II. LITERATURE REVIEWS

1. Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are comprised a group of gram-positive, low-GC, acid tolerant, non-sporulating rods or cocci that are associated by their common metabolic and physiological characteristics (17). The genera belonging to this group can produce organic acids such as lactic acid as the major metabolic end product of carbohydrate fermentation. This trait has historically linked LAB with food fermentations as acidification to inhibit the growth of spoilage agents. Lactic acid and other metabolic products contribute to the organoleptic and textural profile of a food item. The industrial importance is further evidenced by their generally regarded as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces. These microorganisms are considered nonpathogenic and are believed to be beneficial to human health (18, 19). These microorganisms have 3 classes of fermentative metabolism including the obligate homofermentative counterpart, that glucose is fermented predominantly to lactic acid (Figure 1), the facultative heterofermentative counterpart, and the last one is obligate heterofermentative counterpart, that glucose is fermented to equimolar amounts of lactic acid, carbondioxide and ethanol (and/or acetic acid) (Figure 2) (20). The lactic acid bacteria belong to the genera are *Streptococcus*, *Lactococcus*, *Enterococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium* and *Lactobacillus* (21).

1.1 *Streptococcus*

Streptococci were among the earliest bacteria to be recognized as LAB by microbiologist because of their involvement in a large number of human and animal diseases. The genus *Streptococcus* was originally described based on morphological, serological, physiological and biochemical characteristics. It comprised a wide range of organisms including the highly pathogenic bacteria *S. pneumoniae*, *S. pyogenes* and *S. agalactiae*; the intestinal group D streptococci, *S. faecalis* and *S. faecium*; and the

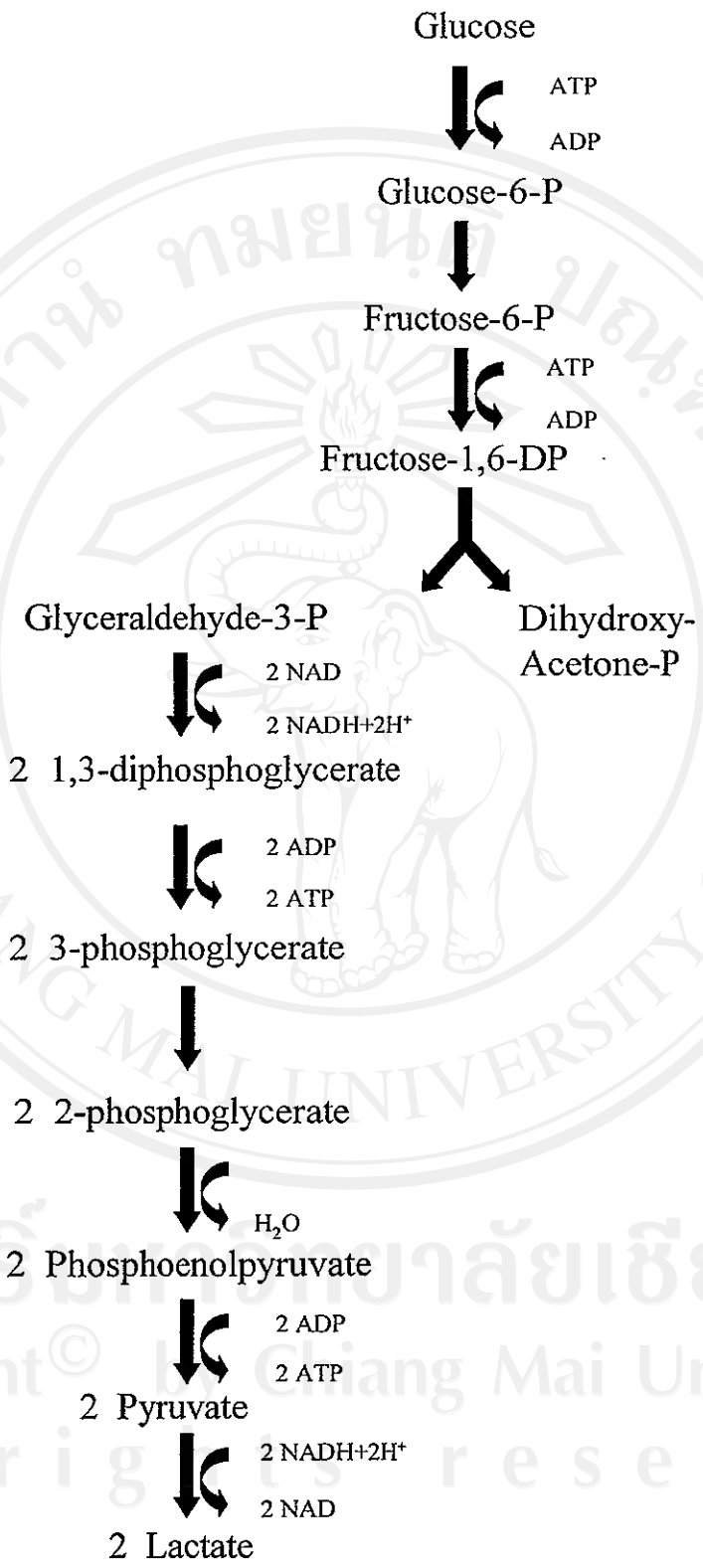

Figure 1 Homolactic fermentation scheme of lactic acid bacteria (20)

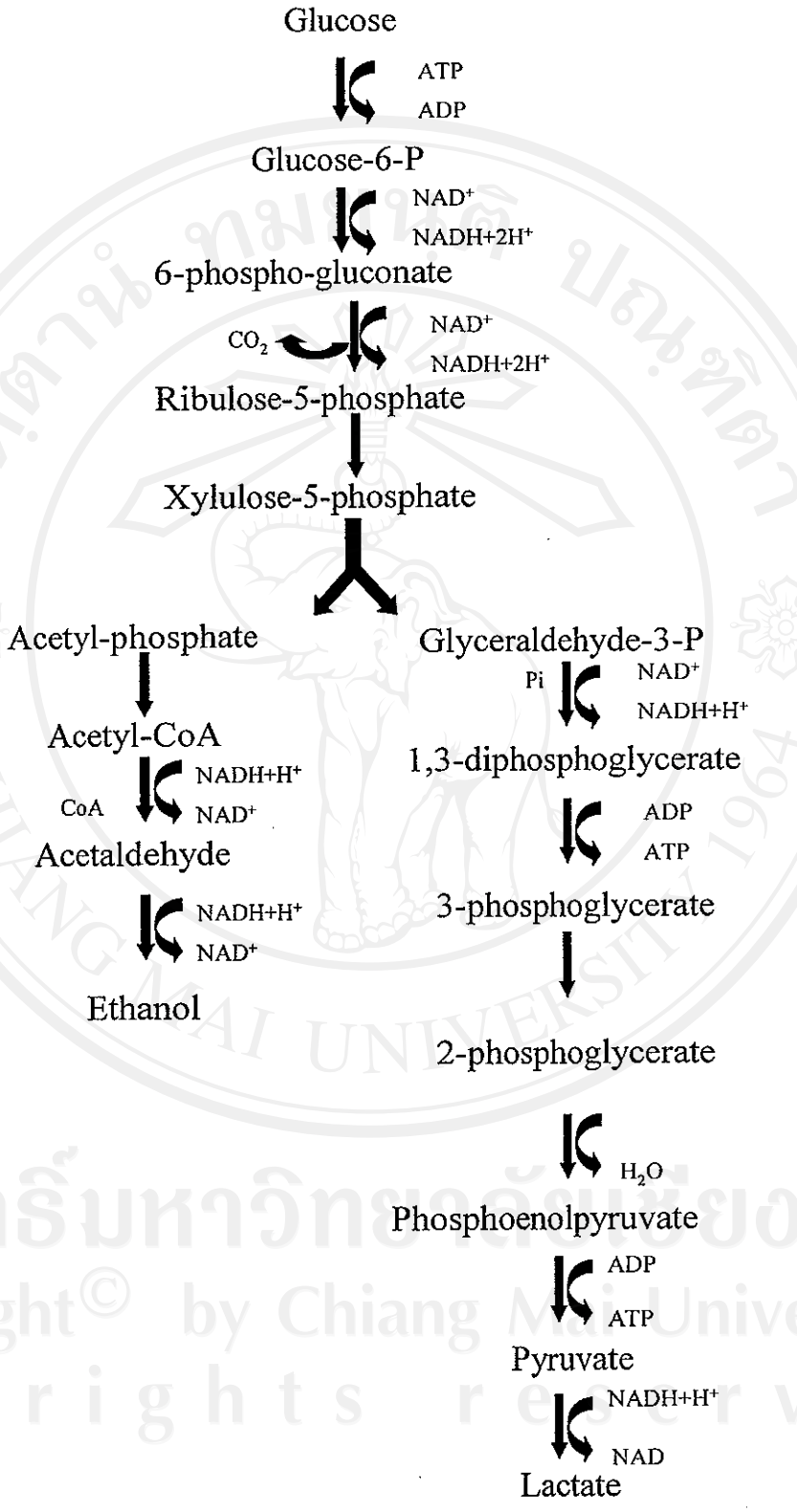

Figure 2 Heterolactic fermentation scheme of lactic acid bacteria (20)

economically important group N starter bacteria for yogurt and cheese manufactures, *S. thermophilus*, *S. cremoris* and *S. lactis*. It does not produce a Lancefield group antigen response. It can grow at 45°C and up to 50°C but not at 15°C, and it is relatively heat resistant (22). The streptococci have complex nutritional requirements and thrive in environments with a good supply of carbohydrate and protein, including tissues and intestinal tracts of animals, milk, dairy products, vegetable material and other foods.

1.2 *Lactococcus*

Most but not all of the Lancefield group N lactic streptococci have been reclassified to the genus *Lactococcus* (22). The genus *Lactococcus* includes several common species: *L. garvieae* associated with mastitis in cows, *L. piscium* from salmonid fish, *L. plantarum* from frozen peas, *L. raffinolactis* from raw milk and *L. lactis* from plant materials and dairy products. The use of *Lactococcus* is widespread in industrial starter culture technology. Genetic studies on lactococci have focused on the lactic fermentation, casein hydrolysis, diacetyl production from citrate, phage resistance and bacteriocin production. Many strains of *Lactococcus lactis* produce a range of bacteriocins. The most important of which is the lantibiotic, nisin, the broad spectrum bacteriocin against gram-positive bacteria, including *Clostridium botulinum* and its spores (23).

1.3 *Enterococcus*

Enterococcus can grow at 10 and 45°C, in 6.5% NaCl and at pH 9.6, survive at 60°C for 30 min and react with Lancefield group D antisera. The L (+)-lactic acid production of enterococci was homofermentatively from glucose and derived energy from degradation of amino acids. It has a phosphoenolpyruvate phosphotransferase system (PEP-PTS) for the uptake of lactose and other carbohydrates, including gluconate (24). The importances of the enterococci in food and public health are related to their enteric habitat, their use as indicators for food safety and their possible involvement in food borne illness. The value of enterococci as indicators of faecal contamination in foods is limited by their ability to survive in the extraenteric environment, their relatively high heat resistance and the fact that they can

predominate in the microbial population of heat treated foods. Enterococci are also used as starter cultures in some foods and are commercially available as probiotics for prevention and treatment of intestinal disorders of humans (25).

1.4 *Carnobacterium*

This genus was proposed by Collins *et al.* (26) that is gram-positive, catalase-negative, non-spore forming rod from poultry meat stored at low temperature. It resembles lactobacillus but it dose not grow on acetate media, and was referred to the "non-aciduric lactobacilli". Comparative 16s rRNA sequence analysis studies of the genus *Carnobacterium* confirmed the similarity of this organism and its distinction from all other LAB (27). The food-associated carnobacteria have a number of physiological features in common with the enterococci, notably their ability to grow at pH 9.5, resistance to thallous acetate, antibiotic resistance and vitamin requirements. The sparse information is available on the association of carnobacteria with foods other than meat, poultry and fish (28).

1.5 *Tetragenococcus*

The genus *Tetragenococcus* is a new genus which separated from *Pediococcus*. In phylogenetic studies of the genus *Pediococcus* using 16s rRNA sequence analysis, it was shown that *Pediococcus halophilus* is clearly separated from the other pediococci. It is more closely related to *Enterococcus* and *Carnobacterium*, which requires 18% NaCl for growth (29).

1.6 *Vagococcus*

The genus *Vagococcus* is the motile group N streptococci (30). It is a newly established genus from a phylogenetic cluster with the genus *Enterococcus*. The studies using 16s rRNA sequences established that they are more closely related to the genus *Enterococcus*, the carnobacteria and *Listeria* than to the genera *Streptococcus* and *Lactococcus* (30, 31).

1.7 *Leuconostoc*

The genus *Leuconostoc* is close to the streptococci, albeit in a separate genus as the heterofermentative cocci which formerly called the betacocci. They produce D-(-) lactate from glucose that is produced by the lactococci and DL-lactate by the heterofermentative lactobacilli with whom, they share many characteristics. In fermented foods of plant origin, *Leuconostoc mesenteroides* is generally as the first organism to grow and it is succeeded by the more acid-tolerant lactobacilli (32, 33). The *Leuconostoc* group has considerable species-specific commercial importances, including spoilage in sugar processing by production of dextrans, the malolactic fermentation in wine making, and production of flavor components from citrate in dairy fermentations that lead to the wide applications in research, industry and medicine.

1.8 *Pediococcus*

Pediococci has tetrad formation and spherical shape served as key characteristics for their early recognition. They are most likely to be confused with micrococci and aerococci because of morphological similarity, pseudocatalase production and salt tolerance. The pediococci are homofermentative lactic acid bacteria. All species can produce DL-lactate from glucose (23). They require a fermentable carbohydrate for growth and grow poorly in milk because lactose is not readily utilized. Fermentation of glucose follows the Embden-Meyerhof pathway with DL- or L-(+)−lactate as the major end product under optimal conditions. Pyruvate can be diverted to other end products and diacetyl/acetoin is often produced by *Pediococcus damnosus*, while *Pediococcus pentosaceus* produces an equimolar amounts of lactate and acetate from pentoses (34). The pediococci are the important starter bacteria in fermented sausages of some regions. Some of the widely used starter strains such as *Pediococcus acidilactici* and *P. pentosaceus* produce bacteriocins (23).

1.9 *Bifidobacterium*

Bifidobacteria are generally characterized as gram-positive, non-spore forming, non-motile and catalase-negative anaerobes. Presently, 30 species are

included in the genus *Bifidobacterium*, 10 of which are from human sources (dental caries, faeces and vagina), 17 are from animal intestinal tracts or rumen, 2 are from waste water and another one is from fermented milk. Bifidobacteria are phylogenetically grouped in the actinomycetes branch of gram-positive bacteria (20). They are the saccharolytic organisms that produce acetic and lactic acids without generation of CO_2, except during on the degradation of gluconate. They distributed in various ecological niches including the human gastrointestinal and genitourinary tracts but exact ratio of which is mainly depended on age and diet. The indigenous microflora of infants is predominated by bifidobacteria, which become established shortly after birth. The numbers of bifidobacteria decrease with increasing age of the individual and eventually become the third most abundant genus accounting (for 25% of the total adult gut flora) after the genera *Bacteroides* and *Eubacterium* (35).

1.10 *Lactobacillus*

Lactobacillus is generally characterized as gram-positive, non-spore forming and non-flagellated rods or coccobacilli. They are either aerotolerant, microaerophilic and strictly anaerobic fermentative and require the complex nutrition. This genus is heterogeneous 33-35 mol% G+C content in the DNA (36). They grow as the resident microflora in many different habitats as shown in Table 1.

Table 1 The habitats of genus *Lactobacillus*

Sources		
Humans	**Food spoilages**	**Other habitats**
oral cavity	Beer	plants and plant materials
intestinal tract	fruit and grain mashes	sewage soil, water and marine
vagina	marinated fish	food fermentation
	sugar processing	(milk, meat and vegetable)
	milk	cereal products
	meat and meat products	silage
	fermented beverages	

They are aciduric or/and acidophilic, producing pH 4.0 in foods containing a fermentable carbohydrate. As a result, they may inhibit growth or kill other bacteria. It is generally accepted that lactobacilli grow up to a maximum pH of 7.2, although exceptions with respect to substrate and strain exist. The classical division of the lactobacilli was based on their fermentative characteristics, namely (23): 1) obligately homofermentative; 2) facultatively heterofermentative; and 3) obligately heterofermentative. The current taxonomy of the genus *Lactobacillus* was based on the classical phenotypic subdivision as shown in Table 2.

Group 1 includes the obligately homofermentative lactobacilli that ferment glucose exclusively to lactic acid and do not ferment pentoses or gluconate. It represents thermobacteria and the important food associated species: *L.acidophilus*, *L. delbrueckii* and *L. helveticus*.

L. acidophilus is used in the production of acidophilus milk. It is considered as the important representative of probiotic bacteria (37). This strain adheres to human fetal intestinal cells *in vitro*, indication its ability of adherence to epithelial cells *in vivo*. This species and other of the *L. acidophilus*-group are studied for their typical probiotics properties (23).

L. delbrueckii and its members in complex including *L. delbrueckii*, *L. bulgaricus*, *L. lactis* and *L. leichmanii*, have 80% DNA homology and have therefore been reclassified as *L. delbrueckii* subsp. *delbrueckii*, subsp. *bulgaricus* and subsp. *lactis* (38).

Group 2 includes the facultatively heterofermentative lactobacilli that ferment hexoses to lactic acid and may produce gas from gluconate but not from glucose. They also ferment pentoses by an inducible phosphoketolase to produce lactic and acetic acids. Important food-associated species in this group include *L.casei* and *L. plantarum*.

Table 2 Taxonomy of genus *Lactobacillus* which based on the phenotypic subdivision

Group 1	Group 2	Group 3
Obligate homofermenters	**Facultative heterofermenters**	**Obligate heterofermenters**
L. acidophilus	*L. acetotolerans*	*L. brevis*
L. amylophilus	*L. agilis*	*L. buchneri*
L. amylovorus	*L. alimentarius*	*L. collinoides*
L. aviarius	*L. bifermentans*	*L. fermentum*
subsp. *araffinosus*	*L. casei*	*L. fructivorans*
subsp. *aviarius*	*L. coryniformis*	*L. fructosus*
L. crispatus	subsp. *coryniformis*	*L. hilgardii*
L. delbrueckii	subsp. *torquens*	*L. kefir*
subsp. *bulgaricus*	*L. curvatus*	*L. malefermentans*
subsp. *delbrueckii*	*L. graminis*	*L. oris*
subsp. *lactis*	*L. hamsteri*	*L. panis*
L. farciminis	*L. homohiochii*	*L. parabuchneri*
L. gallinarum	*L. intestinalis*	*L. parakefir*
L. gasseri	*L. murinus*	*L. pontis*
L. helveticus	*L. paracasei*	*L. reuteri*
L. jensenii	subsp. *paracasei*	*L. sanfrancisco*
L. kefirgranum	subsp. *tolerans*	*L. suebicus*
L. mali	*L. paraplantarum*	*L. vaccinostercus*
L. ruminis	*L. pentosus*	*L. vaginalis*
L. salivarius	*L. plantarum*	
subsp. *salicinus*	*L. rhamnosus*	
subsp. *salivarius*	*L. sake*	
L. sharpeae		

L. casei resides in various habitats including dairy products, silage, human mouth and intestine and sewage. The species was poorly defined and contained five subspecies based on phenotypic characteristics (39). The studies of DNA homology by Collins *et al.* (30) indicated that the majority of organisms designated *L. casei* subsp. *casei*, together with *L. casei* subsp. *alactosus*, subsp. *pseudoplantarum* and subsp. *tolerans*, have high levels of DNA relatedness but they are reported to be distinct from the type strain of *L. casei* subsp. *casei*. This homology group are given species status as *L. paracasei* with subspecies *paracasei* to contain all of the subspecies, except subspecies *tolerans* which was proposed as *L. paracasei* subsp. *tolerans*. The close relationship between these strains was confirmed by 16s RNA sequence homology (36). Dicks *et al.* (40) proposed the reclassification of *L. casei* subsp. *casei* ATCC 393 and *L. rhamnosus* ATCC 15820 as the neotype strain of *L. casei* subsp. *casei*. They also recommended the refection of the species name *L. paracasei*.

L. plantarum is used as a starter organism in some fermented sausages and cereal products. It was considered as the important organism in the natural fermentation of meats and the major colonizer of the human gastrointestinal mucosa (41), although this assumption may be based on the false classification of atypical streptobacteria. The strains classified as *L. plantarum* include two DNA homology groups, *L. plantarum* and *L. pentosus*. Colllins *et al.* (36) reported high 16s RNA sequence similarity between these species, confirming that they are closely related.

The other subgroup of the group 2 lactobacilli includes *L. curvatus* and *L. sake* which have important associations with foods. Most strains show high DNA homology with *L. sake* and some with *L. curvatus*. These bacteria are an important part of the adventitious microflora of modified atmosphere and vacuum packaged meats and meat products that are stored at refrigerated temperature (5°C) (23).

Group 3 includes the obligately heterofermentative lactobacilli that ferment hexoses to lactic acid, acetic acid and/or ethanol and carbon dioxide. The production of gas from glucose is a characteristic feature of these bacteria. The most important obligate heterofermentative *Lactobacillus* associated with food fermentations is *L. sanfrancisco* which convert maltose to lactic and acetic acids and various flavor compounds in sourdough bread (23).

2. Ecology of lactobacilli

They are normal indigenous flora in the oral cavity, small and large intestine and female genital tract (42). Their distributions are affected by several environmental factors including pH, oxygen availability, level of specific substrates and presence of secretions and bacterial interactions. The *Lactobacillus* species which predominated on the human gastrointestinal mucosa include: *L. plantarum*, *L. rhamnosus* and *L. paracasei* spp. *paracasei*, have been isolated from 52%, 26% and 17% of healthy individuals, respectively (41). They are rarely associated with cases of gastrointestinal and extraintestinal infection, and strains used for industrial production are regarded as non-pathogenic and safe microorganisms (20).

3. Probiotics

By definition, probiotics are bacterial cultures or living microorganisms which, upon ingestion certain numbers, exert health benefits beyond inherent general nutrition and enhance the good bacteria in the intestine (43). Presently, probiotics are defined as "viable microorganisms (lactic acid and other bacteria or yeasts applied as dried cells or in a fermented product) that exhibit a beneficial effect on the health of the host upon ingestion by improving the properties of its indigenous microflora (20). The concept of using probiotics for disease treatment and prevention as well as health restoration and maintenance are realized by the Ukrainian-born microbiologist and Nobel laureate Elie Metchnikoff in 1907. He had discovered *L. bulgaricus*, a strain produced sour-milk products which was good for health, and studied lactic acid-producing bacteria in gastrointestinal tract as a means of preventing putrefaction and prolong life (3).

3.1 Probiotics in gastrointestinal tract

Gastrointestinal probiotics include the microflora that able to resist acids and bile, survive during intestinal transit, adhere to the intestinal mucosa, and produce antimicrobial substances, and contribute to the beneficial health effects. They must also have the ability to inhibit gut pathogens, and preserve their stabilities during manufacture and storage which can influence both viable and functional properties (44, 45).

Several mechanisms have been suggested to contribute to the probiotic action. If the intestinal microflora is deficient, antigen transport is increased. Lactobacilli have been shown to normalize an increased permeability. The first step in pathogenesis is the binding of bacteria to the intestinal mucosa or mucus and leads to allow the colonization (46, 47). Probiotics compete with pathogens for binding sites and available substrates (48, 49). The important for the beneficial health effect is their adhesions to the intestinal mucosa (46, 47). Probiotics also seem to diminish the rate of progression from inflammation through dysplasia to colon cancer in experimental animals (50). Probiotics can also activate and modulate the immune system and they have been shown to reinforce the gut defence by immune exclusion, immune elimination and immune regulation (51). The other mechanisms, lactobacilli can produce different antimicrobial substances such as organic acids, hydrogen peroxide and low molecular weight antimicrobial substances, bacteriocins (1).

3.2 Probiotics in urogenital tract

The urogenital tract is the riched place for many bacterial species. The balance between a maintenance of a healthy state and an emergence of infecting bacterial probably involves many factors (6). Bacterial adherence to the urovaginal epithelium is recognized as an important mechanism in the initiation and pathogenesis of urogenital tract infections. The pathogens predominate and colonize in the periurethral region before ascending into the bladder, resulting in symptomatic or asymptomatic bacteriuria. The pathogens display virulence characteristics that enable them to resist the normally efficient defence mechanisms of host (7). It is clear that lactobacilli are the predominant members of the healthy adult female flora and able to coaggregate with other bacteria (52). The coaggregation effect may be one method that the flora neutralize uropathogens and inhibit their infections.

3.3 Probiotics in oral cavity

Lactobacilli represent a characteristic group of oral bacteria which numerically comprised a minor component of the oral microbiota and could be detected in the oral cavity soon after birth. Although, they represent generally less than 1% of the bacterial flora present in saliva, they can play an important role in the maintenance of

oral health by stimulating the natural immunity and contributing to the balance of microflora (4).

4. Antimicrobial activity by lactobacilli against pathogens

4.1 Adhesiveness properties and bacterial interference

Microbial adhesion is a generalized phenomenon and perhaps the most important determinant in this provenance is the survival and replication in tissue surface environment. Adhesion provides the organisms to survive in secretion, resist peristalsis, or voiding motion, depending upon the host site (6). Adherence of lactobacilli to epithelial cells has been shown to be an important factor in the colonization of mucous membranes (53). Adherences to the intestinal epithelium and mucus are associated with stimulation of the immune system (54) and adhesion to the intestinal mucosa is also crucial for transient colonization (55), an important prerequisite for probiotics to control the balance of the intestinal microflora. The microbial adhesion process of lactobacilli and bifidobacteria includes passive forces, electrostatic interactions, hydrophobic steric forces, lipoteichoic acids, and specific structures, such as lectin-covered external appendages or aggregation-promoting factor (APF) protein (7). Bruce *et al.* (56) demonstrated that urovaginal lactobacilli competed with uropathogens. Similarly, lactobacilli have the capacity to interfere with the adhesion of pathogenic bacteria on intestinal cells.

4.2 Immunomodulation

Among the mechanisms suggested by which the selected *Lactobacillus* strains may act against microbial pathogens, recent experimental reports have focused on immune stimulation and modulation of selected strains as the strain-dependent manner (57, 58). Lactobacilli strains have been shown to activate cells to secrete both inflammatory and anti-inflammatory cytokines (7). Rangavajhyala *et al.* (59) reported that one strain of *L. acidophilus* has been shown to induce the production of IL-1α and TNF-α. The bacterial cell walls were capable to induce the production of a proinflammatory cytokine, TNF-α, and an anti-inflammatory cytokine, IL-10. The vaginal lactobacilli can activate TNF-α and IL-1α production, induce NF-κB in THP-

1 cells, and increase TNF-α production by human monocytes, so, it may suggest that the presence in the vaginal fluid of agents derived from indigenous bacteria can affect the physiology of the vagina and host defences (60). On the basis that cytokines secreted by human enterocytes play a critical role in mucosal and systemic immunity, lactobacilli strains developing adhesion onto human intestinal cells have been investigated for their potential to stimulate proinflammatory cytokine secretion such as IL-6, IL-8 and TNF-α (61). Christensen *et al.* (62) reported that the lactobacilli with the greatest capacity to induce IL-12 were those most effective in producing dendritic cell maturation. The Th1/Th2/Th3-driving capacities of the gut dendritic cell may be modulated by the composition of the gut microorganisms, including the lactobacilli. For the humoral immune system, Yasui *et al.* (63) reported that *L. casei* strain Shirota activated the humoral immune systems. Oral administration of lactobacilli to infants increased anti-rotavirus IgA production, and also significantly reduced the frequency of rotavirus shedding in stool samples. Matsuzaki *et al.* (64) reported that *L. casei* strain Shirota enhance innate immunity by stimulating the activity of spleenic NK cells and oral feeding with killed bacteria was able to stimulate the production of Th1 cytokines, resulting in repressed production of IgE antibodies against ovalbumin in experimental mice.

4.3 Acids

Lactobacillus species can produce metabolites such as lactic and acetic acid, and thus lowering the pH, a large number of lactobacilli inhibit the growth of bacterial pathogens (65). Hudault *et al.* (66) reported that the mechanism by which lactobacilli impeded the invasion of host cells by bacterial pathogens was abolished after the *Lactobacillus* culture had been neutralized to pH 7, which suggested a pH-dependent mechanism. Alakomi *et al.* (67) reported that the lactic acid produced by lactobacilli acts as a permeabilizer of the Gram-negative bacterial outer membrane, allowing other antimicrobial substances produced by the host to penetrate and increase the susceptibility of pathogens to these antimicrobial molecules. However, the inhibition of the growth of bacterial pathogen s is not due to pH alone, but there were the presence of other *Lactobacillus*-inhibiting substances that are extracellular and diffusible (5).

4.4 Hydrogen peroxide

Hydrogen peroxide - producing lactobacilli predominate in the normal vagina but are seldom found in the vagina of patients with bacterial vaginosis (2). The production of hydrogen peroxide by *Lactobacillus* spp. may be a non-specific antimicrobial defence mechanism of the normal vaginal ecosystem (8). Optional hydrogen peroxide - producing *Lactobacillus* spp. are found in the vagina of most normal women but much less often in that of women with bacterial vaginosis, whereas anaerobic *Lactobacillus* spp. which do not produce hydrogen peroxide have been found to be increased in women with bacterial vaginosis (68).

4.5 Bacteriocins

Bacteriocins are the bactericidal proteinaceous molecules produced by bacteria. The bacteriocin family includes a wide variety of peptides and proteins in terms of their size, source of production, microbial targets, and mechanisms of action (7). The bacteriocin comprises a large and diverse group of ribosomally synthesized antimicrobial proteins or peptides some of which undergo posttranslational modifications (69). Most bacteriocins from lactic acid bacteria exert their antibacterial effect by permeabilizing the target cell membrane, whereby the cells lose their viability (70, 71). Klaenhammer *et al.* (72) defined 4 distinct classes of lactic acid bacteria bacteriocins elucidated their probable structures and mechanisms of action. These bacteriocins can be defined four classes as shown in Table 3.

Table 3 The examples of bacteriocins

Class	Bacteriocins	Strains	Year	Ref. No.
I	Lactocin S	*L. sake*	1991	(11)
	Bavaricin A	*L. bavaricus*	1993	(73)
	Acidocin B	*L. acidophilus*	1994	(74)
	Plantaricin C	*L. plantarum*	1994	(75)
	Lactocin S	*L. sakei*	2002	(76)

Table 3 (continued)

Class	Bacteriocins	Strains	Year	Ref. No.
II	Acidocin B	*L. acidophilus*	1984	(10)
	Lactacin F	*L. acidophilus*	1991	(13)
	Curvacin A	*L. curvatus*	1992	(77)
	Lactacin F	*L. johnsonii*	1993	(78)
	Lactocin B	*L. acidophilus*	1994	(79)
	Curvaticin FS47	*L. curvatus*	1994	(80)
	Acidocin A	*L. acidophilus*	1995	(81)
	Acidocin J1132	*L. acidophilus*	1996	(82)
	Sakacin P	*L. sake*	1996	(83)
	Plantaricin A, EF and JK	*L. plantarum*	1998	(84)
	Plantaricin 423	*L. plantarum*	1998	(85)
	Acidocin IBB801	*L. acidophilus*	1999	(12)
	Amylovorin L471	*L. amylovorus*	2000	(86)
	Sakacin G	*L. sake*	2002	(87)
	Plantaricin NC8	*L. plantarum*	2003	(88)
	Curvacin A	*L. curvatus*	2004	(89)
	Sakacin P	*L. sakei*	2005	(90)
III	Lactocin 27	*L. helveticus*	1975	(91)
	Helveticin J	*L. helveticus*	1986	(92)
	Acidophilucin A	*L. acidophilus*	1991	(93)
IV	Plantaricin S and T	*L. plantarum*	1993	(94)

Class I lantibiotics, the membrane-active peptides (< 5 kDa), contain the unusual amino acid lanthionine, β-methyl lanthionine, and dehydrated residues such as lactosin S that produced by *L. sake* L45 (11), bavaricin A that produced by *L. bavaricus* M1401 (73) and acidocin B that produced by *L. acidophilus* M46 (74).

Class II, the non-lanthionine containing membrane-active peptides, is small heat stable peptide (< 10 kDa) characterized by Gly-Gly $^{-1 * * +1}$ Xaa processing site in

the bacteriocin precursor. The mature bacteriocins are predicted to form amphiphilic helices with varying amounts of hydrophobicity, β-sheet structure, and moderate (100°C) to high (121°C) heat stability.

Class IIa *Listeria*-active peptides, contain a consensus sequence in the N-terminal of –Tyr-Gly-Asn-Gly-Val-Xaa-Cys-; characterized by curvacin A produced by *L. curvatus* LTH 1174 and sakacin P produced by *L. sake* LTH673 (77).

Class IIb poration complexes peptides, consist two proteinaceous peptides for their activity : lactococcin G, produced by *Lactococcus lactis* LMG 2801 (69).

Class IIc Thiol-activated peptides, require reduced cysteine residues for activity ; lactococcin B, produced by *L. lactis* (95).

Class III large protein, is heat-labile proteins with molecular size more than 30 kDa such as helveticin J produced by *L. helveticus* 481 (92), and acidophilucin A produced by *L. acidophilus* LAPT 1060 (93).

Class IV complex bacteriocins, compose of protein plus one or more chemical moieties (lipid, carbohydrate) required for their activities.

The first *Lactobacillus* bacteriocin purified and characterized was class IV lipocarbohydrate-protein macromolecular complex produced by *L. fermentus* (9). The bacteriocin was relatively hydrophobic and heat-stable (96°C for 30 min). Lactocin 27 was isolated and characterized by Uprete *et al.* (96). This bacteriocin was produced by *L. helveticus* and was a small heat-stable glycoprotein that causes an effux of K^+ ions through the membranes of sensitive cells. Lactacin B was identified and purified by Barefoot *et al.* (10). It was produced by *L. acidophilus* and was a number of heat-stable class II bacteriocin. Helveticin J, the first member of class III bacteriocin, was purified and characterized by Joerger *et al.* (92). It was produced by *L. helveticus* 481 and was a large molecular mass with 37,000 Da and heat-labile protein. Lactocin S, class I lantibiotics, was discovered and characterized by Mørdvedt *et al.* (11). It was produced by *L. sake*.

5. Antimicrobial mechanism of bacteriocins

The mechanisms of bacteriocins for possessing the bactericidal activity include enzyme activity modulation, inhibition of overgrowth of spores and anion carrier activity to the formation of selective or non-selective pores (97). However, the common mechanisms of the activity are membrane insertion and pore formation.

5.1 Membrane insertion

Bacteriocins are usually unstructured in aqueous solution, but have the propensity to form α-helical structure when exposed to structure promoting solvents such as trifluoroethanol or when mixed with anionic phospholipids membranes. Some peptides form loop structures owing to a disulphide bridge or a covalent bond (97). In particular, the presence of intramolecular ring structures is a characteristic feature of class I lantibiotics. For example, nisin A, produced by some strains of *Lactococcus lactis* subsp. *lactis*, has five ring structures termed A, B, C, D and E (98). These ring structures are rigid but interconnected by flexible hinge regions. Due to the presence of these hinges, part of the molecule may insert into the membrane, while the other part remains bound to the membrane surface. In contrast to the lantibiotics, the class II bacteriocin such as leucocin A, produced by *Leuconostoc gelidum* UAL 187 exists as a random coil in water or aqueous DMSO solution. In lipophilic media two domains can be identified. First, a three stranded antiparallel β-sheet domain of residue 2-16, linked by a disulphide bridge, and second an amphiphilic α-helix of residue 17-31. The α-helical part has been suggested to be responsible for the target specificity, whereas the β-sheet exerts the antimicrobial activity (99). Interestingly, in analog to the class I bacteriocins, also class II bacteriocins are amphipathic in two respects. The hydrophobicity of several class II bacteriocins increases gradually from the N-terminal to the C-terminal side of the molecules, while the hydrophobicity of the C-terminal part slightly decreases. Most class II bacteriocins have a hydrophilic and a hydrophobic side perpendicular to the N-terminal-to-C-terminal axis. Such peptides often contain a helix-breaker such as a proline or glycine in the α-helical domain that they may facilitate a membrane insertion or wedge formation (100).

In general, it appears that the bactericidal action of the non-lanthionine-containing bacteriocins against sensitive cells is produced principally by destabilization of membrane functions such as energy transduction rather than by disruption of the structural integrity of the membrane. This effect results from the energy-independent dissipation of the proton motive force (PMF) and loss of the permeability barrier of the cytoplasmic membrane and contrasts with the energy-dependent bactericidal action of the lantibiotics (101). Proton motive force is the driving force for many vital energy-demanding processes in the cytoplasmic membrane, notably the accumulation of ions and metabolites and the synthesis of ATP (70). In addition, these bacteriocins dissipated the proton motive force of the target cells, as shown by their influence on the uptake of amino acids whose influx is mediated by secondary and phosphate-bond-driven transport systems (102).

5.2 Pore formation

Pore formation in the cytoplasmic membrane is a common mechanism of action of those LAB bacteriocins for which the mode of action has been determined. Nisin and a number of the class II peptides have been shown to be membrane-active peptides that destroy integrity via the formation of membrane channels. The biological activity of membrane-active peptides in which amphiphilic α-helices or β-sheets form two faces, one hydrophilic and one hydrophobic (100). In this process, lateral oligomerization of peptide monomers occurs in the membrane with the hydrophobic side facing the membrane and the hydrophilic side forming the pore. Loss of membrane integrity generally leads to ion leakage, loss of the proton motive force and cell death. Nissen-Meyer *et al.* (100) discovered the two-component poration complexes which added an exciting dimension to this model in that different peptides may oligomerize and contributed to pore formation. The simplicity of the peptides which contribute to poration complexes, either singly or in combination with other peptides provided exciting opportunities for genetic engineering of bacteriocins in LAB.

Many bacteriocins may contain a conserved amphiphilic core regions that contribute to pore formation, the host-range specificity of these compounds may be dictated by variable domains in the molecules (103). Class I lantibiotics, nisin acts on

liposomes and exerts general action in both Gram-positive and Gram-negative bacterial membranes. However, the bacteriocin domains that confer binding specificities to lipid, protein, or reactive groups in the cell membranes remain to be elucidated. Various models for pore-formation have been proposed during the years. Nisin seems to orient parallel to the surface of the membrane (104). Both the trans negative and the cis acid are able to induce pore formation, possibly by driving the membrane insertion of nisin domains. The C-terminus inserts deeply into the membrane and it has even been suggested that the entire nisin translocates across the membrane (105). Driessen *et al.* (101) suggested that a wedge-like model for nisin-induced pore formation may involve a proton motive force driven co-insertion of lipids and nisin domains.

Some of the class II peptides (lactococcin A, B, G, lactacin F) form pores in membrane vesicles but not in liposomes, implicating a requirement for a specific protein receptor. Class II bacteriocins are thought to form a bundle of α-helical peptides as a barrel-stave like pore. According to the barrel-stave model, the hydrophilic faces of a bundle of amphipathic α-helical peptides form the inner wall of the water-filled pore and the outer hydrophobic side of these helical bundles will face the fatty acyl chains of the membrane lipids (97).

6. Antimicrobial activity of bacteriocins against the microbial pathogens

There were the several studies of the lactobacillus bacteriocins possessed the antimicrobial activity against the gastrointestinal, vaginal and oral pathogens. These bacteriocins were also reported in Table 4, 5 and 6.

Table 4 The antimicrobial studies of *Lactobacillus* bacteriocins against the gastrointestinal pathogens

Researchers	Year	Ref. No.	*Lactobacillus* species and sources	Tested bacteria
Silva *et al.*	1987	(1)	*L. rhamnosus* strain GG; normal human feces	*Clostridium* spp. *Bacteriodes* spp. *Escherichia coli* *Pseudomonas* spp. *Staphylococcus* spp. *Streptococcus* spp.
van Reenen *et al.*	1998	(85)	*L. plantarum* 423; sorghum beer	*Bacillus cereus* *Clostridium sporogenes* *Enterococcus faecalis* *Listeria* spp. *Staphylococcus* spp.
Miteva *et al.*	1998	(106)	*L. delbrueckii*; Bulgarian yellow cheese	*Listeria monocytogenes* *Staphylococcus aureus* *Enterococcus faecalis* *Escherichia coli* *Yersinia enterocolitica* *Y. pseudotuberculosis*
Zamfir *et al.*	1999	(12)	*L. acidophilus* IBB 801	*Escherichia coli* *Salmonella panama*
Gänzle *et al.*	2000	(107)	*L. reuteri* LTH2584	*Bacillus subtilis* *Bacillus cereus* *Enterococcus faecalis* *Staphylococcus aureus* *Listeria innocua*
Atanassova *et al.*	2003	(108)	*L. paracasei* subsp. *paracasei* strain M3; cheese	*Helicobacter pyroli* *Candida albicans*

Table 4 (continued)

Researchers	Year	Ref. No.	*Lactobacillus* species and sources	Tested bacteria
Lash *et al.*	2005	(109)	*L. plantarum* ATCC 8014	*Staphylococcus aureus* *Escherichia coli* *Listeria innocua* *Pseudomonas aeroginosa*
Hernández *et al.*	2005	(110)	*L. plantarum* TF711; goat's cheese	*Bacillus cereus* *Clostridium sporogenes* *Staphylococcus aureus* *Shigella sonnei* *Klebsiella pneumoniae*

Table 5 The antimicrobial studies of *Lactobacillus* bacteriocins against the vaginal pathogens

Researchers	Year	Ref. No.	Lactobacillus species and sources	Tested bacteria
Muriana *et al.*	1991	(13)	*L. acidophilus* 11088	*Enterococcus faecalis*
Ocaña *et al.*	1999	(14)	*L. salivarius* subsp. *salivarius* CRL 1328; vaginal swab	*Enterococcus faecalis*, *Enterococcus faecium*, *Neisseria gonorrhoeae*
Okkers *et al.*	1999	(111)	*L. pentosus*; posterior fornix secretions of prenatal patient	*Candida albicans*
Vaughan *et al.*	2001	(112)	*L. sakei* 5; malted barley	*Enterococcus faecalis*

Table 6 The antimicrobial studies of *Lactobacillus* against the oral pathogens

Researchers	Year	Ref. No.	Lactobacillus species and sources	Tested bacteria
Michalek *et al.*	1981	(113)	*L. casei*	*Streptococcus mutans*
Sookkhee *et al.*	2001	(15)	*L. rhamnosus* *L. paracasei* subsp. *paracasei*; healthy oral cavities' volunteers	*Streptococcus sanguis* *Streptococcus salivarius* *P. gingivalis* *Actinomyces viscosus* oral *Candida albicans*
Nase *et al.*	2001	(16)	*L. rhamnosus* strain GG ; milk	*Streptococcus mutans*
Ahola *et al.*	2002	(114)	*L. rhamnosus* strain GG ; cheese	*Streptococcus mutans*
Kŏll-Klais *et al.*	2005	(4)	*L. paracasei* *L. plantarum* *L. rhamnosus* *L. salivarius* ; saliva and subgingival sites of chronic periodontitis and healthy subjects	*Streptococcus mutans* *Actinobacillus actinomycetemcomitans* *P. gingivalis* *Prevotella intermedia*

7. Oral microbial ecology

Oral cavity is a moist environment which is kept at a relatively constant temperature (34 to 36°C) and a pH close to neutrality in most areas and thus supports the growth of a wide variety of microorganisms. There are several habitats in the oral cavity, each being characterized by different physicochemical factors and thus supporting the growth of a different microbial community. The oral cavity possesses both hard (teeth) and soft (mucosa) tissues. The tooth can be described as a nonshedding hard surface that offers many different sites for colonization by bacteria below (subgingival) and above (supragingival) the gingival margin. In contrast, the oral mucosa is characterized by a continuous desquamation of its surface epithelial

cells, which allows rapid elimination of adhering bacteria. The mucosa that covers buccal, lingual, gingival and palatal sites including the floor of mouth varies according to the anatomical site (115). The oral microbiota of humans is highly complex and diverse. It is composed of more 700 bacterial species, to which may be added protozoa, yeasts and mycoplasmas (116). The more frequently isolated microorganisms are listed in Table 7. The processes of microbial ecological colonization in oral cavity were as the following sequences; colonization at tooth surface, bacterial multiplication, aggregation with additional bacterial species and embedding themselves in a matrix of extracellular polymers of both salivary and bacterial origin. It leads to the formation of a biofilm on the tooth surface (117). Bacterial coaggregation reactions between different species and the autoaggregation of the same species are associated with the initiation and development of dental plaque and biofilms (118).

Table 7 Oral human microorganisms

	Groups	**Microbial genus**
Gram-positive cocci	**aerobes or facultative anaerobes**	*Streptococcus* *Enterococcus* *Micrococcus*
	obligate anaerobes	*Peptostreptococcus* *Peptococcus*
Gram-positive rods	**aerobes or facultative anaerobes**	*Lactobacillus* *Corynebacterium* *Actinomyces* *Arachnia* *Rothia*
	obligate anaerobes	*Eubacterium* *Propionibacterium* *Bifidobacterium* *Bacillus* *Clostridium*

Table 7 (continued)

	Groups	Microbial genus
Gram-negative cocci	**aerobes or facultative anaerobes**	*Neisseria/Branhamella*
	obligate anaerobes	*Veillonella*
Gram-negative rods	**aerobes or facultative anaerobes**	*Campylobacter*
		Eikenella
		Actinobacillus
		Capnocytophaga
		Haemaophilus
		Simonsiella
	obligate anaerobes	*Bacteroides*
		Fusobacterium
		Porphyromonas
		Prevotella
		Leptotrichia
		Wolinella/Selenomonas
	other microorganisms	*Mycoplasma*
		Candida
		Spirochetes
		Protozoa

In the oral cavity of newborns, streptococci (*Streptococcus mitis* biovar 1, *S. oralis* and *S. salivarius*) are the pioneer organisms (119). Pioneer microorganisms fill the niche of this new environment and modify the habitat, and as a result, new populations may develop. As the process continues, the diversity and the complexity of the microbial community increase. Succession ends when no additional niche is available for new populations. At this stage, a relatively stable assemblage of bacterial populations is achieved. It is called a climax community. On teeth, microorganisms colonize in a dense mass forming dental plaque. Dental plaque consists of microbial communities organized in a complex matrix composed of microbial extracellular products and salivary compounds. Dental plaque develops

preferentially on surfaces protected from mechanical friction, such as the area between two teeth (approximal surface), the subgingival area (gingival crevice), and the pits and fissures of the biting surfaces. The predominant organisms isolated from supragingival dental plaque are gram-positive, facultatively anaerobic bacteria, particularly *Actinomyces* spp. and streptococci. Gram-negative bacteria of the group *Veillonella*, *Haemophilus* and *Bacteroides* are regularly isolated but in lower proportions (120). On the mucosal surface, streptococci constitute the highest proportion of the microbiota in these sites, with the predominance of *S. oralis* and *S. sanguis*. The genera *Neisseria*, *Haemophilus* and *Veillonella* have also been isolated. *Streptococcus* spp. (*S. salivarius* and *S. mitis*) and *Veillonella* spp. were the predominant members of the microbiota (121). Other major groups isolated include *Peptostreptococcus* spp., gram-positive rods (mainly *Actinomyces* spp.), *Bacteroides* spp. and other gram-negative rods. Black-pigmented obligate anaerobic rods and spirochetes, which are closely associated with periodontal diseases, have been recovered in small numbers (122).

8. Oral pathogens

8.1 Caries pathogens

Dental caries is a bacterial disease of the dental hard tissues. It is characterized by a localized, progressive, molecular disintegration of the tooth structure. The demineralization of teeth (enamel, dentine, and cementum) is caused by organic acid produced by the bacterial fermentation of dietary carbohydrates. The frequent ingestion of carbohydrates may lead to the selection of bacteria that are acidogenic and concurrently to a low-pH environment (123). Cariogenic bacteria have a strong tendency to adhere to tooth surfaces (118). There was a report that mutans streptococci including *S. mutans*, *S. sobrinus*, *S. cricetus* and *S. rattus* were shown to be the most cariogenic, and the other cariogenic bacterial species were *L. acidophilus*, *L. casei*, *Actinomyces naeslundii*, *A. naeslunndii* genospecies 2 (formerly *A. viscosus*), *S. salivarius*, *S. sanguis* and *E. faecalis* (123). There is considerable evidence that mutans streptococci (particularly *S. mutans* and *S. sobrinus*) and *Lactobacillus* are involved in the initiation and progression of caries. These two

bacterial groups are able to rapidly metabolize carbohydrates into acid, primarily lactic acid and to tolerate a low-pH environment. The increase in *Lactobacillus* is generally slower, and *Lactobacillus* reaches a high level only after the lesion can be detected clinically (124). In addition to mutans streptococci and lactobacilli, a broad range of microorganisms may be isolated from root lesions and *Actinomyces* occasionally constitutes the predominant species (125, 126). Although different groups have found varying proportions of common oral bacteria in root caries, there is consensus that *Actinomyces* spp., *Lactobacillus* spp. and *Streptococcus* spp. play a major role in this disease process (127).

8.2 Candidiasis pathogens

Candidiasis is by far the most common oral fungal infection in man. Thus, it has been reported that more than 90% of HIV-infected individuals develop oral candidiasis during some point of their disease. It is most common oral manifestation in these patients (128, 129). Candida is presented as a commensal in the oral cavity of healthy individuals. The number of organisms in the saliva of carriers increases in pregnancy, tobacco smokers, and denture stomatitis cases. *Candida* species are opportunistic pathogens, and both general and local predisposing factors are important in the pathogenesis of oral candidal infection.

C. albicans is the most common *Candida* species residing in the oral cavity, in both health and disease (130). All clinical types of oral candidiasis are considered opportunistic. Non-*albicans* species such as *C. tropicalis*, *C. glabrata*, *C. parapsilopsis* and *C. krusei* are also pathogenic to man (131).

It is generally accepted that oral candidiasis can be divided into two broad categories, i.e., primary and secondary oral candidiasis. The disorders where oral candidiasis is a manifestation of generalized systemic candidal infections are categorized as secondary oral candidiasis (132). The primary oral candidiasis is subclassified into three major variants, namely, pseudomembranous, erythematous and hyperplastic, each of which may manifest as acute or chronic lesions.

8.3 Periodontal pathogens

Periodontal diseases are the definition term describing the inflammatory pathologic state of tooth supporting tissues. The diseases can be divided into two categories, namely, gingivitis and periodontitis. Gingivitis is defined as an inflammation of gingival tissues which does not affect the attachment of teeth. Periodontitis involves the destruction of the connective tissue attachment and the adjacent alveolar bone. In periodontitis, the gingival crevice is deepened to form a periodontal crevice along the root surface. The induction and progression of periodontal tissue destruction is a complex process involving plaque accumulation, release of bacterial substances, and host inflammatory response (133, 134). Pathogens may release substances that cause to the tissue destruction, by the direct action of enzymes and endotoxins, or indirectly, by induction of inflammation. Tissue damage may cause from the release of lysosomal enzymes secreted by phagocytes and by the production of cytokines that stimulate connective tissue cells to release metalloproteinases (including collagenases) or cytokines that activate bone resorption.

Periodontal diseases are the multifactorial infections of bacterial complex species. These bacteria interact with host tissues and cells and cause to the release of broad array of inflammatory cytokines, chemokines and mediators, some of which lead to destruction of the periodontal structures, including the tooth-supporting tissues, alveolar bone, and periodontal ligament (135). At the initiation of disease, the complex microbial biofilms were presented (136). In a healthy gingival crevice, the total number of microorganisms is few and the facultative gram-positive bacteria were predominantly found. The number of plaque bacteria associated with gingivitis is 10- to 20-fold more than in healthy sites. The facultative gram-positive bacterial still predominate, but there is an increase in the proportion of obligately anaerobic gram-negative bacteria (137).

Oral diseases demonstrate a significant proportion of the etiology of polymicrobial infections. For example, odontogenic infections are typically polymicrobial and composed of anaerobic gram-positive cocci and gram-negative rods. Their virulent factors and the synergistic interaction of these bacteria have been identified (138). Brook *et al.* (139) reported that the species often included in the

polymicrobial infections encompass *S. aureus*, *Peptostreptococcus* spp., *Prevotella* spp. and *Porphyromonas* spp. The capacity of a variety of bacterial species existing in a complex 'biofilm' contribute to the disease process (140). In the healthy gingival crevice, suspected periodontopathogens such as *P. intermedia*, *A. actinomycetemcomitans*, *P. gingivalis*, and spirochetes are undetectable or found in very small numbers. In the poor oral hygiene, the accumulation of plaque can lead to the inflammation and the increasing flow rate of gingival crevicular fluid. This fluid may provide nutrients for bacteria and favor the growth of fastidious obligately anaerobic gram-negative bacteria implicated in periodontal destruction (141). The characteristics of microbiologic progression from periodontal health to gingivitis (e.g. chronic inflammation of the gingival tissue without tissue destruction), and eventually to periodontal disease are vast and complicated (142). Socransky *et al.* (140) have demonstrated that many of periodontal pathogens species routinely occur together in the subgingival biofilms. The description of specific microbial complexes as representative of various stages in the progression of oral health to periodontal tissue destruction has provided a more organized approach to evaluate the role of the large number of oral species, some of which might contribute to disease. Six bacterial resided clusters were described in subgingival biofilms and related to structural characteristics of the biofilm extending away from the tooth surface (143). They are listed as follows.

The purple complex is composed of *A. actinomycetemcomitans* serotype a joining *A. actinomycetemcomitans* serotype b and *A. naeslundii* genospecies 2 (*A. viscosus*), *Actinomyces odontolyticus* and *Veillonella parvula* (140).

The second complex is *Actinomyces*. These species are thought to be 'early colonizers' and generally considered to express receptors for host ligands, enabling rapid and firm attachment to the host surface.

The yellow complex is composed of species of *Streptococcus* including *Streptococcus sanguis* and *Streptococcus oralis*.

The green complex consist of *Capnocytophaga* spp., *Campylobacter concisus*, *Eikenella corrodens* and *A. actinomycetemcomitans* and appeared to be a group of bacteria that existed in the biofilm milieu, less cognitively associated with other individual bacterial species.

The orange complex is consisted of *Fusobacterium* spp., *Prevotella* spp., *Micromonas micros* (*Peptostreptococcus micros*), *Campylobacter* spp., *Eubacterium* spp. and *Streptococcus constellatus*. These species have been considered 'bridging' species related to both their physiological capabilities to use and release nutrient substances in the biofilms and the recognition that they express cell surface structures and can bind to the early colonizers and to members of the red complex (144;145).

Finally, the red complex consists of three specific bacterial species: *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola*. The cluster is considered the most significant complex in periodontal disease progression because the members of this consortium increase in numbers and prevalence with increasing clinical parameters of periodontal disease. *P. gingivalis*, *T. forsythia* and *T. denticola* are found together in plaque samples often adjacent to the epithelial lining of the periodontal pocket of gingival sulcus (146, 147). It is likely that *P. gingivalis* benefits from what appears to be a nutritional interdependence with *T. denticola*. Both *P. gingivalis* and *T. denticola* have several characteristics that make them prime candidates as pathogens involved in the clinical destruction of periodontal tissues, there were;

- concomitant with the clinical signs of periodontal destruction
- appear closely 'linked' topologically in the developing biofilm
- and their ability to produce a number of outer membrane-associated proteinases (e.g. arginine- and lysine-specific cysteine proteases, a chymotrypsin-like serine protease).

These investigators have suggested that the 'red complex' presents as a portion of the climax community in the biofilms at sites expressing progressing periodontitis. A change in plaque composition appears to affect the habitat, resulting in clinically apparent gingivitis. Other studies indicate that a change in habitat such as the development of gingivitis also affects plaque development. Plaque accumulated much more rapidly after cleaning, at sites that exhibited gingivitis than at sites that were periodontally healthy (148, 149). Thus, it is possible to postulate a scheme of microbial succession followed by reciprocal host-bacterial interaction (Figure 3). Initial colonization appears to involve members of the yellow, green and purple complexes along with *Actinomyces* species. This leads to autogenic succession

in which members of the orange and then red complexes become more predominant. The presence of increased levels of the last two complexes is hypothesized to lead to a change in the habitat, manifested clinically as gingivitis. The gingivitis in turn favors further proliferation by members of not only the orange and red complexes, but probably members of the early colonizing species as well. This cycle could be broken in a number of ways. The way would be to eliminate all plaque. The second would be to eliminate members of the red and/or orange complexes. This would probably limit gingivitis and its feedback effect of greater plaque development. The third would be to decrease gingivitis by a non antimicrobial approach, leading to decreased plaque accumulation and possibly diminished red and orange complex development.

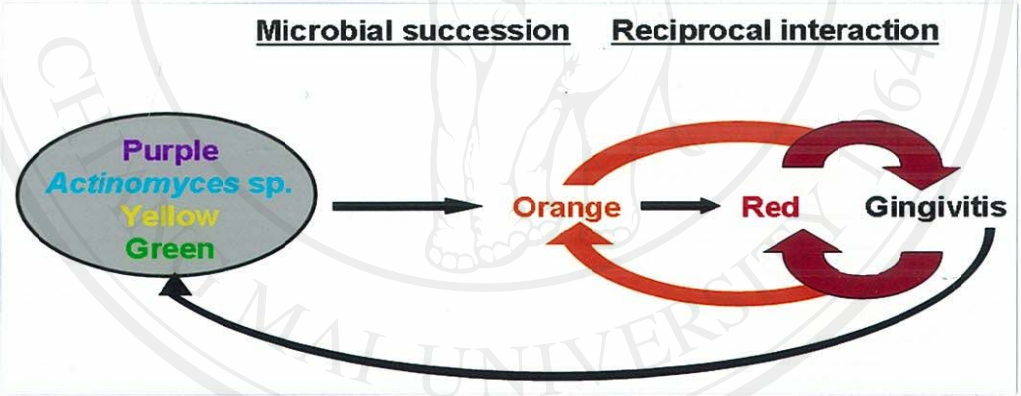

Figure 3 Hypothesized relationship between the addition of species during microbial succession leading to the development of gingival inflammation. In turn, the increased inflammation would result in increased growth of colonizing species (143).

9. *Porphyromonas gingivalis*

9.1 Microbial characteristics

P. gingivalis, a gram-negative black-pigment anaerobe, presents in subgingival plaque. It appears to play a significant role in the progression of chronic

periodontitis (150) and has been identified as a major aetiologic agent of chronic periodontitis (151).

P. gingivalis is an asaccharolytic organism and dependent on nitrogenous substrates for energy production (152). However, sugars such as glucose can be utilized for the biosynthesis of intracellular macromolecules but not converted to metabolic end products (153). Among the potential nitrogenous substrates available in the mouth, *P. gingivalis* has only a limited ability to ferment free amino acids, with the possible exception of aspartic acid and asparagines, which can be metabolized through oxaloacetate, malate, and fumarate to yield succinate. In contrast, peptides are efficiently utilized for growth (152). *P. gingivalis* produces multiple proteases that can degrade a number of potentially important substrates in the gingival crevice, including collagen, fibronectin, fibrinogen, laminin, and keratin (154, 155).

P. gingivalis has an obligate iron requirement for growth. It appears to lack a siderophore system (156) and utilizes hemin (iron protoporphyrin IX) to satisfy this iron requirement (157). A number of hemin-containing compounds such as hemoglobin, haptoglobin, myoglobin, hemopexin, methemoglobin, oxyhemoglobin, albumin, lactoperoxidase, catalase and cytochrome c provide the hemin following proteolytic processing (154, 158-160). Hemin is stored on the cell surface, a feature considered to give rise to the characteristic black-pigmented appearance of *P. gingivalis* colonies (161). The intact hemin molecule can be transported into the cell in an energy-dependent process regulated by the levels of available hemin (162).

9.2 Pathogenesis and virulence factors

9.2.1 Entry into the oral cavity

Initial entry of *P. gingivalis* into the oral cavity is thought to occur by transmission from infected individuals (163). Saliva is considered an important vector for transmission (164). Individauls are colonized by a single (or at least a predominant) genotype, regardless of site of colonization or clinical status. Strains of many different clonal origins, in contrast, are present in different individuals. *P. gingivalis* is essentially an opportunistic pathogen, with virulence not being restricted to a particular clonal type.

9.2.2 Adherence to oral surfaces

The oral cavity provides a variety of surfaces to which *P. gingivalis* can adhere. There are the mineralized hard tissues of the teeth, along with mucosal surfaces including those of the gingival, cheek and tongue. Oral surfaces rapidly become colonized with the early commensal microbiota of the mouth, as a consequence of which organisms such as *P. gingivalis* usually encounter surfaces rich in antecedent bacteria and their products. *P. gingivalis* can adhere to many of these early plaque organisms such as oral streptococci (*S. gordonii*, *S. sanguis*, *S. oralis*, *S. mitis* and *S. crista*) and *A. naeslundii* (165-167). Like other mucosal surfaces, the oral mucosa is rich in secretory immunoglobin A (IgA) and other proteins that may block the initial attachment of pathogens to epithelial surfaces, or may block the activity or absorption of antigenic molecules, such as toxins. The ability of *P. gingivalis* to adhere to subgingival, buccal and crevicular epithelial cells, as well as to the surface of other bacteria, is thus a crucial step in this successful establishment within the oral cavity (168). The initial event in the pathogenicity of *P. gingivalis* is its interaction (adherence) in the oral cavity. As part of the repertoire of *P. gingivalis* virulence factors, it has been shown to possess distinct molecules/structures that are essential to interactions with the host. Specifically, this species has been shown to be capable of adhering to a variety of host tissues and cells, and to invade these cells and multiple (169). The production of proteases, fimbriae, hemagglutinating factors, extracellular membrane vesicles, lipopolysaccharide (LPS) and a polysaccharide capsule (PS) appear to contribute these compositions as follows.

(1) Fimbriae

P. gingivalis fimbriae, a type of adhesion molecules, have been shown to mediate adherence to other bacterial species, to oral epithelial cells and to salivary-pellicle-coated tooth surfaces; the carboxy-terminal region of the fimbrillin. Fimbriae-associated proteins may also be involved in adherence to epithelial cells. *P. gingivalis* fimbriae are critical determinants for each of these processes (170). The fimbriae of *P. gingivalis* can also specifically bind to a domain of certain proline-rich proteins in human saliva. Recent evidence indicates that this domain appears conserved on various oral streptococci, suggestion evolutionary adaptive responses by *P.gingivalis* to enhance its colonization potential in the host (171). The gene

encoding fimbrillin (*fim*A) is present in a single copy in the chromosome and is monocistronic. Protein sequence analysis reveals no significant homology to fimbrial proteins from other bacteria, indicating that *P. gingivalis* fimbriae may represent a unique class of gram-negative fimbriae (172). There is considerable heterogeneity in this gene between *P. gingivalis* strains; these differences may be important in the function and immunogenicity of the fimbriae (173). The presence of more than one type of fimbriae on *P. gingivalis* has recently become apparent. Electron microscopy of *fim*A-inactivated strains has revealed that, in addition to the major fimbriae, *P. gingivalis* possesses shorter fimbriae (174). These structures, designated minor fimbriae, are composed of a protein of 67 kDa that is antigenically distinct from the fimbrillin product of *fim*A. A 72-kDa protein is the constituent subunit of these fimbriae, which are designated PG-II (175).

(2) Hemagglutinin

Hemagglutinin proteins, a type of adhesion molecules, are established virulence factors for a number of bacterial species, and *P. gingivalis* produces at least five hemagglutinating molecules. When expressed on the bacterial cell surface, hemagglutinins may promote colonization by mediating the binding of bacteria to receptors (usually oligosaccharides) on human cells. Since *P. gingivalis* utilizes heme for growth, binding of bacterial cells to erythrocytes may also serve a nutritional function (176). Hemagglutinin is an outer-membrane protein, designated HA-Ag2, that is distinct from fimbrillin and that has a high binding affinity for human erythrocyte has since been identified. Hemagglutinin activities expressed by *P. gingivalis* include those complexed with lipopolysaccharide (LPS), and lipid on the cell surface (177), and a released 40-kDa form of activity designated exohemagglutinin (178).

The first hemagglutinin gene, *hag*A, encodes a protein with a predicted molecular mass of 283.3 kDa (2,628 amino acids) and contains four contiguous direct 440- to 456- aa residue repeat blocks (179). It is likely that each repeat block contains a functional hemagglutinin domain since the polypeptide product of a single repeat clone demonstrated hemagglutinating activity. The *hag*B and *hag*C gene are at distinct chromosomal loci and encode 350-aa polypeptides with inferred molecular masses of 39 kDa that are 98.6% identical. These polypeptides have no sequence

similarities to HagA, and no significant homologies to them can be seen in the protein sequence databases. On the other hand, the HagD polypeptide is 73.8% identical to HagA (180), while HagE contains a 523-aa region with 93% homology to HagA (179). These *hag*A-like sequences are also found at other sites within the *P. gingivalis* chromosome.

(3) Capsule

The capsule of *P. gingivalis* composed of polysaccharide (PS) and lipopolysaccharide (LPS). Polysaccharide material appears to be composed of two distinct carbohydrate-containing antigens. These may be the PS and LPS antigens. Both PS and LPS are believed to be involved in the resistance of *P. gingivalis* to phagocytosis by polymorphonuclear leukocytes (PMNs) (181).

(4) Coaggregation factors

Coaggregation is a phenomenon that describes the specific interaction of pairs of oral bacteria via cognate binding. Many species of oral bacteria have been shown to demonstrate this function, presumably related to the development of the complex biofilms of the oral cavity. Thus, intergeneric coaggragation clearly contributes to the characteristics of the complex microbial ecology of biofilms established in the multiple habitats of the oral cavity (182). The ability of *P. gingivalis* to adhere to other bacteria within the oral cavity may also influence its establishment within the periodontal pocket. The aggregation of oral bacteria is a highly specific process that often involves the interaction of complementary bacterial surface molecules, which act as adhesions and receptors (183). *P. gingivalis* outer-membrane vesicles have also been shown to promote autoaggregation, as well as to mediate attachment of *P. gingivalis* to other bacterial species (184).

Coaggregation therefore appears to be a major colonization strategy of oral microorganisms, involving numerous surface ligands and receptors, including fimbriae of *P. gingivalis*. While a large array of genera and species exists in this complex microbial environment, these interactions show some specificity leading to some structure and organization within the biofilm at various oral cavity microenvironments (182). The vesicles from *P. gingivalis* can aggregate numerous oral bacterial such as *Streptococcus* spp., *F. nucleatum* and *A. naeslundii*. These vesicles also have the ability to 'bridge' non-aggregating species such as *S. aureus*

with *Streptococcus* spp. and certain types of *C. albicans*. Thus, the binding capabilities of *P. gingivalis* may contribute to the formation of interactions between multiple species in the oral cavity (185) and contributes to the ability of the microorganism to effectively colonize the subgingival sulcus. This interaction is altered by heat treatment, various sugars, amino acids, cation chelation, and protease treatment, suggesting a specific ligand-receptor interaction (186).

9.2.3 Colonization to host tissue

After a pathogen has adhered to host tissue, it must scavenge essential nutrients to colonize and grow. In particular, iron plays a crucial role in the establishment and progression of an infection. Within the host, the majority of iron is contained in intracellular complexes with hemoglobin, myoglobin, catalase and cytochrome c or stored in ferritin and hemosiderin, its insoluble degradation product (187). Although there is an abundance of iron in the intracellular tissue fluids of the human, the amount of free ionic iron (10^{-18} M) is far too low to support growth of most bacteria (188). Pathogenic bacteria have developed numerous mechanisms to acquire iron within microenvironments, including endogenous/exogenous siderophores (189), binding host iron proteins (190), and other more limited processes including hemolysins (187). Utilization and transport of hemin and hemin-containing compounds for nutritional iron has been documented for numerous pathogenic bacteria. *P. gingivalis* utilizes, *in vitro*, a broad range of hemin-containing molecules: hemoglobin, myoglobin, hemopexin, methemoglobin, oxyhemoglobin and cytochrome c, as well as inorganic iron and transferrin (158, 191). *P. gingivalis* obtains hemin by proteolysis of hemoglobin and heme-carrying proteins (i.e. haptoglobin, hemopexin and albumin). The transport of heme and heme-derived iron in *P. gingivalis* occurs by an energy-dependent process; an outer-membrane receptor that is specific for the protoporphyrin IX ring appears to be involved in the initial binding of heme to the cell surface (162). The rapid binding of hemin to *P. gingivalis* whole cells was observed in both hemin- and iron-deplete conditions. This hemin accumulation was suggested to occur by an energy dependent binding of the hemin molecule and transported into the bacterial cell. Hemin has been shown to regulate the expression of Omps, fimbriae, vesicles, hemolysins, trypsin-like proteases,

collagenolytic activity, hemagglutination activities, antigenicity and hemin-binding properties of lipopolysaccharide of *P. gingivalis* (192, 193).

The *tla* (TonB-linked adhesion) gene product has a role in hemin acquisition and utilization (194). A hemin-regulated gene, *hem*R, has been isolated from *P. gingivalis* which codes for a protein with significant homology to the iron regulated TonB-dependent outer membrane iron receptor protein of numerous gram-negative bacteria. The TonB-dependent hemoglobin-hemin receptor (HmuR), homologous to HemR, is involved in the utilization of both hemin and hemoglobin in *P. gingivalis* (195). It appears that *P. gingivalis* may possess several iron uptake/storage systems developed for different host iron proteins such as hemin, hemoglobin and transferring. The heme pigment of *P. gingivalis* is composed of micro-oxo bisheme, ($[Fe^{3+}PPIX]_2O$) (160). The loss of pigmentation, or changes in availability of iron from the environment appear to enable *P. gingivalis* to store iron, which can then be used to maintain growth and virulence under iron depleted conditions. The growth of *P. gingivalis* when hemin is limited results in increased virulence. This is probably due to increased protease activity and hemagglutination, as well as to other undefined changes that contribute to the overall pathogenic potential of microorganisms (196).

9.2.4 Encounter with host defense mechanisms

Bacterial colonizers of the periodontal pocket will encounter the cells and extracellular effector molecules of the host immune system. Both innate and acquired defense mechanisms are operational in the periodontal pocket. Of the various immune mechanisms in the periodontal pocket, PMNs appear to play a major role in controlling the overgrowth of periodontal bacteria (150). *P. gingivalis* affects on almost all aspects of PMN recruitement and activity. Neutrophil chemotaxis is inhibited by low-molecular-weight fatty acids, such as succinic acid, that are produced by the organism (197, 198). Succinate may act by reducing the intracellular pH of neutrophils. *P. gingivalis* can also immobilize PMN responses to chemotactic peptides by depolarizing PMN membranes (199). This activity is associated with an outer membrane protein of 31.5 kDa, which may be a porin. Depolarization could cause by translocation of this porin into the PMN membrane, thus producing an ion channel that the PMN would be unable to regulate. Package of the porin in

extracellular vesicles could then be a means by which *P. gingivalis* could act at remote sites to inhibit PMN influx in the periodontal pocket.

The LPS of *P. gingivalis* strongly activates complement, generation the chemotactic product C5a. The activity of cell-associated LPS may be tempered by capsular polysaccharide that can physically mask LPS on the cell surface (200). *P. gingivalis* and its components can induce the expression of a variety of cytokines and chemokines (Table 8). Increases in the levels of proinflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-8 are likely to not only promote inflammation but also stimulate bone and tissue destruction in the periodontal area. The end-products of metabolism may also affect the immune responses. Butyric acid, a volatile fatty acid produced by *P. gingivalis*, can induce B- and T-cell death through apoptosis (198). Penetration of the periodontal tissue by butyric acid could thus suppress immune system function. Within professional phagocytes, the production of superoxide dismutase (SOD) may protect the organisms from oxygen-dependent killing. *P. gingivalis* elaborates a single SOD of 21.5 kDa. The *sod* gene is downstream of the *prt*C (collagenase) gene in *P. gingivalis* (201), raising the possibility of coordinate regulation of these potential virulence factors. Protection from oxidative damage may also be provided by surface accumulations of hemin, which will form μ-oxo dimers in the presence of reactively oxygen compounds (160).

Table 8 Proinflammatory cytokine induction by *P. gingivalis* and its cellular constituents

Inducers	Cell(s) of origin	Cytokine
LPS	fibroblasts	Interleukin-1alpha (IL-1α)
		Interleukin-1beta (IL-1β)
		Interleukin-6 (IL-6)
		Interleukin-8 (IL-8)
		Macrophage chemoattractant protein-1 (MCP-1)

Table 8 (continued)

Inducers	Cell(s) of origin	Cytokine
LPS	monocytes/ macrophage	Tumor necrosis factor-alpha (TNF-α) IL-6, IL-8 Granulocyte macrophage colony-stimulating factor (GM-CSF) Interferon gamma (IFN-γ)
	PMNs	Interferon alpha (TNF-α) IL-8
Lipid A-associated proteins	fibroblasts	IL-6
Fimbriae	fibroblasts	IL-1β
	monocytes/ macrophages	IL-1α, IL-1β TNF-α IL-6, IL-8 Kuffer cells cytokine (KC)
	calvarial bone cells	GM-CSF
	PMNs	IL-6
	T cells	Interleukin-2 (IL-2) TNF-α IFN-γ
75-kDa surface protein	monocytes/ macrophages	Interleukin-1 (IL-1)
12-kDa antigen	monocytes/ macrophages	IL-1β
PS	monocytes/ macrophages	IL-1β
Whole cells	PMNs	IL-8 MCP-1

9.2.5 Invasion and tissue destruction

The direct invasion of host cells, bacterial components may penetrate and directly damage epithelial cells, which is suggested by the local and systemic immune responses that occur to these antigens (202). Collagenase, macromolecular toxins (such as LPS) and outer-membrane vesicles and associated the proteolytic activities may enter gingival tissues, triggering a host response resulting in direct tissue destruction. These compounds have the potential to impinge upon host tissue integrity and cause loss of the alveolar bone and other supporting periodontal tissues, an important feature of advanced periodontal disease.

(1) Proteinases

The proteases are also involved directly in tissue invasion and destruction by bacteria and in evasion and modulation of host immune defenses. Specific examples of tissue degradation and attenuation of host defense mechanisms include the degradation of extracellular matrix proteins, activation of MMPs, inactivation of plasma proteinase inhibitors, cleavage of cell surface receptors, activation or inactivation of complement factors and cytokines, and activation of the kallikrein-kinin cascade (155). *P. gingivalis* cells elaborate a number of proteolytic activities that accomplish all these activities. In the realization that periodontitis is a destructive and inflammatory condition, there naturally has been intense research effort directed toward identifying and characterizing the proteinases produced by periodontal pathogens.

A number of proteases produced by *P. gingivalis* are thiol-dependent enzymes that cleave C-terminal to arginine (Arg-Xaa) or lysine (Lys-Xaa), Arg-X- and Lys-X-specific proteinases, within protein or peptide substrates and thus were designated, on the basis of substrate specificity, "trypsin-like" enzymes. Biochemically, these enzymes are members of the cysteine-proteinase family. In addition to the cysteine proteinase enzymes, *P. gingivalis* produces serine proteinase activities. Enzymes that degrade immunoglobulins, collagen and/or gelatin, complement factors, fibrinogen and fibronectin can almost always be describe to one of these two families (203).

The genes encoding Arg-X-specific enzymes are designated *rgp* and those encoding Lys-X-specific enzymes are designated *kgp*, a classification scheme that would certainly simplify the nomenclature. A number of genes encoding Arg-X- and

Lys-X-specific proteases from different stains of *P. gingivalis* have now been isolated and sequenced (Table 9). The genes within the two Arg-X protease families exhibit some differences in sequence and in organization of the coding regions within the genes. Multiple forms of Arg-X proteases are recovered from the cell surface of *P. gingivalis* and from the culture fluid. These forms are believed to result mainly from posttranslational processing of larger polyprotein precursor products (204).

Table 9 *P. gingivalis* proteinases; substrate specificity and genes

Substrate Specificity	Homologous genes (host strain)
Arg-X	*rgp-1* (HG66), *rgp*A (ATCC 33277), *prp*R1 (W50), *prt*R (W50), *prt*H (W83), *agp* (381), *cgp*R (ATCC 33277)
Arg-X	*rgp*-2 (HG66), *rgp*B (ATCC 33277), *prt*RII (W50), *pr*R2 (W50)
Lys-X	*kgp* (HG66), *prt*K (W50), *prt*P (W12)

The hemagglutinating activities and adherence of *P. gingivalis* cells have both been associated with protease activity (205). Inactivation of a cysteine protease gene in *P. gingivalis* 381 has a direct effect on hemagglutinin activity (206) as well as on the ability of *P.gingivalis* to bind gram-positive bacteria, oral epithelial cells, and extracellular matrix proteins (207). It is now evident that the C-terminal coding regions of the genes encoding the RgpA family of cysteine proteinases encode extensive amino acid blocks that have up to 90% identity to sequences that are also found within the *hag*A, *hag*D and *hag*E genes encoding hemagglutinins (208). It is formally possible that translational initiation occurs internally in the primary mRNA transcripts, effectively generating an additional level of control over hemagglutinin production.

Proteinases and fimbriae, the production and activity of the major fimbriae are modulated by proteolytic activity. This control appears to occure on at least three levels: posttranslational modification, transcriptional modulation, and substrate activation. The proteinase activity was necessary for processing and maturation of fimbrillin, in addition to its role in modifying other cell surface-associated proteins. Both the fimbriae and the Arg-X-specific (205), Lys-X-specific (209) proteinases are

associated with these binding and degradative processes. Hydrolysis of fibronectin or other matrix proteins such as collagen by *P. gingivalis* Arg-X proteinase enhances the binding of fimbriae to these substrates (210).

(2) Nonproteolytic, potentially destructive compounds

P. gingivalis can degrade the glycosaminoglycans hyaluronate, chondroitin sulfate, and heparin (154). The repertoire of enzymes and metabolites that could be detrimental to the host also includes phospholipase A, which can provide the prostaglandin precursors that could stimulate prostaglandin-mediated bone resorption (211); alkaline and acid phosphatases, which may contribute to alveolar bone breakdown (212); Dnase and Rnase (213); sialidase (154); volatile sulfur compounds such as hydrogen sulfide, methylmercaptan, and dimethyl disulfide, which are cytotoxic and can inhibit protein synthesis (214); butyrate and propionate, which are cytotoxic for epithelial cells, fibroblasts, and lymphocytes (215); indole and ammonia, which also exhibit cytotoxicity (216).

9.3 Prevention and treatment

Prevention of gingivitis and periodontitis is based on the control of their causual and risk factors. The most widely accepted risk factor is the periodontal biofilm that forms on the teeth in the absence of effective oral hygiene. Various factors such as smoking, diabetes, ehtnic origin, specific types of gram-negative anaerobic bacteria in the periodontal biofilm, poor education, infrequent dental attendance, genetic effects, increased age, male sex, diabetes, psychosocial stress, and depression have also been shown to be the important considerations in the prevention and treatment of periodontitis (217, 218).

Toothbrushing and the use of dental floss and other devices to remove bacterial plaque from the teeth are the most common ways of disrupting or removing the periodontal biofilm from teeth. The antibacterial drugs have been used as adjuncts for controlling the biofilm. These cominations contain various biocides, surfactants, polymers, or other components that can reduce the biofilm and are generally not associated with the emergence of a resistant microbiota (219).

Treatment for gingivitis and periodontitis should establish periodontal health, arrest the progression of disease, prevent recurrence of disease, and preserve the

dentition in a state of health, comfort, and function. This goal can be accomplished by various non-surgical and surgical therapies, depending on the specific treatment objective. The professional treatment of periodontitis, the corner stone of periodontal therapy is anti-infective non-surgical treatment aimed at controlling the biofilm and other prominent risk factors. Dental plaque and calcus can be removed from tooth-crown and root surfaces (scaling and root planning) by use of various manual or powerd instruments. This non-surgical therapy, combined with improved personal oral hygiene, can reduce tissue inflammation and pocket depths and improve clinical periodontal attachment (220). Antibiotics are used in conjunction with scaling and root planning, but only in patients with refractory disease or in those who have fever and lymphadenopathy. Correction or replacement of defective prostheses and dental restorations that retain dental plaque is also an important part of periodontal therapy. For patients with advanced disease, a variety of types of periodontal surgery are used to reduce the depth of periodontal pockets, gain access for debridement of residual dental calculus and plaque, and stimulate regeneration of lost periodontal support by use of various surgical procedures, grafting materials, and biological substances.

Successful treatment of periodontal disease is dependent on regular maintenance or supportive follow-up therapy after active treatment is completed. For patients with aggressive or refractory disease, retreatment with the adjunctive use of antibiotics dictated by appropriate microbial culture and sensitivity testing might be needed (221). A wide variety of systemic antibiotics in varying doses has been used to treat periodontal disease either alone or in combination with standard non-surgical and surgical periodontal therapy (222). Limited data exist regarding the effect of antibiotic use alone in treating periodontitis (223) and the use of systemic antibiotics for the treatment of periodontal disease has a risk of adverse drug reaction and increased selection of multidrug-resistant organisms. Systemic antibiotics should only be used in conjunction with mechanical debridement and can provide the greatest benefit to patients who do not respond to debridement alone or who have fever or lymphadenopathy (224). Systemic administration of combinations of metronidazole and either amoxicillin or ciprofloxacin has been widely used with great success; however the presence of subgingival yeasts and resistant bacteria can be a problem in some periodontitis patients (225).