum against bacteria, yeast and moulds.

- Diacetyl

Diacetyl (2, 3-butanedione) is produced from excess pyruvate that was converted from citrate. It is more active against Gram-negative than Gram-positive bacteria including yeast. This molecule inhibited all other cultures when the pH was neutral or acidic, whereas inhibitory activity decreased as pH increased above 7.0. Although diacetyl is recognized as safe, relatively large amounts needed to provide antimicrobial activity and its high volatility become limitation.

This agent is well adsorbed on membrane since the inhibition activity disappears with filter sterilized cultural supernatants.

- Hydrogen peroxide

Hydrogen peroxide accumulates in culture of lactobacilli as autoinhibition. It was produced during aerobic growth. The H₂O₂ production is quite high for obligately heterofermentative strains, but considerably lower for the obligately homo- and facultatively heterofermentative strains. Hydrogen peroxide is an inhibitor of *S. aureus* as well as *Pseudomonas* spp. in culture of *L. delbrueckii*, *L. plantarum* and *L. acidophilus* (Leke *et al.*, 1999). Hydrogen peroxide causes reversible inhibitory effect on growth of *P. gingivalis* under poor nutritional conditions by oxidizing effect on lipid membrane and also negatively affect on hemagglutination and Arg-gingipain activity (Wheater *et al.*, 1952).

- Metabolic end products in trace amounts such as acetoin, acetaldehyde, ethanol, and carbon dioxide also contribute to the antimicrobial effect.

To determine spectrum of bacteriocin activity, there have some strategies to prevent the inhibitory effect from the by-products and secondary metabolites. To suppress the effects of pH activity, neutralization the pH of the cell-free supernatant with sodium hydroxide, Tris-HCl, phosphate buffer (Joerger and Klaenhammer, 1986; Sobrino *et al.*, 1992; Jimenez-Diaz *et al.*, 1993; Enan *et al.*, 1996; van Reenen *et al.*, 1998; Suma *et al.*, 1998; Deraz *et al.*, 2005; Todorov and Dicks, 2006) or dialysis against broth or polyethylene glycol (Schillinger and Lucke, 1989; Daeschel *et al.*, 1990) have been done. To exclude potential inhibition by hydrogen

peroxide, culture supernatant were treated with catalase (Joerger and Klaenhammer, 1986; West and Warner, 1988; Daeschel *et al.*, 1990; Sobrino *et al.*, 1991; Jimenez-Diaz *et al.*, 1993; Zamfir *et al.*, 1999) or minimized by the anaerobic incubation conditions (De Martinis and Franco, 1998; Messi *et al.*, 2001). As promising broad antimicrobial activity was obtained from crude products of *L. paracasei*, *L. rhamnosus*, and *L. plantarum* (Messi *et al.*, 2001; Sookkhee *et al.*, 2001; Koll-Klais *et al.*, 2005), however those experiments did not eliminate the effect from all of bioactive components produced by lactobacilli.

1.2.4.3 Bacteriophage

To verify the presence of bacteriophage, the bacteriocin producing strain was streaked onto an agar plate and incubated overnight. The agar was then inverted into the lid of Petri dish and the indicator strain streaked transversely across the original streak. Incubation at appropriate temperature for the indicator strain and inhibition was observed. A lack of growth was a result from the bacteriocin effects (West and Warner, 1988).

1.2.4.4 Other inhibitory substances

Some species also produced antifungal compounds, for example, phenyllactic acid, 4-hydroxyphenyllactic acid, and cyclic dipeptides such as cyclo (Phe-Pro), cyclo (Phe-4-OH-Pro) (Magnusson *et al.*, 2003). Certain strains of *L. reuteri* produce a unique antagonistic activity for example reuterin, and reutericycline.

Reuterin is found in three forms (Fig.1-3a) and produced during anaerobic catabolism (Piard and Desmazeaud, 1992).

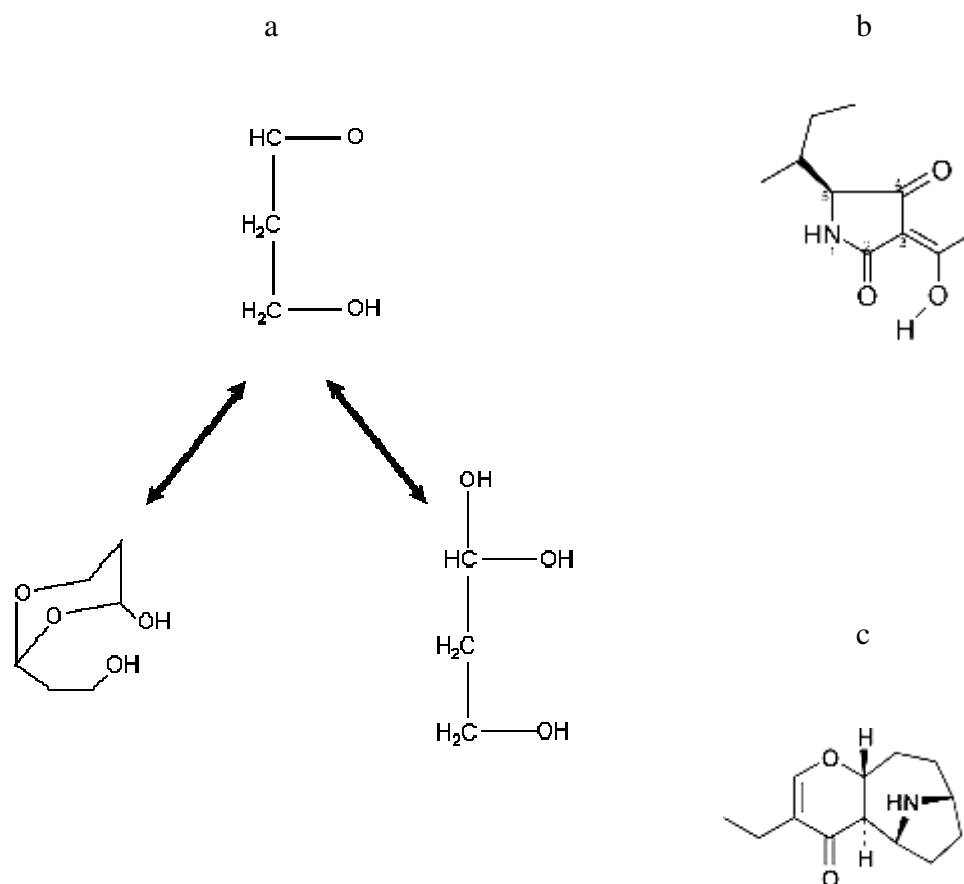


Figure 1-3 Chemical structure of (a) reuterin, an antimicrobial from *Lactobacillus reuteri*, exists in three forms in aqueous solution (monomer, hydrated monomers, and cyclic dimers) (b) reutericyclin, and (c) tenuazonic acid

Reutericyclin (Fig.1-3b) is a novel tetramic acid derivative structurally related to tenuazonic acid (Fig.1-3c). It shares characteristic properties with bacteriocins from LAB,

although its chemical structure is different. It is an amphiphilic molecule as comparable to bacteriocins with a tendency to form aggregates in aqueous solution. Similar to the kinetics of bacteriocin production by LAB, reutericyclin is described by primary metabolite kinetics, and the compound adsorbs on the producer cell walls (Ganzle *et al.*, 2000). It is bacteriostatic or bactericidal to Gram-positive bacteria based on its activity as a proton-ionophore. Gram-negative bacteria are resistant to reutericyclin because of the barrier properties of their outer membrane, and resistance of beer-spoiling lactobacilli towards hop bitter acids provides cross-protection to reutericyclin (Ganzle, 2004).

1.3 Bacteriocins

1.3.1 Bacteriocin definition

Gene-encoded, ribosomally synthesized antimicrobial peptides are being produced as constituents of their innate immune systems in nature by mammals, birds, amphibians, insects, plants and microorganisms, which is a common defense strategy against bacteria (Hancock, 2001; Zasloff, 2002). They are often cationic and amphiphilic, and most of them kill bacteria by permeabilizing bacterial cell membranes. However, a bacteriostatic effect was also reported for some other bacteriocins such as lactocin 27 (Upreti and Hinsdill, 1973), and plantaricin C19 (Atrih *et al.*, 2001). Antimicrobial peptides can be roughly categorized into those that have high content of a certain amino acids, most often proline, those contain intramolecular disulfide bridges, and those with an amphiphilic region in their molecule if they display an α -helical structure (Papagianni, 2003).

Ribosomally synthesized antimicrobial peptides produced by bacteria are generally referred to bacteriocins, which may be either plasmid encoded (Kanatani *et al.*, 1995) or chromosomally encoded (Holck *et al.*, 1994). The producer cells are also immunized at nominal levels of their own inhibitors (Upreti and Hinsdill, 1973). Their net charges, with pIs varying from 8.3 to 10, are common features (Ennahar *et al.*, 1999). Even bacteriocins's structure resembles many of the antimicrobial peptides produced by eukaryotes, they seem to be more potent and much higher specific activity than those produced by animals and plants (Papagianni, 2003). Higher potencies and specificity of bacteriocins were due to their high

affinity binding to specific receptors or docking sites on the target-cell surface (Fimland *et al.*, 2006). The bacteriocins produced by Gram- positive bacteria are often proteins smaller than 6 kDa. In contrast, most of bacteriocin produced by Gram- negative bacteria are often produced peptide bacteriocins larger than 20 kDa. The bacteriocin productions are not necessary linked to one species or restricted to organisms sharing a specific environment. Among the lactobacilli, there has been much interest in *L. acidophilus* and *L. plantarum*, due to its potential application as starter bacteria for the fermentation of dairy foods, vegetables, meat, and fish products.

1.3.2 Differences between bacteriocins and antibiotics

- 1) Bacteriocins differ from traditional antibiotic because of their composition and narrow spectrum of activity, in which they kill bacteria of the same or closely related species. The antibiotics are harmful to the growth of other organism in small concentrations and are broad spectrum of their action. Classical antibiotics usually have a much wider activity, and even when it has activity restricted, it does not show the evidence of effect on closely related strains.
- 2) Antibiotics are secondary metabolite which is not important for growth, but bacteriocins are primary metabolite.
- 3) The producing strain usually shows immunity to the bacteriocin they produce, but antibiotics do not show evidence (Jack *et al.*, 1995). The bacteriocins has not been reported to cause allergic reactions, but antibiotics have.

4) Bacteriocins are not included in peptide antibiotics because peptide antibiotics are not ribosomally synthesized, in spite of multienzyme complexes or sequential enzyme reactions. The peptide antibiotics (aminoglycoside, bacitracin, gramicidin, polymyxin B, lincomycin, and valinomycin) are secondary metabolites, whereas bacteriocins are primary metabolites being produced during the exponential growth phase (Jack *et al.*, 1995).

5) Because of bacteriocins being proteinaceous agents, they are rather rapidly digested by proteases in the human digestive tract than most therapeutic antibiotics.

6) Most peptide antibiotics are synthesized during the late log phase till the stationary growth phase while almost all bacteriocins produced by lactic acid bacteria being produced during the exponential growth phase to the beginning of the stationary phase (Upreti and Hinsdill, 1973).

1.3.3 Bacteriocin classification

The bacteriocins produced by lactic acid bacteria can be divided into 4 major groups on the basis of their mode of action, activity of spectrum, molecular weight, biochemical properties and genetic origin (Table 1-2): lantibiotics (Class I), the nonlantibiotics (Class II), the large heat-labile bacteriocins (Class III), and complex bacteriocins (Class IV) (Klaenhammer, 1993).

Table 1-2 Classification of bacteriocins from Gram-positive bacteria (adapted from Papagianni, M. 2003. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications, *Biotechnol. Adv.*, 21: 478-499.)

Class	Characteristics and subclasses
I Lantibiotics	<p>Ribosomally produced peptides that undergo extensive post-translation modification</p> <p>Small (<5 kDa) peptides containing lanthionine and β-methyl lanthionine</p> <p>Ia. Flexible, elongated molecules compared to Ib</p> <p>Ib. Globular peptides with net anionic or net neutral charge</p>
II Nonlantibiotics	<p>Low-molecular-weight (<10 kDa)</p> <p>Heat stable peptides formed exclusively by unmodified amino acids.</p> <p>Ribosomally synthesized as inactive prebacteriocin and activated by post-translational cleavage of the N-terminal leader peptide.</p> <p>IIa. Anti-listerial single peptides that contain YGNGVN-terminus</p> <p>IIb. Two-peptide bacteriocin</p> <p>IIc. Bacteriocins produced by the cell's general <i>sec</i>-pathway</p>
III Large heat-labile bacteriocins	<p>High-molecular-weight (> 30 kDa)</p> <p>large heat-labile proteins</p>
IV Complex Bacteriocins	<p>Complex bacteriocins carrying lipid or carbohydrate moieties</p>

1.3.4 Mode of action

The structure of bacteriocins composed of 3 domains involving the recognition of specific cell surface receptors, the translocation of the bacteriocin into the target cell, and the killing domain including immunity region on carboxy terminal fragment (Rogelj and Matijasic, 2006). The first domain is the largest sequence. The second domain is located at the N-terminal part. The remainder part is quite short sequence. Bacteriocins would interact with membranes and have antibacterial activity based on two mechanisms: membrane disruptive fashion and non-membrane disruptive fashion (Powers and Hancock, 2003). In both fashions, the mode of action occurs in 3 steps (Andersson *et al.*, 1988). The first step is either the neutralization the charge over patch of cell surface by non-specific electrostatic attraction, or binding to the negatively charged bacterial phospholipids-containing membranes and or acidic bacterial cell walls. Secondly, the bacteriocins is adsorbed to specific receptors on the external surface of sensitive cells, and are then fold and translocated to their specific targets within these cells, whereas their amphiphilic characters enable membrane permeation. However many peptide molecules will insert into the membrane interface and are proposed to cause either aggregate into a micelle-like complex which spans the membrane as micelle-like aggregates or flip-flop across the membrane under the influence of the large transmembrane electrical potential gradient. The net effect is that some monomers translocate into the cytoplasm, dissociate from the membrane and bind to cellular polyanions. Finally, after a time, the last step develops, which results in cell death.

1.3.4.1 Membrane disruptive fashion

Three mechanistic models, “the barrel stave”, “micellar aggregate”, and “carpet” model, have been developed to explain membrane disruption (Andersson *et al.*, 1988; Montville and Bruno, 1994; Abee, 1995; Tahara and Kanatani, 1996; Duche, 2002; Castellano *et al.*, 2003; Powers & Hancock, 2003). Regardless of which model is correct, the net result of membrane disruption would be the rapid depolarization of the bacterial cell leading to rapid cell death, with total killing occurring within 5 minutes for the most active peptides. There have several studies indicate that bacteriocins affect the permeability of the cytoplasmic membrane and the membrane allows the passage of solute as large as glutamate (Tahara and Kanatani, 1996), the efflux of cell metabolites, potassium, or preaccumulated amino acids in sensitive organisms (Abee *et al.*, 1994), and then either by causing the proton motive force depletion (Bruno and Montville, 1993; Tahara and Katani, 1993), or by forming pores (Tahara and Katani, 1993).

- In the barrel-stave or wormhole mechanism, the formation of such amphipathic helices is the critical conformational change require for pore formation. The amphipathic peptides reorient perpendicular to the membrane and align (like the staves in a barrel) in a manner which the hydrophobic regions face outwards into the lipid environment of outer leaflet of the membrane while the cationic residues align inward to form transmembrane pores. Once associated with the membrane surface a number of the ordered bacteriocins could potentially aggregate. The bacteriocin can in principle completely span the membrane thereby forming a transeint pore (Fig.1-4).

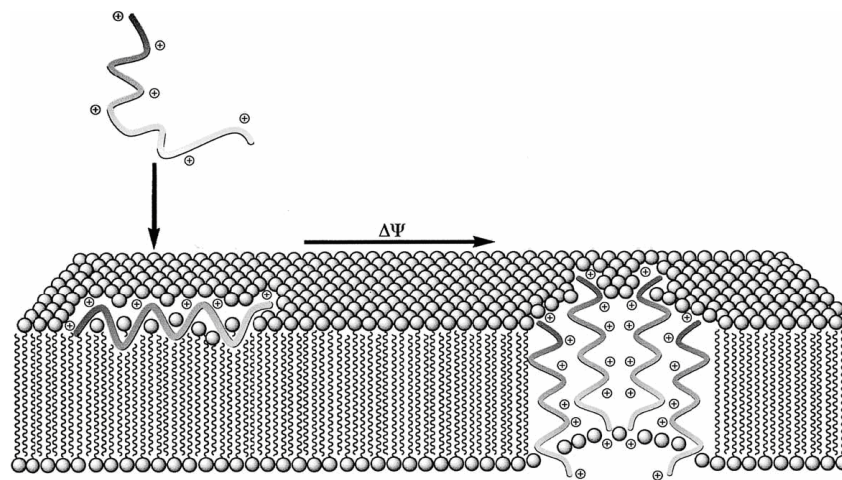


Figure 1-4 Barrel-stave/wormhole mechanism of pore formation by cationic peptides which do not require a receptor

- In the micellar aggregate model suggests that the peptide reorient, binding and associate in an informal membrane-spanning micellar or aggregate-like arrangement of monomers in the membrane leading to formation of poration complexes and further indicates that collapse of these micellar aggregate can explain translocation into the cytoplasm. The micelle-like aggregate are proposed to have water associated with them, and this provides channels for the movement of ions across the membrane and possibly leakage of larger water-soluble molecules, mainly through efflux of potassium, and leakage of inorganic phosphate (Klaenhammer, 1993). These aggregates would be variable in size and lifetime and will dissociate into monomers that may be disposed at either side of the membrane. Therefore, the concentration of bacteriocin should determine the size exclusion limit the pores. Indeed, the requirement for a high concentration of the bacteriocin to allow the passage of large molecule as glutamate or high-molecular weight dextrans was reported (Tahara and Kanatani, 1996).

Class IIa bacteriocins are believed to insert into the target membrane via their hydrophobic and/or amphiphilic C-terminal domain, and aggregate to form water-filled pores. These compounds are bactericides that disrupt the integrity of the cytoplasmic membrane, producing ionic imbalance and leakage of organic phosphate to exert their killing action. Unlike lantibiotics, which totally dissipate both $\Delta\phi$ and ΔpH , class IIa bacteriocins readily provoke a total dissipation of ΔpH , but only a partial dissipation of $\Delta\phi$ (Ennahar *et al.*, 1999). The amphiphilic region has been thought to allow the peptide monomers to oligomerize into membrane-spanning pores, with the hydrophobic side facing the membrane and the hydrophilic side forming the pore. The hydrophobicity of bacteriocin provided by neutral amino acid residues will promote interaction with targeting cell membranes and aid the presence of transmembrane potentials, when amphipathic nature also act as surfactant-like activity on the cell membranes. These structures could participate in the formation of transmembrane pore (Turner *et al.*, 1999).

- In the carpet model, the peptides do not insert into the membrane but align parallel to the bilayer, remaining in contact with the lipid head groups and effectively coating the surrounding area. This orientation leads to a local disturbance in membrane stability, causing the formation of large cracks, leakage of cytoplasmic components, disruption of the membrane potential and, ultimately, disintegration of the membrane.

1.3.4.2 Non-membrane disruptive fashion

Bacteriocins that do not appear to act on membranes are thought to act on cytoplasmic targets. They differ in their lethal action and can initiate reactions which can inhibit energy production and synthesis of protein or nucleic acids.

Translocation across membranes is proposed to occur by a process related to the micellar aggregate mechanism. Once present in the bacterial cytoplasm, cationic peptides are thought to interact with DNA, RNA and/or cellular proteins and to inhibit synthesis of these compounds. In addition, specific enzymatic targets have been identified for certain bacteriocin. For these bacteriocins, loss of viability is much slower than with membrane-acting bacteriocins, which exert their antimicrobial effects within minutes.

1.3.5 Structure –activity relationship

The structure of bacteriocins have been reported in 4 forms: β -sheet, α -helical, loop and extended peptides, with the first two forms being the most common in nature. For clarity, representative structure from each one are indicated in Fig.1-5. A common trait shared amongst the bacteriocin is the ability to fold into amphipathic or amphiphilic conformation with opposing hydrophobic and positively charged faces, often induced by interaction with bacterial membrane or membrane mimics. The structural motifs, that could form amphiphilic α -helices or β -sheets on N-terminal part, often exist as transmembrane region in pore-forming peptides.

1.3.5.1 β -sheet peptides

This form is characterized by the presence of an antiparallel β -sheet, generally stabilized by disulfide bonds. Larger bacteriocins within this form may also contain minor helical segments.

The disulfide bonds impart a stabilizing force to the overall molecule and allow the bending (hinge-like) to occur and that this structural flexibility in what has been traditionally thought of as rather rigid β -hairpin conformation permits or drives translocation across membrane.

1.3.5.2 α -helical peptides

α -Helical conformation often contains a slight bend in the center of the molecule.

1.3.5.3 Extended peptides

The extended peptides lack classical secondary structure, generally due to their high proline and /or glycine contents. Indeed, these peptides form their final structure not through interresidue hydrogen bonds but hydrogen bond and Van der Waals interaction with membrane lipids. The conformation of these groups is dependent on its environment. For example, in both lipid environments (anionic SDS or zwitterionic dodecylphosphocholine (DPC)), indolicidin (13 residues) exists in an extended conformation, however, in neutral DPC micelles, the molecule takes on a more bent conformation due to half-turns about residues 5 and 8. This fashion possesses reasonable antimicrobial activity but does not have a high affinity for lipopolysaccharide (LPS) when compare to β -sheet peptides and α -helical peptides.

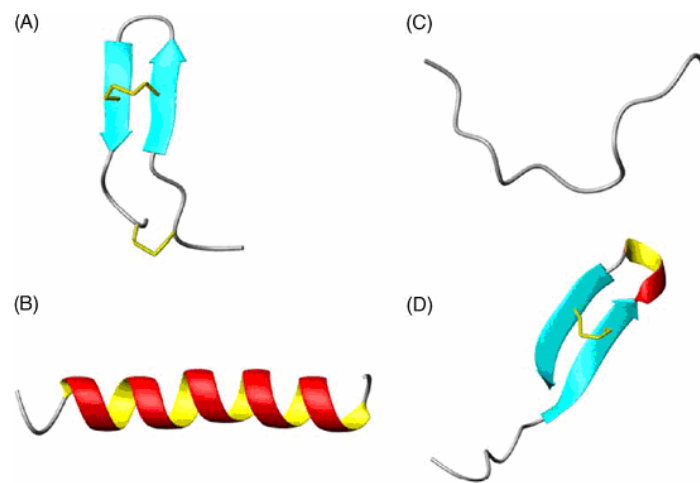


Figure 1-5 Structural conformation of antimicrobial peptides: (A) β -sheet, tachyplesin I; (B) α -helical, magainin 2; (C) extended, indolicidin; (D) loop, thanatin: disulfide bonds are indicated in light big ribbon (Adapted from Powers, J.P., and Hancock, R.E.W. 2003. The relationship between peptide structure and antibacterial activity, *Peptides*, 24: 1681-1691.)

1.3.5.4 Loop peptides

These peptides are characterized by their loop structure imparted by the presence of a single bond (either disulfide, amide or isopeptide).

It has been postulated that bacteriocins with fewer amino acid residues would tend to have a relatively broader antibacterial spectrum than those with larger numbers of residues. Furthermore, bacteriocins with slight differences in their structure and bacteriocins from the same bacterium naturally display antibacterial spectra with insignificant differences or no difference at all. Surprisingly, also exhibit spectra with only slight differences or no difference at all, although their respective structures are less related as compared to other members of the group (Ennahar *et al.*, 1999). The bacteriocins have the great variety of their chemical structures

which affect the biological properties. They are generally accepted about targeting at the membrane. Both specificity and potency of the activity of bacteriocins are apparently influenced by the lipid composition of target membranes which would determine the occurrence and the degree of interactions with bacteriocin molecules (Ennahar *et al.*, 1999). The net charge of whole live bacteria affect the susceptibility of antimicrobial peptides that the strains with highly negative charge tend to express more sensitive to the peptides (Zasloff, 2002; Ouhara *et al.*, 2005).

1.4 Role of *P. gingivalis* in periodontal diseases

1.4.1 Dental biofilm development

In oral cavity, dental plaque (supragingival and subgingival plaque) is a complex and dynamic microbial ecosystem comprising hundreds of species that exist in a dental biofilm. Environmental factors (such as pH, nutrients, oxygen concentration and metabolic properties of their bacteria) and communication among those microorganisms (include metabolite exchange, cell to cell recognition, genetic exchange, and signaling molecules) play a vital role in the biofilm formation (Filoche *et al.*, 2004; Takahashi, 2005).

Streptococcus sanguinis, *Streptococcus salivarius*, and *Streptococcus mitis* (a Gram-positive *streptococcus*) are the first organisms to colonize the crown surface at 24 h, followed by Gram-positive rods and filamentous bacteria of the *Actinomyces* species. Such organisms are facultative. The supragingival plaques consist of the stable environment of the tooth surface coated with salivary components. This continuous supply of saliva acts as a nutrient for the microorganisms while the carbohydrate derived from foods is also intermittently supplied and is a source of receptors recognized by the primary colonizers, for example, mucins, agglutinin, proline-rich proteins, phosphate-rich proteins, and enzymes (Fujimura and Nakamura, 1979; Hillman *et al.*, 1985). These species compete the initial phase of adherence, even *P. gingivalis* is capable of binding the pellicle proteins (Rudney and Chen, 2004). *Streptococcus* and *Actinomyces* species are saccharolytic and catalytic carbohydrates derived from foods to form lactic, formic, acetic, succinic and other organic acids, and concomitantly consume oxygen by

NADH oxidase. The acidification contributes demineralization of the tooth surface (dental caries), and results in partial mortality occurring in non-mutans streptococci (Takahashi, 2005).

Those aerobic indigenous microfloras are able to increase the Eh by oxidizing surrounding environment, which is not suitable for periodontal pathogen (Leke *et al.*, 1999). Furthermore, *S. sanguinis* and *S. uberis* have the ability to produce hydrogen peroxide by converting oxygen, to promote periodontal health by keeping the numbers of potentially pathogenic organism (*P. gingivalis*, *P. intermedia*, *Fusobacterium nucleatum*, *Eikenella corrodens*, *Capnocytophaga sputigena*, and *A. actinomycetemcomitans*) below the threshold level necessary to initiate disease (Hillman *et al.*, 1985; Leke *et al.*, 1999). In general, plaque from the healthy periodontium volunteers contains significantly higher proportions of inhibitors to the various test strains than plaque from periodontitis patients (Hillman *et al.*, 1985). As plaque matures during the development of gingivitis and the subgingival biofilm develops, these activities create acidic and anaerobic conditions. Then the conditions facilitate colonization of the supragingival plaque by more aciduric and oxygen-labile bacteria like mutans streptococci and lactobacilli (carious pathogen), or the appearance of subgingival colonization (Filoche *et al.*, 2004). On the subgingival area, the flora shifts toward a Gram-negative rather than a Gram-positive flora, and becomes more motile and anaerobic. Interestingly, *S. sanguinis* and *S. uberis* inhibit the growth of periodontal pathogen, *S. intermedius* and *S. mutans* enhance the growth of *P. gingivalis* and *Wolinella recta*. Additionally, *A. actinomycetemcomitans* has the ability to produce an inhibitory factor to the growth of certain streptococcal species (Hillman *et al.*, 1985;

Takahashi, 2005). It results in a reduction of local hydrogen peroxide production, which in turn permits the outgrowth of various periodontal pathogens. The pocket flora is largely anaerobic, Gram-negative and heavily motile, with spirochaetes populating the biofilm base (Takahashi, 2005).

1.4.2 Characteristics of *P. gingivalis*

Members of the genus *Porphyromonas* are 0.5-0.8 by 1.0-3.5 μm in diameter. *P. gingivalis* species are nonmotile, asaccharolytic, obligatively anaerobic Gram-negative coccobacilli exhibiting smooth, raised colonies. When grown on a blood agar surface, the colonies are initially white to creamy color. These colonies darken upon time (4-8 days) from their edge towards the center and a deep red to black color, which correlates with the concentration of protoheme, see also in section 1.5.3; proteolytic enzymes. The species produce a large number of enzymes, proteins and end-products of their metabolism that are active against a broad spectrum of host proteins and provide mechanisms for evasion of host defense. These latter compounds include proteinase inhibitors, immunoglobulins inhibitors, iron-containing proteins, bactericidal proteins, extracellular matrix proteins, and proteins intimately involved in phagocytic functions, such as complement fixation and coagulation. The ability of *P. gingivalis* to secrete a cysteine proteinases in a host provides them with distinct advantages for its survival and growth, including the ability to use large host proteins for their growth and metabolism. Since these proteinases

cleave both synthetic and host proteins after arginine and lysine residues, their growth is significantly enhanced in the presence of protein hydrolysates (Holt *et al.*, 1999).

The macromolecules associated with *P. gingivalis* that might function in the inflammatory and destructive events of periodontal disease (Holt *et al.*, 1999), called virulence factors. Through the modulation of their cell surface components, bacterial pathogens can enhance their survival and suppress host defenses such as complement system and phagocytosis (Schifferle, 1994).

1.4.3 Bacterial membrane composition of *P. gingivalis*

The membrane structures of *P. gingivalis* contain capsule, adhesins, vesicles, lipopolysaccharides (LPS), and other outer membrane proteins.

The capsule is extracellular, hydrophilic, and negatively charged polysaccharides (Tortora *et al.*, 1998). Thick capsular hydrophilicity decreases ability to activate an alternative complement pathway and masks the lipopolysaccharide of the outer membrane, and therefore the bacteria are protected from opsonization and phagocytosis (Holt *et al.*, 1999). Its encapsulation is associated with the presence of K antigens. The K antigens are thermostable, negatively charged, sensitive to periodate degradation and resistant to proteinase K treatment (Laine *et al.*, 1997).

P. gingivalis's adhesin involves peritrichous fimbriae (Fig.1-6) and a cell-bound hemagglutinating adhesion. The fimbriae are constructed from fibrillin subunits, which form a mature oligomeric protein of 67 kDa with hydrophobicity, and function similar to the fimbriae of

other Gram-negative bacteria (Holt *et al.*, 1999). The receptor molecules for fimbriin include salivary molecules such as proline-rich proteins, proline-rich glycoproteins, and statherin (Rudney and Chen, 2004; Takahashi, 2005). The cell-bound hemagglutinating adhesion (antigenic determinant) is associated with polypeptides with molecular mass of about 33 and 38 kDa (Mouton *et al.*, 1989).

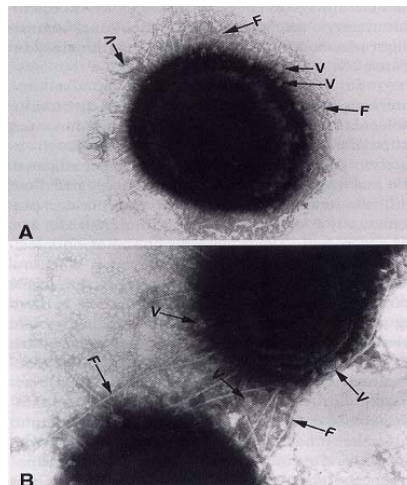


Figure 1-6 Transmission electron photomicrographs of negatively stained cell surfaces of *Porphyromonas gingivalis* strain ATCC 33277, Numerous thin fibrils or fimbriae (F), Numerous outer membrane vesicle (V) (Adapted from Holt, S.C., Kesavalu, L., Walker, S., and Genco, C.A. 1999. Virulence factors of *Porphyromonas gingivalis*, Periodontol. 2000, 20:180.)

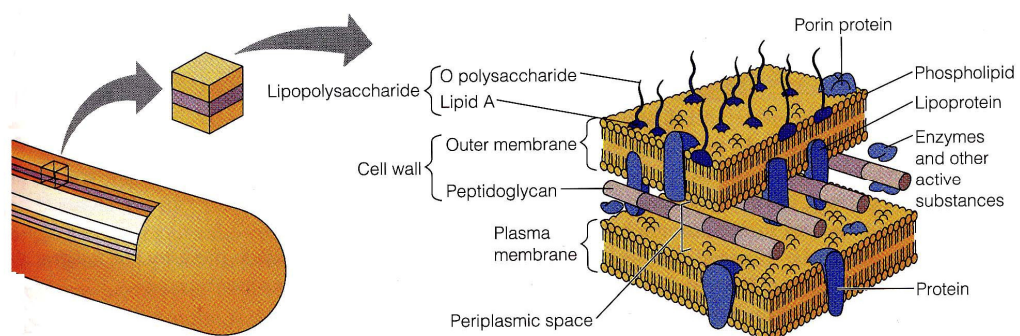


Figure 1-7 A Gram-negative cell wall

(Adapted from Tortora, G.J., Funke, B.R., and Case, C.L. 1998. Microbiology:an introduction. 6th ed. Benjamin/Comings Publishing Company:Melo, California.pp.87.)

LPS is a major component of the outer membranes having two important characteristics of Gram-negative bacteria (Fig.1-7). The first is the polysaccharide portion composed of sugar, called O polysaccharides. It functions as an antigen and is useful for distinguishing species of Gram-negative bacteria. The second is the lipid portion of the lipopolysaccharide, called lipid A, and is referred to as endotoxin (Tortora *et al.*, 1998). The *P. gingivalis* strain may produce a population of lipid A with different levels of acylation depending on the growth conditions (Paramonov *et al.*, 2005).

The proteolytic enzyme synthesized by *P. gingivalis* is associated with the capsule and outer membrane vesicles, which are blebs of outer membranes released from the bacterium. They are two forms of cysteine proteinase specific for arginyl-X bonds (50 and 95b kDa) and lysyl-X bond specific enzyme (105 kDa), and are referred to as Arg-gingipain and Lys-gingipain, respectively. They are capable in the subversion of the opsono-phagocytic capacity to complement and destroy immunoglobulins, iron-binding proteins, and antimicrobial peptides (Jagels *et al.*, 1996; Paramonov *et al.*, 2005).

The plasma membrane consists primarily of phospholipids and proteins (Fig.1-8). As a result, plasma membranes have selective permeability. Large molecules (such as proteins) cannot pass through the plasma membrane, probably because these molecules are larger than the channels in integral proteins, whereas the smaller molecules such as ions usually pass through easily. Substances that dissolve easily in lipids enter and exist more easily than other substances (Tortora *et al.*, 1998). The porins and integral proteins are key factors to regulate a

transport mechanism (Lamont and Yilmaz, 2002). In addition, phospholipid bilayer of plasma membrane has selective permeability. Besides, the function of cytoplasmic membrane is the site of carrier-mediated transport. The others are sites of ATP generation and cytochrome activity, sites of cell wall synthesis and chromosome attachment (Tortora *et al.*,1998).

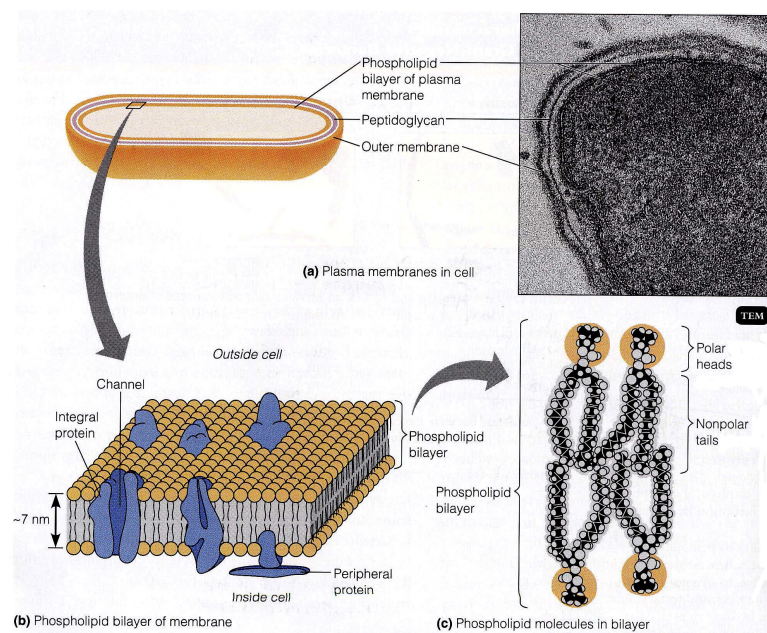


Figure 1-8 Plasma membrane (a) A diagram and micrograph showing the phospholipid bilayer (b) A portion of the inner membrane showing the phospholipid and proteins. (c) Space filling models of several molecules as they are arranged in the phospholipid layer.

(Adapted from Tortora, G.J., Funke, B.R., and Case, C.L., 1998. Microbiology: an introduction.6th ed. Benjamin/Commings Publishing Company:Melo, California. pp.90.)

1.4.4 Periodontal disease(s)

The word *periodontal* literally means "around the tooth." Periodontal disease is a chronic bacterial infection that affects the gums and bone supporting the teeth. The term "periodontal disease" in this sense refers to inflammatory lesions of gingivae (gingivitis) and those affecting

the underlying structures of the periodontium (periodontitis). Gingivitis is a direct immune response to the dental microbial plaque building up on the teeth. Then the periodontitis is developed and influenced by the individual's immune and inflammatory response. Periodontitis is characterized (Fig.1-9) by a significant breakdown of tooth supporting tissues, including the periodontal ligament and alveolar bone (Kinane, 2001; Lamont and Yilmaz, 2002; Slots, 2002; Southard and Godowski, 1998). Clearly, gingivitis precedes periodontitis and this implies that prevention of gingivitis is also a primary preventive measure for periodontitis.

There are various forms of periodontitis, which can be classified according to localization, duration, and population. The most common form of periodontitis is adult periodontitis. For histopathological characteristics, there have periodontal pocketing, location of junctional epithelium apical to the cemento-enamel junction, loss of collagen fibers subjacent to the pocket epithelium, alveolar bone loss, numerous polymorphonuclear leukocytes in the junctional and pocket epithelium, and a dense inflammatory cell infiltrate with plasma cells, lymphocytes, and macrophages (Kinane, 2001).

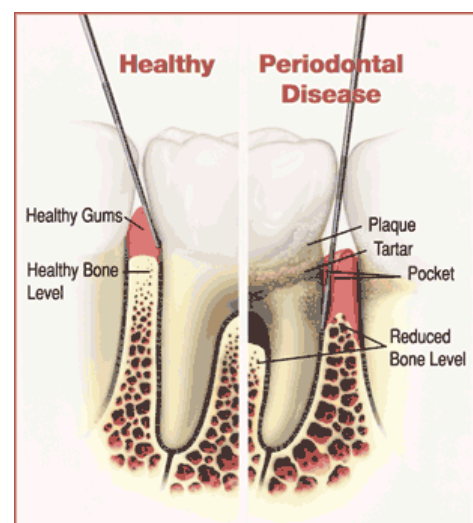


Figure 1-9 Healthy gingiva and periodontal diseases

(Adapted from <http://www.perio.org/consumer/2a.html#causes>)

1.4.5 Pharmacotherapy (focusing on microbial factor)

The goals of periodontal therapy are to alter or eliminate the pathogenic organism in contact with periodontal tissue and get rid of contributing risk factor for periodontal diseases (Drisko, 2001; Petersilka, 2002). Mechanical therapy consists of debridement of the roots by the meticulous use of hand or power-driven scalers to remove plaque, endotoxin, calculus and other plaque-retentive local factors (Fig.1-10). The term mechanical therapy refers to both supragingival and subgingival scaling as well as root planning. Chemotherapeutic approaches include topical application of antiseptics or sustained-release local drug delivery agents that are designed to prevent plaque accumulation and to disinfect the root surfaces and adjacent periodontal tissues. Systemic approaches encompass the selective use of antibiotics and host modulation of tissue destructive enzymes. Once the infection and inflammation are controlled by surgical or nonsurgical methods, periodontal health can be sustained for extended periods of time with daily plaque control by patient and periodic professional maintenance by dentist and dental hygienist, see in Fig.1-11 (Drisko, 2001).

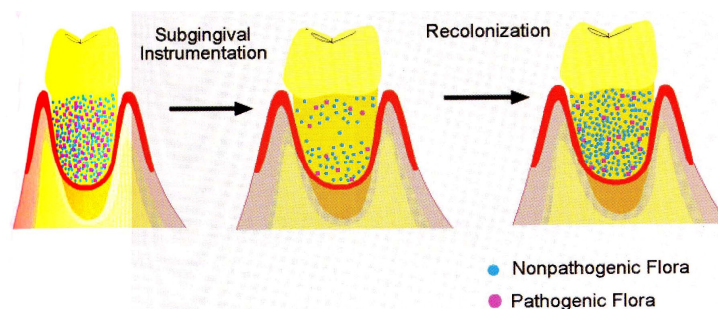


Figure 1-10 The reduction and recolonization pattern in subgingival debridement (Adapted from Petersilka, G.J., Ehmke, B., and Flemmig, T.F. 2002. Antimicrobial effects of mechanical debridement, *Periodontol.* 2000, 28: 56-71.)

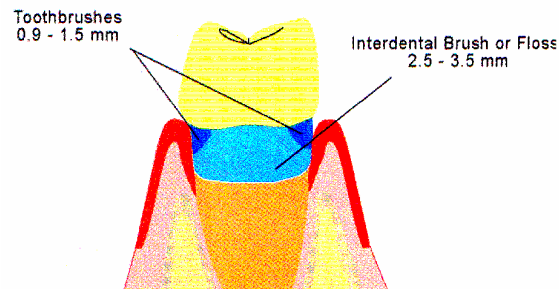


Figure 1-11 Mean range of penetration depths of toothbrushes and interdental hygiene devices (Adapted from Petersilka, G.J., Ehmke, B., and Flemmig, T.F. 2002. Antimicrobial effects of mechanical debridement, *Periodontol.* 2000, 28: 56-71.)

When manual instrumentation or sonic/ultrasonic scalers are used for the treatment of the subgingival pockets, profound shifts in the composition of the microbial flora are observed. They found recolonization of subgingival plaque that it takes several months to repopulate the pocket following a thorough scaling and root planing in the presence of good daily oral hygiene by the patient (Fig.1-11) However, with poor supragingival plaque control, the microbiota may reestablish itself within 40-60 days following subgingival debridement (Drisko, 2001). Beside, location of *P. gingivalis* in inaccessible areas, such as furcations or the base of periodontal pockets, is probably responsible for the failure of mechanical therapy to remove the organism (Slot and Ting, 1999).

If a pocket is deeper than 5 mm after conventional treatment, surgical procedures have been suggested (Lindhe *et al.*, 1982). However there is a possible alternative to do periodontal surgery with effective drug concentrations in local delivery routes. In clinical trials, subgingival delivery of antibiotics reduced the pocket depths and gained of attachment level

(Loesche, *et al.*, 1991; Southard and Godowski, 1998), but in some studies local drug delivery failed (Drisko, 2001; Slots, 2002).

1.4.5.1 Topical antiseptics

Bacteria are primary etiological agents in periodontal diseases and oral antiseptics have been shown to provide benefits in combating dental caries and gingival diseases due to dental biofilm maturation. Antiseptics have a considerably broader spectrum of activity than antibiotics and, in contrast to antibiotics, often have multiple intracellular targets which reduce the likelihood of resistance development.

Mouthrinses are the simplest vehicles for antiplaque agents such as chlorhexidine gluconate (0.12% and 0.2%), PeridexTM, PerioguardTM, Listerine[®], PlaxTM, and povidone-iodine mouthrinse (Bouwsma *et al.*, 1996; Southard and Godowski, 1998; Chang *et al.*, 2001), however, mouthrinses do not penetrate the periodontal pocket. Major applications of mouthrinses are in reducing plaque and gingivitis (Bouwsma *et al.*, 1996; Slots, 2002).

Chlorhexidine chips (PerioChipTM) (Fig.12) for subgingival placement may be capable of reducing mean probing depth in deep periodontal pockets and may not cause noticeable reduction in periodontal pathogens compared to thorough scaling and root planning. 0.12% and 0.2% chlorhexidine are not effective for subgingival irrigation, and cause less change in the subgingival microbiota than low strength (0.05%) of povidone-iodine. Using 2% chlorhexidine irrigation may provide more effective killing of subgingival pathogens (Slots,

2002). However, even low concentrations of chlorhexidine may be toxic to gingival fibroblasts and reduce the production of collagen and non-collagen proteins, potentially impeding periodontal healing. An undesirable sequel associated with chlorhexidine usage is teeth staining, since chlorhexidine enhances accumulation of a sulfur-containing component of plaque that in turn interacts with iron to produce the stain materials. Chlorhexidine and copper in combination were 10 to 100 times more effective than when chlorhexidine was used alone (Bouwsma *et al.*, 1996).

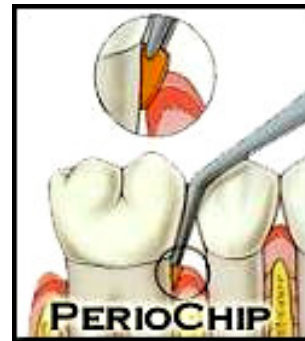
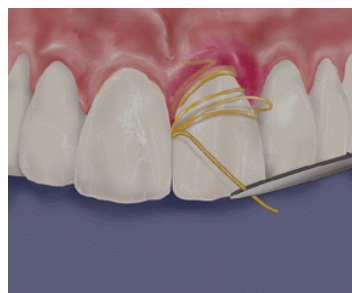


Figure 1-12 PerioChip™ and Actisite® Periodontal Fiber

(Adapted from http://www.dentalcare.com/soap/pr_forum/periodon/actuse.htm)

Listerine, an essential oil mouthrinse, was as effective as an amine and stannous fluoride rinse; however chlorhexidine reduced plaque more than both of them.

Plax, a sodium benzoate-containing rinse, used as a prebrushing rinse has an application reducing tooth stain (Bouwsma *et al.*, 1996).

Povidone-iodine employs microbicide to a wide variety of bacterial, fungal and viral infections. Short durations of povidone-iodine contact with various periodontopathic bacteria provides effective *in vitro* killing. Emergence of povidone-iodine resistance

microorganisms has not been reported to date. Povidone-iodine is water-soluble, does not irritate healthy or diseased oral mucosa, and exhibits no adverse side-effects, such as discoloration of teeth and tongue and change in taste sensation, as seen with chlorhexidine. Despite its advantage, povidone-iodine has the potential to induce hyperthyroidism due to excessive incorporation of iodine in the thyroid gland and should therefore be used only for short periods of time. Contraindications are pregnant patients and nursing women in order to protect the infant. For subgingival irrigation, an effective concentration is 10% povidone-iodine applied repeatedly to obtain a contact time of at least 5 min. This is generally performed upon completion of each session of scaling and root planing, but may also be done prior to mechanical debridement to reduce the risk of bacteremia, particularly in medically compromised individuals and in patients with severe gingival inflammation. For use in ultrasonic scalers, 10% povidone-iodine is diluted by mixing 1 part solution with 9 parts or less of water, dependent upon patient acceptance. A controlled release device for subgingival application of povidone-iodine has been developed. Due to rapid microbial killing by povidone-iodine, a short-term application of the agent alone may produce an adequate antimicrobial effect. Povidone-iodine mouthrinse/gargle has shown a reduction in gingival surface bacteria. Investigators have also reported on favorable clinical outcome after treating advanced periodontitis with combination of subgingival povidone-iodine and systemic antibiotics. In periodontal lesions exceeding 6 mm in probing depths, treated with 0.5% povidone-iodine subgingival irrigation gained in probing attachment (Slots, 2002).

Triclosan, a chlorinated bisphenol, is a mild antiseptic like chlorhexidine and cetylpyridinium chloride. It has been reported to be effective against Gram-positive and most Gram-negative bacteria, to have variable or poor activity against *Pseudomonas* species, and to be active against fungi. The combination of triclosan and polyvinylmethylether-maleic acid copolymer provides a greater antimicrobial effect than that of triclosan alone, because the combination enhances uptake of triclosan to enamel and buccal epithelial cells. A triclosan-copolymer dentrifice significantly reduces plaque and gingivitis. Triclosan and zinc citrate combination were marked complementary and additive inhibitory effects, especially against some Gram-negative species.

Meridol, an amine and stannous fluoride rinse, provides significant improvement of the Gingival Index, Sulcular Bleeding Index, and Plaque Index after a 7 month-period testing. It has been observed that a significant decrease in proportion of rods and other plaque bacteria and a significant increase in cocci percentage occurred in the supragingival plaque treated with the amine and stannous fluoride rinse. The use of this agent resulted in tooth staining, but significantly less than chlorhexidine (Bouwsma *et al.*, 1996).

Sodium hypochlorite (household bleach) has ideal properties of antimicrobial agent, including broad antimicrobial activity, rapid bactericidal action, and relative non-toxicity at concentrated use. There is neither color nor staining. There is ease of access and the cost is low. The active species is undissociated hypochlorous acid (HOCl). Hypochlorite is lethal to most bacteria, fungi and viruses. Activity is reduced by the presence of organic material, heavy metal

ions and low pH. Hypochlorite solutions will gradually lose strength, so that fresh solutions should be prepared daily, especially if the solution is not stored in closed brown opaque containers. Disadvantages include irritation of mucous membranes when used in high concentrations, bleaching of colored fabrics and corroding effect on some metals. No contraindications exist. A sodium hypochlorite solution for subgingival irrigation can be prepared from household bleach that usually contains 5.25–6.0% of available chlorine. If 1 part bleach is combined with 49 parts water, the resulting solution will contain an appropriate working concentration of about 0.1% or 1000 p.p.m. of available chlorine. In actual use situations, a working bleach solution can be obtained by adding 1 teaspoon (5 ml) household bleach to 250ml water (approximately 2 large drinking glasses), and deliver the bleach solution subgingivally via a commercial oral irrigator at a high pressure setting. The lowest concentration of chlorine that reliably inactivates test bacteria in vitro is 0.01%. In experimental biofilms with various periodontal pathogens, the highest bactericidal activity was obtained with 2.25% sodium hypochlorite and 10% povidone-iodine followed by 0.2% chlorhexidine. At low concentrations (0.1%), sodium hypochlorite can be used as topical antibacterial agent without inhibiting fibroblast activity and is recommended as subgingival sodium hypochlorite irrigation in the maintenance phase of periodontal therapy. Subgingival irrigation with 0.5% sodium hypochlorite (Dakin's solution) caused significantly greater and longer lasting reduction in plaque and gingivitis than irrigation with water (Slots, 2002).

1.4.5.2 Topical antibiotics

A variety of locally delivered antimicrobial agents have commercially emerged in various countries. They are designed to enhance healing and stabilize periodontal health. In disease sites that are more difficult to control, local drug delivery devices such as tetracycline hydrochloride (Actisite[®] Periodontal Fiber) (Fig.1-14), metronidazole gel or strip (Elyzol[™]), Minocycline microspheres, 10% doxycycline gel (Atridox[™]), or ofloxacin may be placed directly adjacent to the infected site (Slots, 2002). The rationale for using an antimicrobial agent is to chemically kill or reduce the plaques within the biofilm in the pocket. By placing an antibiotic or antiseptic in direct contact with the root surface, pathogenic organisms that are not accessible to mechanical removal by hand or power-driven instruments can be reduced or eliminated. However, if disease activity is generalized and there are many sites losing attachment, local delivery may not be the treatment of choice (Drisko, 2001).

Considering potential problems with selectivity of antimicrobial action and possible development of bacterial resistant and adverse host reactions, topical antibiotic therapy seems to constitute a less desirable choice than the use of a broad-spectrum antiseptic agent with low potential for adverse reactions. Also, commercial topical antibiotic devices tend to carry high financial costs. When choosing between equally effective and safe drug therapies, preference should usually be given to the one having the lowest cost. If dental practitioners desire to utilize antibiotics topically in periodontal therapy despite the propensity of most antibiotics to induce bacterial resistance, then the choice of antibiotics should be restricted to those that are too toxic to

be administered systemically (bacitracin, polymyxin B, neomycin) or are unlikely to develop resistance (metronidazole). Double or triple antibiotic combinations may then be used to provide an adequate spectrum of antibacterial activity. However, periodontal sites having yeast infections may respond adversely to antibiotic medications directed against bacterial pathogens (Slots, 2002).

1.4.5.3 Systemic antibiotics

While periodontal debridement with or without locally applied chemotherapeutic agents is usually affective in controlling early to moderate periodontal lesions, more advanced cases may require antibiotics following periodontal debridement if the sites have not responded as well as expected (Drisko, 2001). We summarized the systemic antibiotics that are used for periodontal therapy and also affect to *P. gingivalis*: penicillins, tetracycline, metronidazole, tetracycline plus metronidazole, amoxicillin plus metronidazole, spiramycin-metronidazole, ciprofloxacin, clindamycin, and doxycycline

Location of *P. gingivalis* in inaccessible areas, such as furcations or the base of periodontal pockets is probably responsible for the failure of mechanical therapy to remove the organism. The most effective control of periodontal *P. gingivalis* seems to be achieved by employing a combination of resective periodontal surgery systemic antibiotic therapy and good oral hygiene. Regeneration of periodontal connective attachment is contingent upon the absence of *P. gingivalis* and other periodontal pathogens in treated sites (Slots and Ting, 1999).