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EVALUATION OF THE CLINICAL AND IMMUNOMODULATORY EFFECTS OF THE PROBIOTIC LOZENGES OR SUB-ANTIMICROBIAL DOSE DOXYCYCLINE AS ADJUNCTS TO NON-SURGICAL PERIODONTAL THERAPY IN CHRONIC PERIODONTITIS

PhD Thesis

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ONAY

Bu tez Yeditepe Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliğinin ilgili maddeleri uyarınca yukarıdaki jüri tarafından uygun görülmüş ve Enstitü Yönetim Kurulu'nun .16./.12./2015 tarih ve 31-3 sayılı kararı ile onaylanmıştır.

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I. SUMMARY

The effect of probiotics or sub-antimicrobial dose doxycycline (SDD) as an adjunct to scaling and root planing (SRP) on inflammatory parameters in chronic periodontitis (CP) patients needs further investigation because of the controversial data. Therefore, the aim of the present study was to evaluate the clinical and immunomodulatory efficacy of *Lactobacillus reuteri* (*L. reuteri*) containing lozenges (Probiotic) or SDD (Doxycycline hyclate) as adjuncts to non-surgical periodontal therapy.

A total of 45 patients, with at least 2 teeth having one approximal site with a probing depth (PD) of 5-7 mm and gingival index (GI) of ≥2 in each quadrant, were selected and randomly divided into 3 groups. Group I received SRP + Probiotic lozenges ($2x10^8$ cfu/ml), Group II received SRP + SDD (doxycycline hyclate) tablets, whereas Group III received SRP + placebo. Plaque index (PI), GI, PD, bleeding on probing (BoP) and attachment gain were measured and gingival crevicular fluid (GCF) sampling was performed at baseline and day 90. GCF analysis were assessed by enzyme linked immunosorbent assay (ELISA). GCF volume, MMP-8 and TIMP-1 levels were also evaluated at baseline and at day 90.

Intra-group comparisons of all parameters were evaluated with paired sample t test while inter-group comparisons of all clinical and biochemical parameters were evaluated with Oneway Anova test. Tukey test was used for the double comparisons of the groups. The statistical significance was set at $p < 0.05$. At the end of the observation period, statistically significant improvements in all evaluated parameters were observed within each group. Inter- group comparisons of mean differences of both clinical and biochemical parameters revealed statistically significance in favour of both SRP + Probiotic and SRP + SDD between days 0-90 at the end of day 90 ($p < 0.05$). However, no siginificant differences were observed between SRP + Probiotic and SRP + SDD in terms of both clinical and biochemical parameters. ($p > 0.05$)

In conclusion, the results of the present study revealed that the adjunctive usage of *L. reuteri* containing lozenges and SDD tablets significantly reduced inflammation when compared to SRP + placebo in CP patients.

Key Words: SRP, Chronic Periodontitis, Host Modulation, Sub-antimicrobial dose Doxycycline, Probiotics

II. ÖZET

Bu çalışmada, kronik periodontitisli (KP) hastalarda başlangıç periodontal tedavi (BPT)'ye yardımcı olarak kullanılan *L.reuteri* içeren probiyotik tabletlerin ve sub-antimikrobiyal doz doksisiklin (SDD) uygulamasının etkinliklerinin klinik ve biyokimyasal olarak değerlendirilmesi amaçlandı.

Çalışmaya her kadranda sondalama derinliği (SD) ≥ 5 mm, gingival indeksi (Gİ) ≥ 2 olan en az iki tek köklü dişe sahip, yaşları 35 ile 50 arasında değişen, 21 kadın ve 24 erkek toplam 45 KP'li hasta dahil edildi ve rastgele 3 gruba ayrıldı. 1. gruba diş yüzey temizliği ve kök yüzey düzleştirilmesi (SRP) ile birlikte *L.reuteri* içeren probiyotik tabletler ($2x10^8$ cfu/ml) 3 ay süre ile günde 2 defa (SRP + Probiyotik), 2. gruba SRP ile SDD (20 mg doksisiklin hiklat) içeren tabletler 3 ay süre ile günde 2 defa (SRP + SDD) ve 3. gruba sukraloz içeren plasebo tabletler SRP ile birlikte yine 3 ay boyunca günde iki defa olmak üzere reçete edildi. Biyokimyasal örnekleme SD ≥ 5 mm ve Gİ ≥ 2 olan tek köklü diş bölgesinden tedavi öncesi (0. gün) ve sonrası (90. gün) alındı. Bunu takiben plak indeks (Pİ), Gİ, SD, rölatif ataşman seviyesi içeren klinik ölçümler yapıldı. Biyokimyasal değerlendirme ELISA testi ile yapıldı ve DOS hacmi, MMP-8 ve TIMP-1 seviyesi değerlendirildi. Gözlem süresinin sonunda, tüm ağza ait klinik parametrelerin hepsinde grup içi değişimlerde istatistiksel olarak anlamlı azalmalar tespit edildi. Klinik ve biyokimyasal parametrelere ait fark ortalamalarının gruplar arası karşılaştırmalarında istatistiksel olarak anlamlılık tespit edildi ($p<0.05$). Elde edilen bu farklılığın 2'li karşılaştırmada SRP + Probiyotik ve SRP + SDD grupları lehine olduğu belirlendi ($p<0.05$).

Bu çalışma, KP'li hastalarda BPT'yi destekleyici olarak olarak 3 ay süreyle probiyotik tablet ve SDD kullanımının, sadece BPT uygulanan gruba göre inflamasyonun çözülmesinde oldukça etkili olduğunu destekler niteliktedir.

Anahtar Kelimeler: Kök Yüzey Düzleştirme, Kronik Periodontitis, Konak Modulasyon, Sub-antimikrobiyal Doz Doksisiklin, Probiyotik

III. DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

Sadberg CİHANGİR HAMUD

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V. CONTENTS

VI. ABBREVIATIONS

A.a:	*Aggregatibacter Actinomycetencomitans*
AAP:	American Academy of Periodontology
ADA:	American Dental Association
AL:	Attachment Loss
BMP:	Bone Morphogenetic Protein
BoP:	Bleeding on Probing
CAL:	Clinical Attachment Level
CFU:	Colony Forming Unit
CP:	Chronic Periodontitis
DBPC:	Double- blind, randomized, placebo controlled
DM:	Diabetes Mellitus
ECM:	Extracellular Matrix
ELISA:	Enzyme Linked Immunosorbent Assay
EMP:	Enamel Matrix Protein
FAO:	Food and Agriculture Organization
FDA:	Food and Drug Administration
FMSRP:	Full Mouth Scaling and Root Planing
GCF:	Gingival Crevicular Fluid
GF:	Growth Factor
GI:	Gingival Index
HMT:	Host Modulatory Therapy
IFMA:	Immunofluorometric Assay
IFN- γ:	Interferon gamma
Ig:	Immunoglobulin
IL:	Interleukin
kDa:	Kilo dalton
LGG:	*Lactobacillus Rhamnosus GG*
LPS:	Lipopolysaccharide
mg:	miligram
ml:	milliliter
mm:	millimeter
MIP:	Macrophage inhibitory protein
MMP:	Matrix Metalloproteinase

MPO:	Myeloperoxidase
mRNA:	Messenger Ribonucleic acid
MRONJ:	Medicine related osteonecrosis of jaw
ng:	Nanogram
nm:	nanometer
NOS:	Nitric Oxidase Synthase
NSAIDs:	Non-steroidal Anti-inflammatory Drugs
OHI:	Oral Hygiene Instruction
OSFMD:	One-stage, full-mouth disinfection
PBI:	Papilla Bleeding Index
PBS:	Phosphate Buffered Saline
PD:	Probing Depth
Pg:	*Porphyromonas gingivalis*
PGE_2:	Prostaglandin E_2
PI:	Plaque Index
Pi:	*Prevotella intermedia*
PMN:	Polymorphonuclear leukocytes
QWSRP:	Quadrant Wise Scaling and Root Planing
rRNA:	Ribosomal Ribonucleic Acid
RAL:	Relative Attachment Level
RIA:	Radioimmunoassay
rpm:	Revolutions per minute
RT-PCR:	Reverse Transcription Polymerase Chain Reaction
s:	Second
SDD:	Sub-antimicrobial Dose Doxycycline
SRP:	Scaling and Root Planing
TGF-β:	Transforming Growth Factor beta
TIMP:	Tissue Inhibitors of Metalloproteinase
TNF- α:	Tumor Necrosis Factor alpha
t-PA:	Tissue Plasminogen Activator
TVC:	Total Viable Count
WHO:	World Health Organisation
µl:	microliter

VII. FIGURES and TABLES LIST

1. INTRODUCTION and AIM

Periodontal disease is a biofim associated inflammatory disease and develops as a result of an imbalance between dental plaque biofilm organisms and host defence mechanisms that is characterized by attachment and alveolar bone loss (1). Bacteria are therefore a necessary prerequisite for disease to develop, but insufficient to cause periodontal disease alone. For periodontal disease to develop, a susceptible host is also required. Excessive production of destructive enzymes (such as matrix metalloproteinases [MMPs]) and inflammatory mediators (e.g. interleukins [IL]) and prostaglandins) are released during the cascade of destructive inflammatory events that occur as a part of the inflammatory response.

Periodontal treatment comprises non-surgical debridement followed by a re-evaluation. The main aim of the treatment includes reducing the bacterial load and altering the microbial composition towards a flora more associated with health (2). Therefore without the surgical reflection of the soft tissues surrounding the teeth, the number of biofilm microorganisms reduces and the ecology of microbial biofilm is disrupted. Non-surgical periodontal treatment results in reductions in the total microbiota, however, the efficacy varies in different situations. In order to improve the outcomes of the non-surgical periodontal therapy, local and systemic antibiotics, lasers and antmicrobial photodynamic therapy have been proposed. The development of increasing levels of bacterial resistance and many side effects of the antibiotics may limit their usage in the periodontal treatment. On the other hand, lasers and photodynamic therapy still needs further improvements and investigations in terms of clinical efficacy (3, 4). In the recent decade, host modulation therapy (HMT) has been proposed as a treatment strategy in the field of periodontology.

HMT is a treatment concept that aims to reduce tissue destruction and stabilize the periodontium by downregulating or modifying destructive aspects and ⁄or upregulating protective or regenerative components of the host response. There are many agents that have been used for this purpose in the treatment of periodontal diseases such as; non-steroidal anti-inflammatory drugs (NSAIDs), bisphosphonates, growth factors (GF) and SDD.

SDD is a 20-mg of doxycycline hyclate that is taken twice daily for a period 3-9 months as an adjunct to root surface instrumentation in the treatment of periodontitis. It has the ability to down-regulate MMPs, reduce osteoclastic activity and bone resorption, stimulate fibroblastic collagen production (5). Recently, probiotics have been drawn

attention in medicine and periodontal therapy as well. A probiotic approach, in periodontology might be of interest and we may see several possibilities for the success of this supportive approach in the future. Probiotics are defined as 'Live microorganisms which when administered in an adequate amount confer a health benefit on the host' by World Health Organisation (WHO). The suggested possible mechanisms of action are; inhibition of pathogens, the promotion of beneficial species and immuno-modulation (6, 7). It has been taught that immuno-modulation activity can be occured via inhibition of collagenases and reduction of inflammation-associated molecules, induction of expression of cyto-protective proteins on host cell surfaces, modulation of pro-inflammatory pathways induced by pathogens, prevention of cytokine-induced apoptosis, modulation of host immune response (6).

Regarding the periodontal literature, only few studies have been evaluated the effects of probiotics on the host modulation. From this standpoint, we aimed to evaluate the host modulatory effects of both *L.reuteri* containing probiotic lozenges and SDD agents as adjuncts to non-surgical periodontal therapy in CP patients on the clinical and biochemical parameters in a 3-month follow up period.

2. LITERATURE REVIEW

2.1. Periodontal Diseases and Pathogenesis

Periodontitis is an inflammatory disease that is characterized by the breakdown of periodontal ligaments and alveolar bone loss, develops as a result from a complex interplay between the subgingival biofilm and the host immune–inflammatory events (8).

Oral microflora has a harmonious and positively beneficial relationship with the host. The composition of bacteria remains stable over time and exists in a state of dynamic equilibrum. In these circumstances, subgingival plaque is controlled through the innate immune system. However, the host inflammatory and immune response can be overwhelmed by excessive plaque accumulation, by plaque independent host factors (e.g. immune disorders, changes in hormonal balance or systemic diseases such as diabetes) or by environmental factors (e.g. smoking, diet and stress) (9, 10). The term 'innate immunity' refers to the elements of the immune response that are determined by inherited factors, that have limited specificity, and they are "fixed", that they do not change or improve during an immune response or as a result of previous exposure to a pathogen (8). In order to cause damage, bacteria must either colonize the gingival crevice by evading host defenses or damaging the crevicular epithelial barrier by producing substances. Bacterial products which includes the bacterial enzymes (e.g. collagenase, proteases) can be divided into proteolytic enzymes and hydrolytic enzymes, that breakdown extracellular matrices (ECM), such as collagen and host cell membrane substances, in order to produce nutrients for their growth and subsequent tissue invasion (11). Surface components of subgingival bacteria are involved in adhesion to epithelial cells at the beginning of the colonization, biofilm formation and also in bacterium-host cell cross-talk. *Porphyromonas gingivalis (Pg), Aggregatibacter actinomycetemcomitans (A.a.)* and other periodontal pathogens possess multiple virulence factors, such as cytoplasmic membranes, peptidoglycans, outer membrane proteins, lipopolysaccarides (LPS), capsules (9, 10).

The microbial challenge presented by subgingival plaque results in an upregulated host immune-inflammatory response in the periodontal tissues that is characterized by the excessive production of inflammatory cytokines (e.g. ILs, tumor necrosis factor-α [TNF-α]), prostanoids (e.g. prostaglandin E_2 [PGE_2]) and enzymes including MMPs. These pro-inflammatory mediators are released from leukocytes, fibroblasts or other leading to the clinical signs and symptoms of disease (5). Collagen

structure of periodontal tissues are degraded by proteases for further leukocyte infiltration. Destructive inflammatory events, such as production of destructive enzymes and inflammatory mediators from infiltrating neutrophils and resident periodontal tissue cells are part of the natural host response to infection, in periodontal disease and other chronic inflammatory diseases. There is an imbalance between pro-inflammatory mediators and destructive enzymes, anti-inflammatory mediators and enzyme inhibitors (5).

Periodontitis appears to behave as a multifactorial disease, therefore bacteria are a necessary prerequisite for a disease to develop but insufficient to cause periodontal disease alone (5, 12). Acquired and environmental risk factors, such as diabetes mellitus (DM), cigarette smoking and stress, as well as genetically transmitted traits, such as IL-1 gene polymorphisms, may accentuate the host inflammatory response to the bacterial challenge and eventually the susceptibility to the disease (13, 14). The severity of the outcome and disease occurrence are determined by these factors (14).

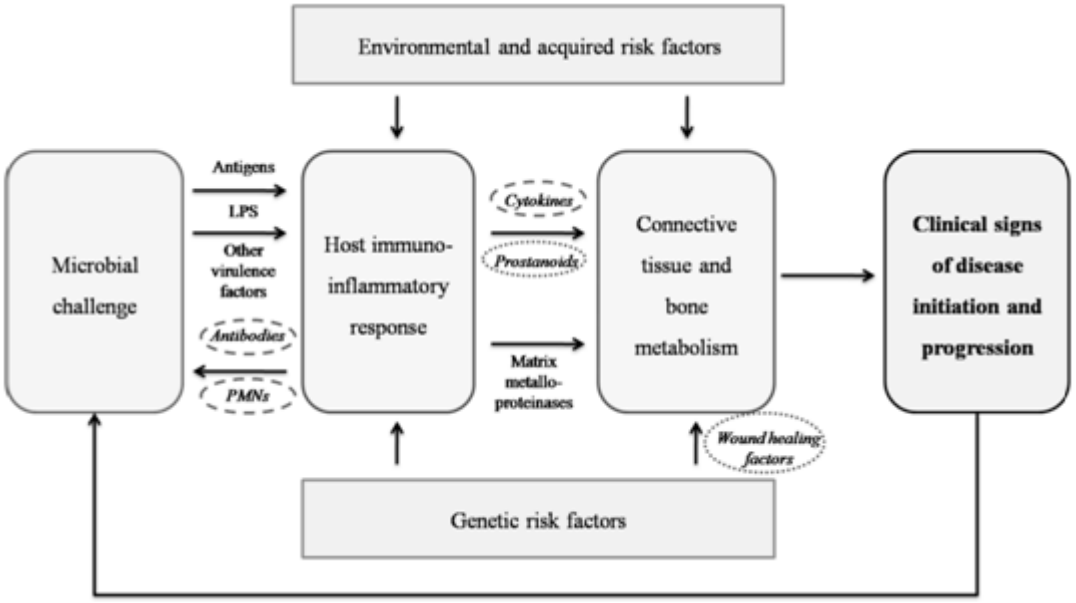

Figure 1. Pathogenesis of human periodontitis (14)

2.2. Chronic Periodontitis

CP is the most commonly occurring form of periodontal disease, and has been defined as "an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss, and bone loss." (15). According to the American Academy of Periodontology's (AAP) classification in 1999, CP is associated with the accumulation of plaque and calculus and it generally demonstrates the characteristics of a slowly progressing inflammatory disease. However, systemic and environmental factors (e.g., DM, smoking) may modify the host's immune response to the dental biofilm so that periodontal destruction becomes more progressive (15). The major clinical and etiologic characteristics of the disease: microbial biofilm formation (dental plaque-calculus), periodontal inflammation (e.g., gingival swelling, BoP, pocket formation), attachment and alveolar bone loss (16, 17, 18).

Overall characteristics of CP are as follows:

- CP is considered a site-specific disease. As a result of the site-specific nature, the number of teeth with clinical attachment loss classifies CP as localized when < 30% of sites are affected and generalized when this level is exceeded.
- Disease severity may be described based on the degree of attachment loss as mild (1-2 mm), moderate (3-4 mm) an severe (≥ 5 mm).
- CP is prevalent in adults but may occur in children. Increases in prevalence and severity with age is observed, and it generally affects both genders equally. Periodontitis is an age-associated (not an age-related) disease.
- CP does not progress at an equal rate in all affected sites throughout the mouth. Some involved areas may remain static for long periods, whereas others may progress more rapidly. It is associated with areas of greater plaque accumulation and inaccessibility to plaque-control measures, local predisposing factors, smoking, stress, and systemic risk factors.
- The subgingival biofilm harbors variety of bacterial species, and the composition of the biofilm may vary between subjects and the sites (16, 19).

2.3. Treatment of Chronic Periodontitis

CP is an inflammatory disease initiated by microorganisms existing in a biofilm community. Prevention of disease initiation is related to biofilm formation prevention and/or removal of microbial biofilm. Periodontal treatment traditionally comprises non-surgical mechanical instrumentation followed by a re-evaluation period,

than if there is any need for further treatment, surgical (phase II) therapy is conducted (2).

Non-surgical periodontal therapy is also referred by the names, **phase I therapy, initial therapy, cause related therapy** and **etiotropic phase of therapy**. Non-surgical therapy aims to eliminate both living bacteria in the microbial biofilm and calcified biofilm microorganisms from the tooth surface and the adjacent soft tissues. In addition, non surgical periodontal treatment aims to create an environment in which the host can more effectively prevent pathogenic microbial recolonization using personal oral hygiene methods (20). Elimination of etiologic and contributing factors in periodontal treatment are achieved by complete removal of calculus, correction of defective restorations, treatment of carious lesions and education of comprehensive daily plaque control. Local contributing factors are oral conditions that increase an individuals' susceptibility to periodontal infection to specific sites. They don't initiate either gingivitis or periodontitis but only act to contribute to the disease process previously initiated by bacterial plaque. These local factors increase plaque retention, plaque pathogenicity and also can damage periodontium. Control or elimination of contributing local factors includes the following therapies (21):

- Complete removal of calculus
- Correction of faulty dental restoration
- Restoration of carious lesions
- Orthodontic tooth movement
- Treatment of occlusal trauma
- Correction of developmental defects
- Correction of personal habits
- Extraction of hopeless teeth
- Treatment of food impaction areas

Non-surgical therapy consists of supra and subgingival surface debridement combined with oral hygiene instructions (OHI). SRP is the primary therapy of choice and it is considered as the 'gold standard' for treating periodontitis. SRP reduces the bacterial load and alters the microbial composition towards a flora more associated with health. In turn, these microbiologic changes result in lower levels of inflammation and relative stability in periodontal attachment levels (2). Elimination or adequate suppression of periodontopathic bacteria in subgingival microbiota, is essential for

periodontal healing. Mechanical root debridement is a prerequisite for controlling periodontal infections, and clinical improvement following mechanical root debridement is directly related to the degree of the removal of pathogenic subgingival microbes. However, conventional mechanical root debridement does not usually eradicate all periodontopathic bacteria from the subgingival ecosystem. Sites with deep periodontal pockets, grooves, furcations, and concavities are difficult to access with periodontal instruments. Periodontopathic bacteria may remain in those sites. In addition, periodontal bacteria have been detected on the mucosa, tongue, tonsils, and gingiva, from where they may colonize dental plaque. Also, *Pg*, *A.a*, and spirochetes are capable of invading gingival epithelial cells, subepithelial connective tissues, and dentinal tubules. Mechanical therapy alone may not completely eliminate the periodontal pathogens residing in these extradental oral sites. Due to the multifactorial etiological nature of periodontitis, many adjunctive treatment approaches have been proposed. However, treatment of periodontal disease with adjunctive therapies may not be sufficient, mechanical instrumentation should routinly be performed to disrupt biofilm and to remove the bulk of bacterial deposits prior to adjunctive therapies (21, 22, 23, 24).

2.4. Host Modulation

HMT is a treatment concept that aims to reduce tissue destruction and stabilize the periodontium by downregulating or modifying destructive aspects and⁄ or upregulating protective or regenerative components of the host response (5). The concept of host modulation was first introduced to dentistry by Williams (25) and Golub et al (26) and then expanded on by many other scholars in the dental profession. In 1990, Williams (27) concluded “there are compelling data from studies in animals and human trials indicating that pharmacologic agents, that modulate the host responses believed to be involved in the pathogenesis of periodontal destruction, may be efficacious in slowing the progression of periodontitis.” In 1992, Golub and colleagues (26) discussed “host modulation with tetracyclines and their chemically modified analogues (28).

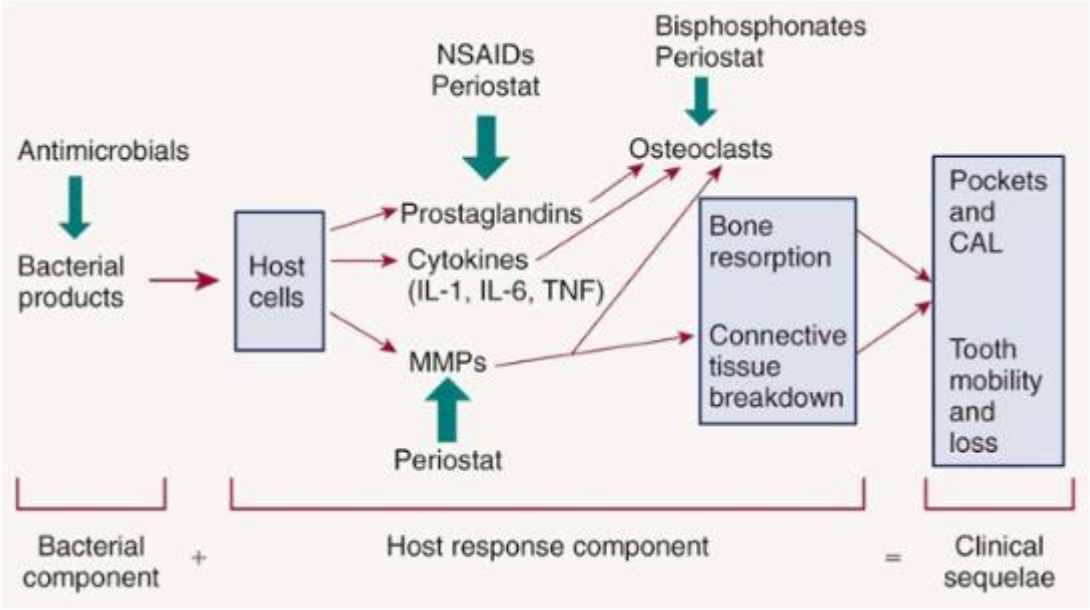

Figure 2. Potential adjunctive therapeutic approaches (28)

In periodontitis, the host is responsible for most of the tissue breakdown that occurs, leading to the clinical signs of disease. The major component of soft- and hard-tissue destruction associated with periodontal disease is, the result of activation of the host's immuno-inflammatory response to the bacterial challenge. The underlying biological mechanisms of this response are characterized by the expression of endothelial cell and intercellular adhesion molecules and by the production of host-derived inflammatory mediators including cytokines and lipids by neutrophils, monocytes, lymphocytes and fibroblasts. Host response modulators offer the potential for modulating or reducing this destruction by ameliorating excessive or pathologically elevated inflammatory processes to enhance opportunities for wound healing and periodontal stability. A variety of drug classes have been evaluated as host response modulators, including the NSAIDs, bone sparing agents (bishosphonates), anti-proteinases (tetracyclines), enamel matrix proteins (EMP), GF, and bone morphogenetic proteins (BMP) (29). NSAIDs inhibit the formation of prostaglandins, including PGE_2, which is produced by infiltrating cell types in the periodontium (including neutrophils, macrophages, fibroblasts and epithelial cells) in response to LPS. PGE_2 is a key inflammatory mediator in periodontal disease as it upregulates osteoclastic bone resorption, and PGE_2 levels are significantly increased in the tissues and GCF of

patients with periodontal disease compared to healthy controls. NSAIDS are associated with significant unwanted effects, including gastrointestinal problems, hemorrhage (as a result of decreased platelet aggregation), and renal and hepatic impairment. Also, once patients cease taking NSAIDs, a return to, or even acceleration of, the rate of bone loss seen prior to drug therapy occurs, sometimes referred to as a 'rebound effect' (30). The bisphosphonates disrupt osteoclastic activity and thereby inhibit bone resorption. A recent development has been the publication of several case reports of avascular necrosis of the jaws, particularly the mandible, following bisphosphonate therapy, with an increased risk of bone necrosis following dental extractions. This has been termed medicine related osteonecrosis of the jaw (MRONJ) and is a significant and clinically serious complication of bisphosphonate therapy (8). Therefore, only one systemic medication has been licenced specifically as a host response modulator in periodontal treatment which is SDD.

2.4.1. Subantimicrobial Dose Doxycycline

SDD is the only systemic host response modulator specifically indicated as an adjunctive agent for the treatment of periodontitis. SDD is approved by the US Food and Drug Administration (FDA), American Dental Association (ADA), the UK Medicines and Healthcare products Regulatory Agency and introduced with a brand name Periostat®. It is a 20-mg dose of doxycycline hyclate that is taken twice daily for periods of 3-9 months as an adjunct to root surface instrumentation (31). The rationale for using doxycycline at subantimicrobial doses as a host response modulator is that; it inhibits the activity of MMPs by a variety of synergistic mechanisms including reductions in cytokine levels, stimulation of osteoblastic activity and new bone formation by upregulating collagen production independent of any antibiotic properties. Doxycycline was shown to be more effective than other tetracyclines in reducing collagenase activity in the GCF of CP patients. It was also confirmed as being a more effective inhibitor of MMPs than either minocycline or tetracycline and has a much lower inhibitory concentration therefore much lower dose of doxycycline is necessary. Furthermore, doxycycline has also been found to be more effective in blocking neutrophil collagenase activity (MMP-8) than fibroblast collagenase activity (MMP-1), suggesting that doxycycline can provide a safe method of reducing pathologically elevated collagenase levels without interfering with normal connective tissue turnover (32). However, a major concern with the long-term administration of doxycycline was the possibility of development of antibiotic resistance. Subsequent studies of relatively

short duration (1–3 months) indicated that this dosage regimen could prevent periodontitis progression without the emergence of doxycycline resistant organisms or other typical antibiotic side effects. In conclusion, down-regulation of destructive events in the periodontium by doxycycline results from modulation of a variety of different pro-inflammatory pathways(5);

- Inhibition of oxidative activation of latent MMPs
- Down-regulation of expression of key inflammatory cytokines (IL-1, IL-6 and TNF-α) and PGE_2
- Inhibition of production of reactive oxygen species produced by neutrophils
- Inhibition of MMPs and reactive oxygen species thereby indirectly reducing tissue proteinase activity
- Stimulation of fibroblast collagen production
- Reduction of osteoclast activity and bone resorption
- Inhibition of osteoclast MMPs

Several studies with different clinical trial periods were conducted to evaluate the use of SDD as adjunct to SRP in CP patients. Emingil et al. (33) aimed to examine the effectiveness of SDD in combination with non-surgical periodontal therapy, compared to non-surgical periodontal therapy alone, on GCF MMP-8 levels and clinical parameters over a 12 month period in CP patients. GCF samples were collected and clinical parameters PD, clinical attachment levels (CAL), GI, PI were recorded. 30 CP patients divided into two group, SDD and placebo groups. The SDD group received 20 mg of doxycycline b.i.d for 3 months with SRP, while the placebo group was given placebo capsules b.i.d for 3 months with SRP. The patients were evaluated every 3 months during the 12 month period. In each visit, all clinical parameters and GCF sampling were repeated. GCF MMP levels were determined by a time-resolved immunoflouresence assay. Significant improvements were observed in all clinical parameters in both groups over the 12 month period. The SDD group showed significantly greater reduction in mean PD scores at 9 and 12 months, and in mean GI scores at all time points than the placebo group. From baseline to 12 months GCF MMP-8 levels were significantly reduced in both groups. The GCF MMP-8 level in the SDD group was significantly lower than the placebo group at 6 months. These data indicated that SDD therapy in combination with SRP may reduce GCF MMP-8 levels and clinical periodontal parameters in CP patients. In a different study Emingil et al.

(34) investigated the impact of adjunctive usage of SDD on the local inflammatory response through cytokine and chemokine levels in GCF samples from patients with CP. Cytokines are considered the major regulators of the host's immune reaction at different stages of inflammation. Cytokine release is closely controlled by chemokine-mediated recruitment of inflammatory cells. Chemokines facilitate the migration and subsequent activation of specific types of leukocytes in response to the bacterial infection. 46 patients with CP received SRP with or without adjunctive SDD. GCF samples were collected and PD, CAL, GI, PI were recorded every 3 months for 12 months. GCF TNF-α, IL-6, IL-4, IL-10, IL-13, IL-17, macrophage inhibitory protein 1-α (MIP 1-α), MIP1-β, monocyte chemoattractant protein 1, and regulated on activated normal T-cell expressed and secreted protein levels were determined by xMAP multiplex immunoassay. Significant improvements were observed in all clinical parameters in both groups over 12 months, whereas the SDD group showed significantly better reduction in GI, PD and attachment gain compared to the placebo group. Decrease observed in IL-6 levels in the SDD group was significantly higher when compared to the placebo group at 6 and 9 months in deep pockets, whereas TNF-α was significantly reduced in moderately deep pockets. SDD showed a stable IL-4 and IL-10 response while reducing the monocyte chemoattractant protein 1 levels at 3 months. Authors concluded that SDD, as an adjunct to non-surgical periodontal therapy, stabilizes the inflammatory response by promoting the suppression of pro-inflammatory cytokines and increasing the anti-inflammatory cytokines. The chemokine activity would account for the regulation of the inflammatory response to SDD therapy.

To examine the effectiveness of SDD in combination with non-surgical therapy on GCF tissue plasminogen activator (t-PA) levels and clinical parameters, Emingil et al. (32) conducted a study in CP patients over 12-month period. The plasminogen activating system plays an important role in controlling proteolytic events in the ECM. This system is involved in tissue proteolysis in several physiological and pathological events including cell migration, local inflammatory reactions, tissue repair and remodeling. GCF samples were collected, PD, CAL, GI and PI were recorded at day 0 and at the months 3, 6, 9, 12. CP patients were randomized to SDD or placebo groups. SDD group received SDD (20 mg) b.i.d for 3 months with SRP, while placebo group was administered placebo capsules b.i.d for 3 months with SRP. GCF t-PA levels were determined by ELISA. Significant improvement was observed in all clinical parameters

in both groups over 12-month period. SDD group had lower PD, CAL and GI scores than placebo group at 6, 9 and 12-months. GCF t-PA levels were reduced in both groups over 12-month period. SDD group had lower GCF t-PA levels than placebo group at months 6 and 9. Findings of this study stated that these results provide an additional information about usefulness of SDD therapy as an adjunct to non-surgical therapy in long-term management of periodontitis (32).

Gürkan et al. (35) evaluated the effect of adjunctive SDD therapy on clinical periodontal parameters and GCF transforming growth factor-beta1 (TGF-β1) levels in patients with severe, generalized CP over a 6-month period. 35 patients with severe, generalized periodontitis and periodontally healthy subjects were included in the present study. Patients received full-mouth supragingival debridment at baseline and randomized to take either SDD b.i.d. or placebo b.i.d. for 3 months. Patients received root planing and OHI once a week for four consecutive weeks. PD, CAL, papilla bleeding index (PBI) and PI and GCF sampling were performed at baseline, 3 and 6 months. The GCF TGF-β1 levels were analysed by ELISA. TGF-β1, a cytokine generally known for its potential effects on repair and regeneration, has important effects on regulation of MMPs. It inhibits the release of pro-collagenase and suppresses collagenase production by fibroblasts and macrophages. Moreover, it down-regulates the transcription of MMP genes and enhances the expression of TIMP-1 and plasminogen activator inhibitor. TGF-β1 is a key mediator not only in MMP regulation, but also in limitation and resolution of inflammation. 13 patients in both study groups completed the 6-month trial. Following SRP + SDD and SRP + placebo therapy significant improvements in clinical periodontal parameters of both groups were observed. In the SDD group a significantly higher percentage (%73.4) of deep pockets resolved when compared with placebo group (%49.7) at 6 months. At baseline there were no significant differences in GCF TGF-β1 levels between the groups. Both total amount and concentration of GCF TGF-β1 in SDD and placebo groups increased when compared with baseline at 3 months. However, only GCF TGF-β1 levels of SDD group was significantly higher than baseline and placebo group at 3 months. At 6 months GCF TGF-β1 levels of both groups were similar to baseline levels. This study indicated that combination of SDD with non-surgical therapy improves clinical parameters of periodontal disease and increases GCF TGF-β1 levels together with a decrease in prevalence of residual pockets in patients with severe, generalized CP.

Needleman et al. (36) investigated the effect of SDD as an adjunct to non-surgical periodontal therapy in smoking CP patients. 34 CP patients were included and divided into test and control groups. The test group consisted of 16 patients whereas 18 patients were included in the control group. After all patients received SRP, SDD (20 mg) or placebo tablets were administered to the test group and control group, twice a day for 3 months. PI, GI, PD and CAL were investigated at baseline, 3 months and 6-month follow up periods. At the end of the study period, no significant differences were found between both groups. The authors reported no significant benefit of using SDD as an adjunct to SRP in smoking CP patients.

In a study by Gorska and Nedzi-Gora (29), the effect of SDD were assessed as an adjunct to SRP on clinical and biochemical parameters in 66 CP patients. The patients were divided into SRP + SDD group or SRP only group respectively. SRP was performed in all patients and SDD were administered twice a day for 3 months. Clinical parameters [PI, bleeding index (BI), PD and CAL)] were recorded at baseline and 3 months. Biochemical parameters (MMP-8, MMP-9 and TIMP-1 levels) were obtained from saliva and peripheral blood and assessed by ELISA. SRP + SDD group showed significant improvements in all clinical and biochemical parameters when compared to SRP only group. The results of this study were confirmed the modulating effect of doxycycline on the host response in CP patients.

Novak et al. (37), evaluated the adjunctive usage of SDD for the host modulation therapy in severe CP patients. 30 subjects ≤ 45 years of age were included. Subgingival debridement and OHI were given each week for 4 weeks to all patients and then SDD or placebo tablets were administered. Periodontal status was monitored at baseline and at 1, 3, 5.25 and 8.25 months. Maintenance therapy was performed at 1, 3, 5.25 and 8.25 months for both groups. Adjunctive SDD usage resulted in significant reductions in deep pockets (≥7 mm) compared to placebo group. A significant clinical response was detected in both groups however more statistically greater clinical response was seen in favor of the SDD group. The researchers reported that the adjunctive usage of SDD was more effective in preventing further increases in PD parameter when compared to placebo group.

Lee et al. (38), aimed to evaluate the efficacy of SDD as an adjunct to SRP. 41 moderate CP patients were randomly allocated to receive SDD or placebo tablets twice a day for 2 weeks after SRP. PD, CAL, GCF levels and MMP-8 and MMP-13 levels were determined throughout the study. The significant reductions in PD and CAL levels

were found in both groups, however, greater reduction was seen in SDD group. The decreases in MMP-8 and MMP-13 levels were detected significantly higher than the SDD group when compared to placebo. The results of this study suggested that SDD + SRP can be used effectively in the long-term management of CP patients.

2.4.2. Probiotics

The concept of beneficial-for-health microorganisms dates back to the ideas of Nobel Prize laureate Ilya Metchnikov in the early years of the 20th century. Working at the Pasteur Institute in Paris, the Ukranian bacteriologist studying the flora of the human intestine developed a theory that senility is caused by poisoning of the body by the products of some of these bacteria (39). To prevent the multiplication of these organisms he proposed a diet containing milk fermented by *Lactobacilli*, which produce large amounts of lactic acid and for a time this diet became widely popular (6). The currently used consensus definition of probiotics was put forward by the World Health Organization (WHO), and the Food and Agriculture Organization (FAO) of the United States. They defined probiotics as ‘‘Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host’’ (40). According to the FAO and WHO guidelines (FAO/ WHO, 2002), probiotic microorganisms must be able to survive through the gut passage, must be able to resist low pH and organic acids. Also they must proliferate and colonize the gastrointestinal tract. Furthermore, they must be non-pathogenic, non-toxic and effective. In order to maintain effective concentrations, probiotics should be ingested on a regular basis (41). No studies have been performed to investigate the concentration of probiotic bacteria in terms of administration, but it is generally accepted that to be effective in the gastrointestinal tract, the concentration of bacteria in the delivery system should be at least 10^6 colony-forming units/ml (cfu/ml).

Probiotics, which are regulated as dietary supplements and foods, consist of yeast or bacteria. Their products can contain a single microorganism or a mixture of several species. They are available as capsules, tablets, packets, or powders and are contained in various fermented foods, most commonly yogurt or dairy drinks. The most widely used probiotics belong to lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium* species, *Propionibacterium* and *Streptococcus* (Table 1). The yeast *Saccharomyces boulardii* also was reported to have health benefits (42).

Table 1. Microorganisms used as probiotics (41)

Lactobacillus species	*Bifidobacterium* species	Other microorganisms
L. acidophilus *L. bulgaricus* *L. casei* *L. crispatus* *L. fermentum* *L. gasseri* *L. johnsonii* *L. lactis* *L. plantarum* *L. reuteri* *L. rhamnosus GG*	*B. adolescentis* *B. animalis* *B. bifidum* *B. breve* *B. infantis* *B. lactis* *B. longum*	*Bacillus cereus* *Enterocccus faecalis* *Enterococcus faecium* *Escherichia coli Nissle* *Propionibacteria* *Streptococcus thermophilus* *Saccharomyces boulardii*

'Replacement therapy' also called 'bacteriotherapy' or 'bacterial interference' could be confused with probiotics. Nevertheless both approaches use live bacteria for the prevention or treatment of infectious disease, there are some slight differencies in between (Table 2) (43).

Table 2. Differences between 'replacement' and 'probiotic' therapy (43)

Replacement therapy	**Probiotic therapy**
Effector strain is applied directly on the site of infection	Generally used as dietary supplements
Colonization of the site by the effector strain is essential	Able to exert beneficial effect without permanently colonizing the site
Involves dramatic and long term change in the indigenous microbiota	Rarely a dramatic and long term microbiological change
Directed as displacing or preventing colonization of a pathogen	
Has a minimal immunological impact	Exerts beneficial effects by influencing the immune system

2.4.2.1. Probiotic Products

Probiotics are provided in products in one of four basic ways (44):

- As a culture concentrate added to a beverage or food such as fruit juice.
- Inoculated into prebiotic fibres.
- Inoculated into a milk-based dairy product such as milk, milk drink, yogurt, yogurt drink, cheese, kefir and bio-drink.
- As concentrated and dried cells packaged as dietary supplements such as nondairy products such as powder, capsule, lozenge and gelatine tablets.

Table 3. Probiotic products in the world. (44)

Strain	Carrier	Country produced
Bifidobacterium bifidum	Infant formula	Turkey
B. animalis DN- 173010	Yogurt	France
B. infantis 35624	Dietary supplement	Japan
B. breve	Drink	Japan
B. lactis	Infant formula	Israel
	Research	Switzerland
	Drink	South Africa, Chile
B. longum	Infant formula	Turkey
B. longum SBT-2928	Milk	Japan
B. longum BB536	Milk	Japan
B. sp	Drink	UK
Lactobacillus acidophilus	Yogurt	Chile, USA
	Drink	UK
	Yogurt drink	Austria
L. acidophilus 5	Yogurt	UK
L. acidophilus 7	Drink	Austria
L. acidophilus Lat 11/83	Research	Russia
L. acidophilus NCFB 1748	Yogurt	Denmark
L. acidophilus SBT-2062	Milk	Japan
L. bulgaricus	Drink	France, Austria
L. casei DN-114 001	Drink	France, Austria
L. casei Shirota	Drink	Argentina, Australia, Belgium, Brazil, Brunei, China, Germany, France, Hong Kong, Indonesia, Japan, Korea, Luxembourg,Mexico, Netherlands, Philippines, Singapore, Taiwan, Thailand, Uruguay,
L. casei	Drink	UK, USA
	Yogurt	USA
	Kefir	USA
		USA, Austria
L. helveticus	Milk	Finland
	Drink	Iceland
L. johnsonii La1	Yogurt	Switzerland, Germany, Japan, Austria
L. lactis L1A	Yogurt	Sweden

L. plantarum	Kefir	USA
L. plantarum 299v	Fruit drink	Sweden

	Ice cream	Sweden
	Recovery drink	Sweden
	Oat mixture	Sweden
L. plantarum JI:1	Research	Sweden
L. reuteri	Infant formula	Israel
	Cheese	Spain, Portugal, Finland
	Milk	Japan, Finland
	Yogurt	USA, Finland
	Yogurt drink	UK
	Ice cream	Finland
	Fruit drink	Finland
	Lozenge	Sweden
	Straw	Sweden
L. rhamnosus ATCC53103	Yogurt	Australia, Papua New
(L. rhamnosus GG)		Guinea, Indonesia,
		Finland, Latvia, Estonia,
		Croatia, South Korea,
		Bosnia-Herzegovina,
		Slovenia, Ecuador,
		Israel, Italy, Netherlands,
		Japan, Norway,
		Switzerland
	Yogurt drink	Australia, Finland,
		Sweden, Croatia,
		Bosnia-Herzegovina,
		Slovenia, Ecuador,
		Uruguay, Netherlands,
		Taiwan, Norway
	Fruit yogurt	Finland, Sweden
	Milk	UAE, Israel, Italy
	Milk drink	Germany, Portugal,
		Japan, Iceland,
		Greenland, Spain,
		Estonia, Ireland, Israel,
		South Korea
	Fruit drink	Finland
	Cheese	Finland
	Kefir	Latvia
	Drink	Finland, Estonia,
		Sweden, Switzerland
	Buttermilk	Finland
	Whey-based drink	Finland
	Quark	Switzerland
L. rhamnosus	Drink	Finland, Sweden, Chile,
		South Africa
L. rhamnosus LB21	Yogurt	Sweden
L. rhamnosus 271	Drink	Sweden
L. salivarius U CC 118	Research	Ireland

L. rhamnosus VTTE-97800	Research	Finland
Streptococcus salivariusK12	Lozenge	New Zeland
S. thermophilus	Drink	France, Austria
	Yogurt drink	Austria
	Infant formula	Turkey
Enterococcus faecium	Yogurt	Denmark
E. faecium Fargo 688	Research	USA

2.4.2.2. Prebiotics

'Prebiotics' are generally defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, and thus effect host health. These prebiotics include inuline, fructo-oligosaccharides, galacto-oligosaccharides and lactulose (43). Regarding the effectiveness, prebiotics must resist to digestion in the upper gastrointestinal track and released in the lower track and used by beneficial microorganisms, mostly *Bifidobacteria* and *Lactobacilli*. The concept of prebiotics essentially has the same aim as probiotics, which is to improve host health with modulation of the intestinal flora, although by a different mechanism. Prebiotics may be beneficial for the probiotic, especially with regard to *Bifidobacteria*. This is known as the ' synbiotic concept'. Synbiotics are defined as 'mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract of the host'(45). However, the use of prebiotic concept in order to stimulate the putative probiotic strains to remain longer in the mouth still needs further investigation.

2.4.2.3. Mechanisms of Action of Probiotics

Although the exact mechanisms of action of probiotics are not known, several mechanisms have been proposed. The effects of probiotics can originate from three main modes of action: modulation of host defenses including the innate as well as the acquired immune system, production of antimicrobial substances against periodontopathogens and competitive exclusion mechanisms (46). *Lactobacillus* and *Bifidobacterium* species produce lactic acid, acetic acid, and propionic acid, which lower the intestinal pH and suppress the growth of various pathogenic bacteria, thereby re-establishing the balance of the gut flora. Also lactic acid bacteria produce various substances, such as hydrogen peroxide, organic acids, adhesion inhibitors, bacteriocins, and biosurfactants, which are toxic to pathogenic microorganisms. Probiotics also decrease pathogenic organisms in the urinary and intestinal tracts by competitively blocking their adhesion to the epithelium (41).

Several studies revealed the immuno-modulatory effect of probiotics and they concluded that the probiotic strains augment the immune response by stimulating the phagocytic activity of lymphocytes and macrophages. Probiotics also increase immunoglobulin A (IgA) and stimulate cytokine production by mononuclear cells.

Therefore anti-inflammatory and pro-inflammatory cytokine production is regulated (42, 46).

2.4.2.4. Safety of Probiotics

The issue of safety is of special concern during the past few years due to the increased probiotic supplementation of different food products. From the safety point of view, the putative probiotic microorganisms should not be pathogenic, should not have any growth-stimulating effects on bacteria causing diarrhea, and should not have an ability to transfer antibiotic resistance genes. The probiotics should rather be able to maintain genetic stability in oral microflora. The increased probiotic consumption leads to increased concentrations of these species in the host organism. *Lactobacillus* bacteremia is a rare entity, with highly variable clinical characteristics, ranging from asymptomatic to septic shock-like symptoms. However, it is evident that careful monitoring is needed in this regard in the future. Clinical characteristics of *Lactobacillus* bacteremia are highly variable, ranging lead bacteremia, however, especially in patients with severe underlying diseases such as diabetes, cardiovascular diseases, gastrointestinal disorders, malignancies, organ transplant patients or in immunocompromised state (39).

FAO and WHO proposed an approach to evaluate the putative probiotics. Depending on this scheme (Figure 3), the genotype and phenotype of probiotic strains should be first established. After that, assessment of safety and efficacy and functional characterization of probiotics should be performed with in vitro assays and animal studies. In vitro assays can be used to obtain knowledge of probiotic strains and mechanisms of their effects. If possible, in vitro effects should be confirmed in animal models. Then, probiotics have to be tested using standard methods in two clinical evaluations: phase 1 (safety assessment) and phase 2 (efficacy assessment) studies. If these clinical studies confirm the efficacy and the safety of a probiotic strain, then that strain can be marketed as a probiotic food. When a claim is made that a probiotic can alter a disease state, then a phase 3 study must be performed (47, 48)

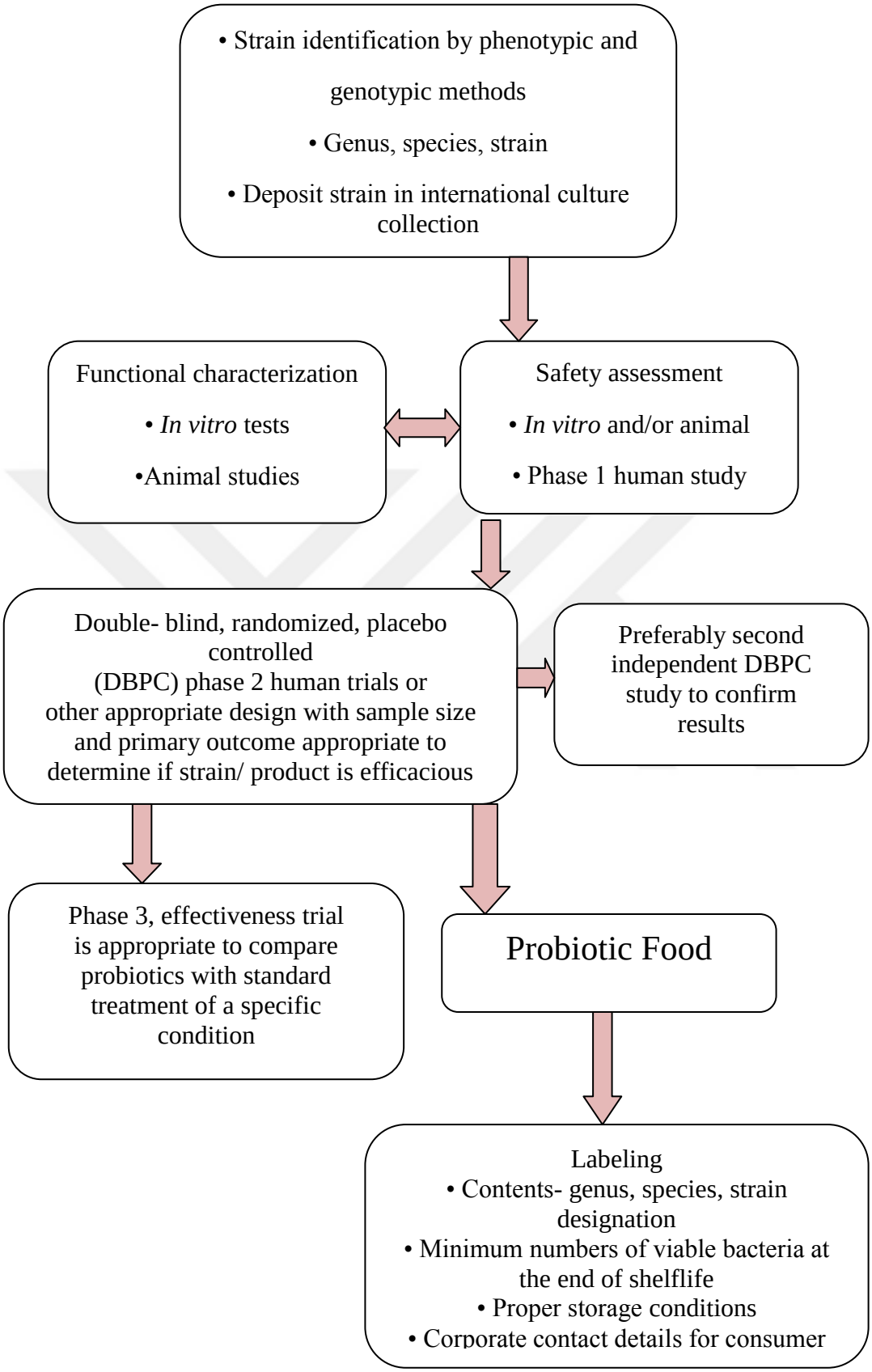

Figure 3. FAO and WHO guidelines for the evaluation of probiotics for food use (47)

2.4.2.5. Probiotics and General Health

The human body lives in a heavily contaminated bacterial environment, and the symbiosis with these microorganisms seems to be a condition for survival. The use of probiotics constitutes a purposeful attempt to modify the relationship with our immediate microbial environment in ways that may benefit general health. A number of potential indications of probiotics have been proposed, including:

- **Gastrointestinal disturbances:** Probiotics have traditionally been used to treat gastrointestinal tract**.** The most widely used species are *Bifidobacteria* and *Lactobacillus*. Probiotic bacteria containing β-galactosidase can be added to food to improve lactose maldigestion. Also probiotic bacteria prevent antibiotic-associated diarrhea, rotavirus associated diarrhea. Mechanism that may drive the protective effects are production of acids, hydrogen peroxidase, antimicrobial substances, competition for nutrients or adhesion receptors, antitoxin actions and stimulation of the immune system. Both curative and preventive effects of probiotics have been shown for ulcerative colitis, Crohn's disease and pouchitis (7).
- **Reduction the risk of colorectal cancer:** The hypothesis is based on the observation that selected *Lactobacilli* reduce the activity of certain fecal enzymes that convert pro-carcinogens into carcinogens and on some epidemiological studies which suggest that regular consumption of fermented diary products are related to a lower risk for certain types of cancer (43).
- **Treatment of urogenital infection:** The existence of *Lactobacilli* in the urogenital microbiota of healthy women, and the obliteration of *Lactobacilli* in patients who develop urinary tract infections, bacterial vaginosis and other genital infections, has led to a focus on *Lactobacilli* as potential probiotics for the prevention of urogenital disease (43).
- **Atopic disease:** Clinical effects have been seen as a significant improvement in the course of atopic eczema in infants given *Lactobacillus rhamnosus GG (LGG) or B. Lactis BB-12*. The preventive potential of *LGG* in atopic disease has been demonstrated recently. *Lactobacilli* has the ability to reverse increased intestinal permeability, enhance gut-specific IgA responses, promote gut barrier function through the restoration of normal levels of microbes, and enhance

TGF-β and IL-10 production as well as cytokines that promote the production of IgE antibodies (43).

- **Treatment of oro-pharangeal infections:** Acute otitis media, *Streptococcal* pharyngotonsillitis may be prevented by the consumption of probiotic containing products. Colonization of the esophageal side of the voice prosthesis by bacteria and yeasts causes either leakage or increased airflow resistance, which impedes fluent speech, respiration and swallowing. Therefore in several studies, buttermilk containing *Lactobacillus lactis* and *Lactococcus lactis ssp. cremoris*, and a fermented milk drink containing *L. Casei Shirota*, were used to evaluate the effectiveness of probiotics to decrease the amount of both bacteria and yeasts on voice prostheses. The studies resulted with the significant reduction of bacterial and yeast prevalance (43).
- **Immuno-modulatory effects:** In experimental animal studies it is demonstrated that probiotics have a beneficial effect on reduction of allergies, and respiratory infections (44).

There are also studies indicating the effect of probiotic consumption on the reduction in blood pressure and regulation of hypertension, serum cholesterol concentration and increased response to inflammation (44).

2.4.2.6. Probiotics and Oral Health

The bacterial biofilm that forms on all hard and soft oral tissues is considered to be the principal etiologic agent in many pathological conditions of the mouth. The accumulation of bacteria within the biofilm, facilitated by poor oral health maintenance, predisposes to allogenic shifts in the microbial community (6). Gastro-intestinal or vaginal microbiota and dental biofilms are considered to be difficult therapeutic targets, because of the encouraging effects of probiotics in different fields of health care, probiotic consumption for oral healthcare is introduced recently.

Biofilms are the communities of microorganisms that allow survival of the community as a whole, and exhibits metabolic cooperation (49). Microbial shift also defined as ‘dysbiosis’, refers that diseases are due to the decrease in the number of beneficial types or an increase in the number of pathogens. Beneficial bacteria can affect the pathogenic species in different ways and thus modify the disease process as follows: by passively occupying a niche that may be colonized by pathogens, by actively limiting a pathogen’s ability to adhere to appropriate tissue surfaces, by adversely affecting the vitality or growth of a pathogen, by affecting the ability of a

pathogen to produce virulence factors; or by degrading the virulence factors produced by the pathogen (50).

An essential requirement for a microorganism to be an oral probiotic is, its ability to adhere to and colonize the surfaces in the oral cavity and resist the defense mechanisms and oral environmental conditions. For the microorganism to be able to exert probiotic properties in the oral cavity, it is essential to adhere to saliva-coated surfaces, to colonize and grow in the mouth, and to inhibit oral pathogens. The putative probiotic species also needs to be safe for the host (6).

Studies have been investigated the pattern of adhesion of different probiotic strains to oral epithelial cells and most of the experiments on adhesion have been carried out with strains mostly used as probiotics in dairy products such as yogurt and cheese (39). *LGG* is one of the most widely studied probiotic bacterial strain. The benefits of *LGG* treatment in gastrointestinal disorders are well documented. Yli-Knuuttila et al. (51) investigated whether *LGG* can be detected in the oral cavity after discontinuation of administration of a product prepared with this bacterium. After the 14-day trial period, the occurrence of *LGG* in the oral cavity has been found to decrease gradually, which indicated that no permanent colonization had occurred and that the oral persistence of *LGG* was only temporary. A potential candidate probiotic strain, *Weissella cibaria*, is isolated from humans and animals, as well as from fermented foods. Kang et al. (52) evaluated the ability of *Weissella cibaria* isolated from the oral cavity to coaggregate with *Fusobacterium nucleatum (F. nucleatum)*, and the adhesiveness of these strains to epithelial cells In conclusion, the three *Weissella cibaria* isolates strongly coaggregated with *F. nucleatum* and efficiently adhered to the epithelial cells (52). *Lactobacilli* species have co-aggregation abilities and form a barrier that prevents colonization of pathogenic bacteria, due to the production of a micro-environment around these pathogens in which inhibiting substances were generated by *Lactobacillus* species (53).

Lactobacillus and *Bifidobacterium* strains have different commercially available probiotics in the dairy industry. Haukioja et al.(54) evaluated the oral colonization potential of these probiotic strains in vitro. Test strains demonstrated 24 hours of survival rates in saliva however they showed great variations in their binding capacity to the saliva-coated surfaces. *Lactobacilli* have been shown to have better adherence than *Bifidobacteria*. Thus, *Lactobacilli* may compete for the same binding sites on saliva coated hydroxylapatite with *F. nucleatum* which indicates that probiotics might affect

the formation of oral biofilms and modify resident microflora. With another study, same researchers defined a novel mechanism whereby *Lactobacilli* and *B. lactis* Bb12 affected the composition of salivary pellicle on hydroxyapatite and thereby inhibited *S.mutans* adherence in vitro (55).

2.4.2.7. Probiotics and Periodontal Health

The bacterial biofilm is the principal etiologic agent in many pathological conditions of the mouth. The accumulation of bacteria within the biofilm, facilitated by poor oral health maintenance, predisposes to allogenic shifts in the microbial community, leading to the onset of periodontal inflammation. The current view on the etiology of plaque-related periodontal inflammation considers three factors that determine whether disease will develop in a subject: a susceptible host; the presence of pathogenic species; and the reduction or absence of so-called 'beneficial bacteria' (46). Due to the difficulties of influencing the host response without the risk of serious side-effects periodontal therapy especially envisages the reduction of the bacterial threat. The worldwide treatment strategy applied for periodontal disease is based on mechanical subgingival debridement, in combination with improved oral hygiene. This shifts the subgingival flora to a less pathogenic composition, characterized by high proportions of gram-positive aerobic species. Although reductions in the total subgingival microbiota of up to two-log values can easily be achieved, a re-colonization, primarily by less pathogenic bacteria, towards baseline numbers occurs within 1–2 weeks. This shift is only temporary, with the re-establishment of a more aggressive microbiota within weeks to months (23, 56) The dynamics of this re-colonization depends on the level of oral hygiene, the efficacy of the subgingival debridement and the residual PD (57). The use of antibiotics or antiseptics, either locally or systemically, does not really improve the long-term effect of periodontal therapy. Therefore, some authors start to focus on the third etiological factor for plaque-related periodontal inflammation, namely; the reduction or absence of so-called 'beneficial bacteria' (46, 58).

In recent years, the researchers have been investigated the use of probiotics as an adjunct to SRP (6, 43, 46). Probiotic microorganisms do not act exclusively by affecting the microbiota. They can also exert effects either by modulating immunological parameters, epithelial permeability and bacterial translocation, or by providing bioactive or regulatory metabolites. The latter effects are appealing for periodontal healthcare because current evidence shows that the destruction of the periodontium is substantially mediated by the host and driven by the bacterial challenge. Therefore, probiotics might

not only suppress the emergence of endogenous pathogens or prevent the superinfection with exogenous pathogens but also they might also protect us through the promotion of a beneficial host response (43, 46).

Probiotic bacteria and their metabolites can be recognized by epithelial and immune cells of the host. Several publications indicated that, some *Streptococci*, such as *Streptococcus cristatus, Streptococcus salivarius, Streptococcus mitis* and *Streptococcus sanguinis* are able to reduce the IL-8 response triggered by periodontopathogens as *F. nucleatum* and *A.a.* on epithelial cells. Della Riccia et al. (59) analyzed the anti-inflammatory effects of *Lactobacillus brevis* extracts on periodontitis patients. The use of the probiotic bacteria led to a significant decrease in inflammatory markers in the saliva such as MMP and nitric oxide synthase activity (NOS), PGE_2 and interferon gamma (IFN-∂) levels. With the production of antimicrobial agents such as lactic acid, hydrogen peroxide, bacteriocin and bacteriocin-like inhibitory substances, probiotics can inhibit bacterial proliferation (60). Homofermentative *Lactobacilli* produce higher concentration of lactic acid in comparison with heterofermentative *lactobacilli,* therefore they inhibit the growth of *Pg*, *Pi* and *A.a.* With the production of hydrogen peroxide, probiotics inhibit the growth of pathogenic species. *S. salivarius* produces two potent bacteriocins, the cationic peptides with a narrow spectrum of antimicrobial activity, salivaricin types A and B. This strain has been used to prevent dental caries caused by *Streptococcus sobrinus* and *Streprococcus mutans*. Salivaricin B was found to be effective to treat halitosis caused by *Prevotella* spp. and *Micromonas micra* (61, 62). Probiotics has another mechanism of actions, 'The competitive exclusion' principle shows that two species that compete for the same resources can not stably exist. One of the two competitors will have a slight advantage over another that leads to extinction of the second competitor or a shift of this species to another niche. The competitive exclusion mechanism can be also used by beneficial bacteria, by preventing the adhesion of pathogenic bacteria as well as competing for the same nutrients. Several bacterial strains, mostly *Streptococci* can impede the colonization of periodontopathogens to hard and soft tissue surfaces by production of bio-surfactants that prevent adhesion. On the other hand, probiotics have been shown to inhibit adhesion by modifying the protein composition of the binding site. The mechanisms of probiotic species might positively affect periodontal health are demonstrated in Figure 4 (6).

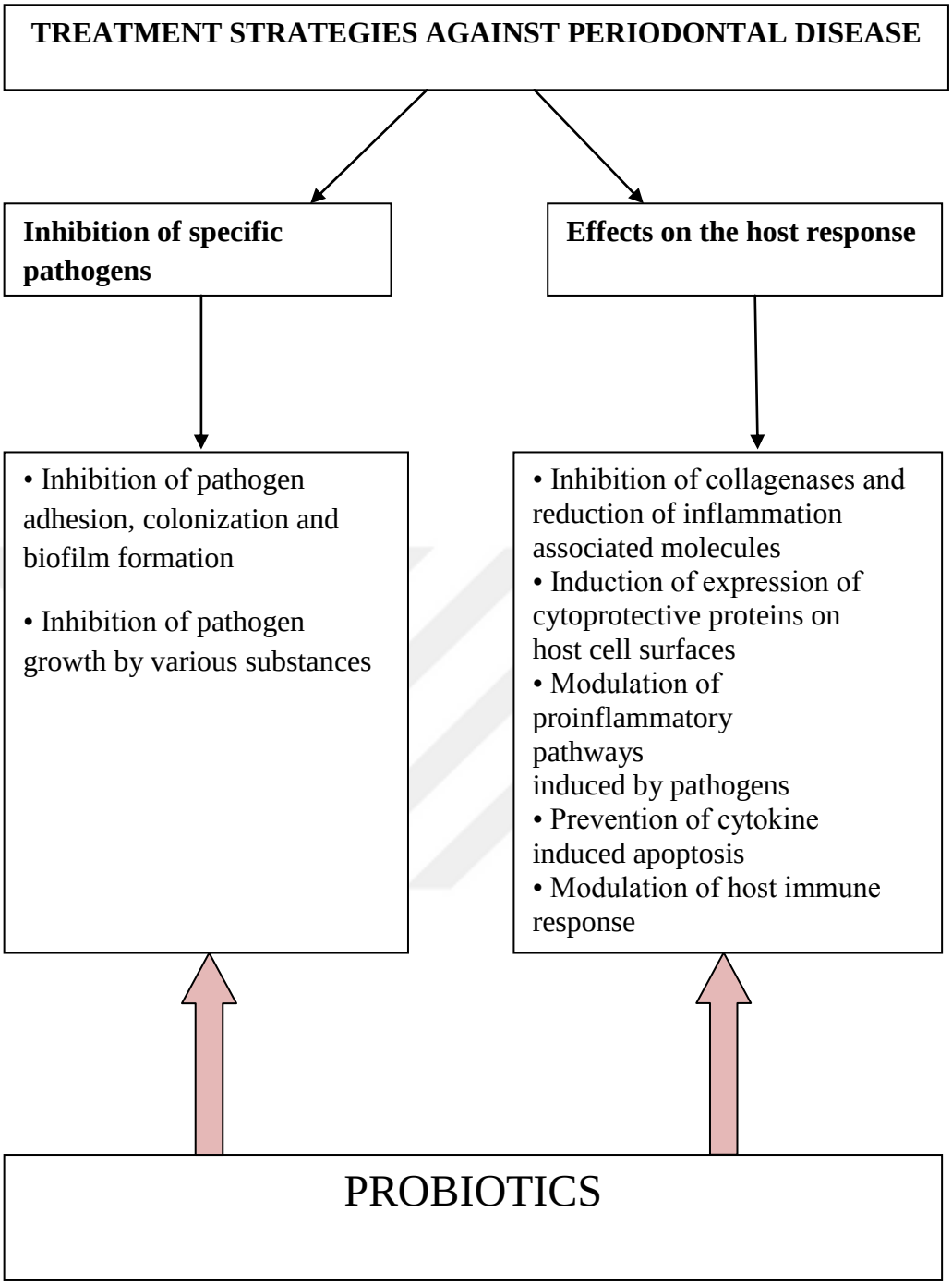

Figure 4. Theoretical possibilities for probiotics to affect periodontal health (6)

In 1954, a beneficial effect of lactic acid bacteria on inflammatory infections of the oral mucosa was reported (43). In 1990s, the use of a Russian probiotic preparation called 'Acilact', a complex of five live lyophilized lactic acid bacteria, with or without *Bifidumbacterin* is claimed that it improves both clinical and microbiological parameters in patients with gingivitis and mild periodontitis (43). Recently, a periodontal dressing consisting of collagen and *L. casei* 37 was reported to exert a beneficial effect on the subgingival microbiota of periodontal pockets (43, 63, 64).

The first well-substantiated and large-scale research effort on the applicability of probiotics in periodontitis was initiated in the late 1970s by Socransky's group. This group of researchers found that subgingival plaque samples of healthy patients contained organisms that could inhibit the growth of *A.a.* and other periodonto-pathogens. Since the beginning of the 21st century, the beneficial oral microbiota and their use in the prevention and treatment of plaque related periodontal inflammation has undergone a revival. In Japan, a *L. salivarius* strain is currently being investigated regarding its potential to suppress periodontopathogens and improve periodontal health. *L. salivarius* is an obligatory homofermentative *Lactobacillus* that is far less aciduric than *L. acidophilus*, which has a possible role in caries development. Ishikawa et al. (66) in this study searched for the inhibiton of *Pg, Pi* and *P. nigrescens* after 6-12 hours in coculture with *L. salivarius* TI 2711 in vitro and also in vivo. 76 patients were included and no pretreatment was performed. Two test groups received *L. salivarius* TI 2711 in tablets either $2x\ 10^7$ cfu/day or $1x\ 10^8$ cfu/day, five times a day for 8 weeks. the number of black pigmented anaerobic rods were significantly reduced, but there was not any significant change in total number of bacteria, in saliva *Streptococcus mutans* or *Lactobacilli* detected.

Krasse et al. (67) assessed the effect of *L. reuteri* in the treatment of gingivitis. 59 patients with moderate to severe gingivitis were included in the studyand given one of two different *L. reuteri* containing gums (LR-1 or LR-2) at a dose of 2×10^8 cfu per day, or a corresponding placebo. At baseline and at day 14, PI, GI were measured and saliva samples were collected. Gingival and plaque scores were significantly reduced in probiotic group but there was no change observed in placebo group. The authors stated, *L. reuteri* is efficacious in reducing gingivitis and plaque scores even though the differences were rather small (67).

Teughels et al. (46) performed series of studies in vivo and in vitro to identify beneficial bacteria that can retard and prevent periodonto-pathogen re-colonization after

SRP. *S. sanguinis* KTH-4, *S. salivarius* TOVE and *S. mitis* BMS were found to be the bacterial species that were most effective in inhibiting *in vitro* periodontopathogen colonization. Even though the application of beneficial bacteria did not exclude pathogen recolonization, it delayed the recolonization process. Nackaerts et al. (68) evaluated the impact of replacement therapy by monitoring bone density changes and alveolar bone level in periodontal pockets in a dog model and resulted study indicates the potential effect of a subgingival application of beneficial species in periodontal pockets.

Shimauchi et al. (69) investigated the effect of probiotic intervention using *Lactobacilli* on the periodontal condition of patients without severe periodontitis. Freeze-dried *L. salivarius WB21 (WB21)*- containing tablets or a placebo were given to volunteers in the study. 66 volunteers were randomly assigned to receive probiotic tablets with xylitol or xylitol alone three times a day for 8 weeks. Periodontal clinical parameters were measured and whole saliva samples were obtained at baseline , at week 4, and at week 8. Salivary lactoferrin levels were measured by ELISA. *Lactobacilli* in saliva and plaque samples was detected by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) using 16S rRNA primers. Periodontal clinical parameters were improved in both groups after an 8-week intervention. Current smokers in the test group showed a significantly greater improvement of PI and PD from baseline when compared with those in the placebo group. Salivary *Lactoferrin* level was also significantly decreased in the test group smokers. Also they reported a significant decrease on the numerical sum of the five selected periodontopathogenic bacteria including *A.a, Pi, Pg, Treponema denticola, and Tannerella forsythia.* Results showed that probiotics could be useful in the improvement/maintenance of oral health in subjects with a high risk of periodontal disease.

Della Riccia et al. (59) conducted a study to analyze the anti-inflammatory effects of *Lactobacillus brevis* extracts on periodontitis patients and to investigate the involved mechanisms in vitro on activated macrophages. 8 healthy subjects and 21 patients with CP were enrolled to analyze the effect of *L. brevis*-containing lozenges on periodontitis-associated symptoms and signs. Saliva samples, collected before and after treatment, and were analyzed for MMP and NOS activity, IgA, PGE_2 and c-interferon (IFN-c) levels. The treatment resulted in significant decrease of all clinical parameters. This was paralleled to a significant decrease of nitrite/nitrate, PGE_2, MMP, and IFN-c

levels in saliva samples. Authors stated that, probiotic treatment suggest innovative, simple and efficacious therapeutical approach of periodontal disease.

The aim of this study was to determine the effect of a probiotic milk containing which contained *Lactobacillus casei* strain Shirota drink on gingival health and the development of experimental gingivitis, Staab et al. (70) administered a probiotic drink once a day for the test group and, the control group did not receive any product to drink. After 8 weeks, individual mechanical plaque control was interrupted for 96 h. The clinical measurements were performed at baseline, at week 8 and again 96 h later. At the same time points, GCF had been collected for analysis of PMN elastase, myeloperoxidase (MPO) and MMP-3. Clinical indices were not different between the groups. In the test group, elastase activity and MMP-3 amount were significantly lower after the intake of the probiotic milk drink. There was a significant increase of MPO activity in the control group. This study suggested a beneficial effect of the probiotic mil drink on gingival inflammation.

Twetman et al. (71) investigated the effect of a chewing gum containing probiotic bacteria on gingival inflammation and the levels of IL-1b, TNF-a, IL-6, IL-8 and IL-10 in GCF. The chewing gums contained two strains of *L. reuteri*: ATCC 55730 and ATCC PTA 5289. The subjects were instructed to chew the gums for 10 min over the course of 2 weeks. BoP and GCF sampling were conducted at baseline and at week 1, 2 and 4. BoP improved and GCF volume decreased in all groups during the chewing period. The levels of TNF-α and IL-8 decreased significantly in probiotic groups compared with baseline and at week 1 and 2, respectively. A non-significant decreasing tendency was also observed concerning IL-1β during the chewing period. The levels of IL-6 and IL-10 were unaffected in all groups at week 1 and 2. This study concluded that, probiotic treatment could be a valuable approach for combating inflammation in the oral cavity by reduction of pro-inflammatory cytokines in GCF.

L. reuteri is an obligate heterofermentative bacteria in the gastrointestinal tracts of humans, and it is reported to produce various compounds such as reuterin and reutericyclin, which are water soluble, broad-spectrum antimicrobials, effective over a wide pH range, and resistant to proteolytic and lipolytic enzymes (72, 73).

Iniesta et al. (74) conducted a study to investigate the effects of an orally administered probiotic on the oral microbiota. 40 gingivitis subjects received daily oral tablets either containing *L. reuteri* or placebo. Unstimulated saliva and subgingival samples were collected and analysed by culture and PCR. Clinical and microbiological

outcome variables were compared between and within groups. There were no significant changes between and within the groups in the clinical variables. In saliva, total anaerobic counts after 4 weeks and counts of *P.i.* after 8 weeks, showed reductions in the test group. In subgingival samples, significant reductions in the changes baseline to 4 weeks were observed for *Pg* counts. With PCR, *L. reuteri* ATCC-PTA-5289 was more frequently detected than *L. reuteri* DSM-17938. The effect of *L. reuteri* administered in tablets resulted in a reduction in the number of selected periodontal pathogens in the subgingival microbiota, without an associated clinical impact.

A survey of the literature so far reveals that there are four studies evaluating the efficacy of probiotic lozenges used as an adjunct to initial periodontal treatment and they are included in the present thesis. The first one is conducted by Vivekananda et al. (75). The aim of this study was to evaluate the effects of *L. reuteri* (Probiotic) alone and in combination with SRP in CP patients. The study period was 42 days. 'Split-mouth' design was used for the SRP, which was performed on day 0; two quadrants were treated with SRP whereas the remaining two quadrants were left untreated. The participants received OHI. *L. reuteri* containing probiotic lozenges or the corresponding placebo lozenges were taken twice daily from day 21 to day 42. PI, GI, GBI, PD, CAL and microbiological levels of the pathogens *A.a, Pg*, and *Pi*. Assessments were made on day 0 before SRP treatment, on day 21 before administration of the lozenges, and on day 42. At day 42, the PI, GI were significantly reduced by all treatment modalities. For PD and CAL, the best result was obtained with the SRP + Probiotic treatment. Probiotic, either alone or following SRP, reduced *A.a, Pi*, and *Pg*. The SRP placebo combination did not significantly affect the levels of the pathogens. *L. reuteri* containing lozenges can be recommended during non-surgical therapy and the maintenance phase of periodontal treatment. Considering the beneficial effects of probiotics, this therapy could serve as a useful adjunct or alternative to periodontal treatment when SRP might be contraindicated.

Teughels et al. (76) aimed to evaluate the effects of *L. reuteri*-containing probiotic lozenges as an adjunct to SRP. 30 CP patients and monitored clinically and microbiologically at baseline, 3, 6, 9 and 12 weeks after therapy. All patients received one-stage full-mouth disinfection (OSFMD) and the lozenges either probiotic containing or only xylitol containing were used twice a day for 12 weeks. At week 12, all clinical parameters were significantly reduced in both groups, while there was significantly more PD reduction and attachment gain (AG) in moderate and deep

pockets, more Pg. reduction was observed in the SRP + probiotic group. This study indicated the oral administration of *L. reuteri* containing lozenges could be a useful adjunct to SRP in CP.

İnce et al. (77) included 30 CP patients in their study and divided into 2 groups. Test group received SRP and probiotic containing lozenges. Control group received SRP and placebo lozenges. PI, GI, BoP, PD and attachment gain were measured. GCF was sampled for the analysis of MMP-8 and TIMP-1 with ELISA. All evaluations were performed at baseline and on days, 21, 90, 180 and 360. Intergroup comparisons of PI, GI, BoP, and PD was found to be significant in favor of the test group at all time points. Decreased GCF MMP-8 levels and increased TIMP-1 levels were found to be significant up to day 180. Mean values of attachment gain were significantly higher in test group compared with control group on days 90, 180 and 360. *L. reuteri* containing lozenges may be a useful supplement in moderately deep pockets of CP patients. Low MMP-8 and high TIMP-1 levels may indicate the role of the lozenges in reduction of inflammation-associated markers up to day 180.

To evaluate the effects of lozenges containing *L. reuteri* as an adjuvant treatment to initial periodontal therapy for CP patients and to detect the level of *L. reuteri* colonisation in the periodontal pockets of treated patients Tekçe et al. (78) conducted a study with 40 patients. One group received SRP plus *L. reuteri*-containing lozenges, and control group received SRP plus placebo. PI, GI, BoP, PD, and RAL were measured. Microbiological sampling was performed at baseline and on days 21, 90, 180 and 360 and were analysed by culturing. After treatment, the measured PI, GI, BoP, and PD as well as the total viable counts (TVC) and the proportions of obligate anaerobes were significantly lower in test group compared with control group at all time points. Similar observations were made for the TVC counts and the proportions of obligate anaerobes with the exception of the day 360. *L. reuteri*-containing lozenges may be a useful adjuvant agent to slow re-colonization and improve clinical outcomes of CP. Further studies are needed to clarify the optimal dose of the lozenges.

Vicario et al. (79) conducted a study to assess the clinical effect of the administration of *L. reuteri* containing lozenges as a probiotic agent in the treatment of initial to moderate CP and potential side-effects of the probiotic agent. Twenty systemically healthy, non-smoking subjects with initial-to-moderate CP were enrolled in this 1-month clinical trial. Subjects were randomly assigned to receive tablets containing *L. reuteri* lozenges or placebo once a day for 30 days. Clinical parameters

were collected at baseline and 30 days of post-treatment. Periodontal clinical parameters were improved in the test group after a 30-day intervention. The test group demonstrated a statistically significant reduction in all the periodontal parameters included in the study while the control group treated with placebo did not show any statistically significant change in periodontal parameters. These data indicated that oral administration of *L. reuteri* containing lozenges improved the short-term clinical outcomes in non-smoking patients with initial-to-moderate CP.

2.5. Gingival Crevicular Fluid

GCF is an inflammatory exudate, emerges between the surface of tooth and the epithelial integument, contains a complex mixture of substances derived from serum, leukocytes, structural cells of the periodontium and oral bacteria. These substances possess a great potential for serving as indicators of periodontal disease and healing after therapy (80, 81).

Several techniques have been employed for the collection of GCF and the technique chosen will depend upon the objectives of the study as each technique has advantages and disadvantages. The techniques can be divided into three basic strategies (80);

1. Gingival washing methods: In this technique, the gingival crevice is perfused with an isotonic solution, usually of fixed volume. The fluid collected then represents a dilution of crevicular fluid and contains both cells and soluble constituents such as plasma proteins.

2. Capillary tubing or micropipettes: After isolation and drying of a site, capillary tubes of known internal diameter are inserted into the entrance of the gingival crevice. GCF from the crevice migrates into the tube by capillary action and the volume of fluid collected can be accurately determined by measuring the distance, which the GCF has migrated. This technique appears to be ideal as it provides an undiluted sample of native GCF whose volume can be accurately assessed. However, it is difficult to collect an adequate volume of GCF in a short period, unless the sites are inflamed and contain large volumes of GCF.

3. Absorbent filter paper strips: The methods of collection with the absorbent filter paper strips, may be broadly divided into the intra-crevicular and the extra-crevicular techniques. The intra-crevicular method is the most frequently used method and can be further sub-divided depending upon whether the strip is inserted just at the entrance of the crevice or periodontal pocket or whether the strip is inserted to the base

of the pocket or until minimum resistance is felt. The principal variations of this technique are not only the method and timing of sample collection, but also the means of estimating the volume of sample collected. The advantages of the technique are that it is quick and easy to use, can be applied to individual sites and, possibly, is the least traumatic when correctly used (82).

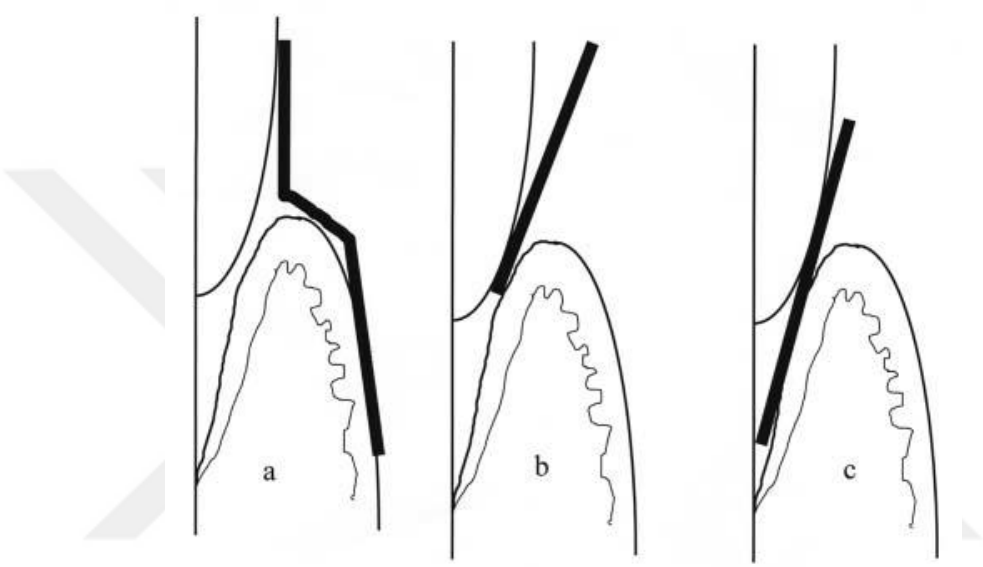

Figure 5. (a) extra-crevicular method; (b) intra-crevicular method 'superficial', (c) intra-crevicular method 'deep' (80)

GCF is a mixture of molecules originating from the blood, host tissues and subgingival plaque including; electrolytes, small organic molecules, proteins, cytokines, specific antibodies, bacterial antigens and enzymes of both host and bacterial origin (83). While the periodontium is clinically healthy, early colonizing bacteria are confined to the gingival margin. In this state, the flow of GCF is relatively low and the fluid itself composed mainly of a transudate of plasma proteins. However, 2–4 days after plaque accumulation the initial lesion of gingivitis commences. As the disease process continues, the marginal gingiva will begin to show clinical changes indicative of inflammation concurrent with an apical advancement of the plaque front and increase in

plaque mass. One of the initial responses of the host to bacterial plaque is an increase in the vascular permeability of the subepithelial blood vessels, thus allowing the escape of plasma from the circulation, leading to edema in the gingival corium (2, 80). As this fluid escapes into the gingival crevice with the junctional epithelium, it results in increased crevicular fluid flow. Since this exudate is essentially a growth medium that supports the hosts' cells and tissues, the GCF can also act as an excellent source of nutrients for subgingival microbes and may actually contain factors that are necessary for the proliferation of some pathologic bacterial species. In addition, this growing microbial mass also releases large quantities of metabolites that diffuse throughout the dentogingival space and penetrate into the junctional epithelium and can contribute to further bacterial colonization. By the progression of periodontal disease, proteolytic activity of inflamed tissues have been identified in GCF at levels that correlate with disease activity (80, 81, 82, 83).

2.6. Extracellular Matrix

The ECM is a vital structural component of the periodontium which regulates the activities and functions of the resident cells. There are three major types of macromolecules that predominate in the ECM namely; the collagens, proteoglycans and glycoproteins (elastin, fibronectin, laminin, osteocalcin, osteopontin, bone sialoprotein, osteonectin and tenascin). These molecules are involved in numerous biological processes during development and regeneration of the periodontium (84). The break-down of collagen occurs during inflammation, tissue break-down, remodeling and tissue repair or wound healing. Bacteria present in subgingival biofilms and their products and components mediate the pathological breakdown of ECM, which is the characteristic of CP (49).

Host-bacterial interactions adjust the destruction of bone and connective tissues, including collagens, proteoglycans, and other components of the ECM. Macrophages and neutrophils release enzymes and cytokines that activate osteoclasts and osteoblasts to stimulate bone degradation with the initiation of host-inflammattory process induced by a microbial biofilm (85). Various bone-associated by-products such as glycosaminoglycans and pyridinoline cross-links traverse the tissue and exit through the periodontal pocket and can be measured in the GCF (Figure 6).

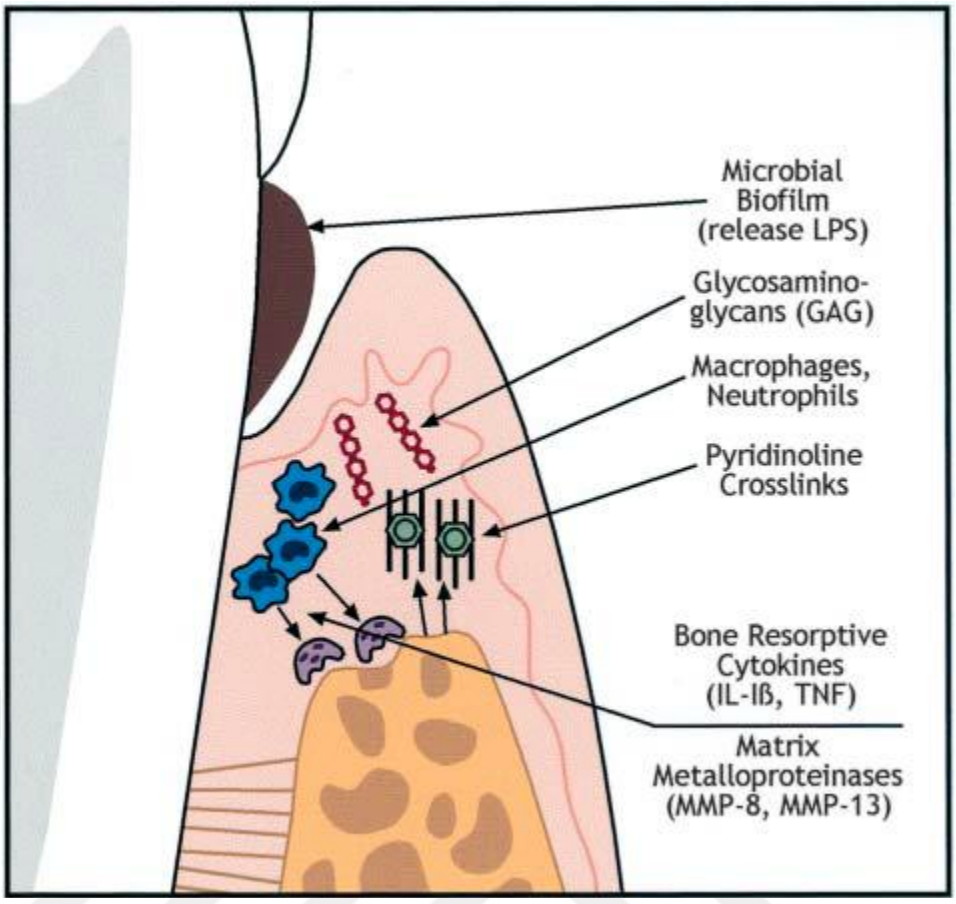

Figure 6. Release of mediators of alveolar bone resorption during active periodontitis (85)

The breakdown of collagen can occur by two different pathways: an intra-cellular and an extra-cellular route (86, 87). Under non-pathological conditions, phagocytosis and intracellular digestion of collagen fibrils is a process observed at a high level in dynamic soft connective tissues such as gingiva and periodontal ligament. During pathological conditions of periodontal disease, the balance between synthesis and degradation is disrupted. In this pathological condition, collagen degradation is likely to occur via four distinct pathways. Matrix components may be dissolved by extra-cellular MMP-dependent or plasmin (Pln)-dependent cleavage reactions, and that larger fragments of matrix may be disposed of by a phagocytic pathway by way of cleavage by lysosomal proteinases. Mineralized matrices appear to be degraded by a complex extra/pericellular process mediated by osteoclasts (osteoclastic pathway)

which relies on degradation by lysosomal proteinases in a narrow pericellular compartment (86).

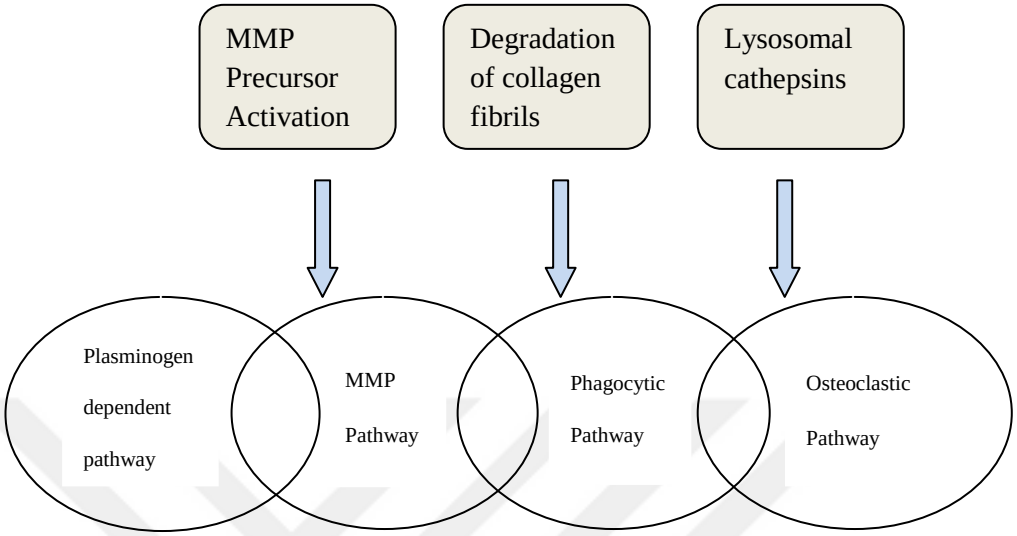

Figure 7. Pathways for degradation of the ECM (86)

2.7. Matrix Metalloproteinases

MMPs are members of a large subfamily of zinc- and calcium- dependent proteolytic enzymes (proteinases), responsible for remodeling and degradation of ECM macromolecules, including interstitial and basement membrane collagens, fibronectin, elastin, laminin and the proteoglycan core protein (88). These potent enzymes are also called ‘matrixin’, secreted as a pro-enzyme form and requires extra-cellular activation. They are mainly but not exclusively synthesized by connective tissue cells. MMPs can also be synthesized by hemopoietic cells, including monocytes and macrophages, keratinocytes, endothelial cells and many types of tumors (88). MMPs play an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction, tissue remodeling and wound healing. They also participate in many pathological processes such as arthritis, osteoporosis, cancer and cardiovascular disease (87, 88).

The MMP family has major subgroups based on the substrate specificity, sequence similarity, and domain organisation (88):

1. The interstitial collagenases
- Collagenase-1 (fibroblast type collagenase; MMP-1)
- Collagenase-2 (neutrophil type collagenase; MMP-8)
- Collagenase-3 (MMP-13)

2. The gelatinases (type IV collagenases)
- MMP-2
- MMP-9

3. The stromelysins
- MMP-3
- MMP-10
- MMP-11

4. The membrane-bound group
- MMP-14
- MMP-15
- MMP-16
- MMP-17

5. Matrilysin
- MMP-7

6. Metalloelastase
- MMP-12

Regulation of MMPs is exerted at many levels and involves both transcriptional and pos-ttranscriptional mechanism. Many different factors have been shown to influence the transcription of MMPs, including active proenzymes, cytokines, growth factors and hormones. Mainly MMP activation against ECM substrates is regulated at 4 "gates": 1) by transcriptional regulation of MMP genes; 2) by precursor activation; 3) by differences in substrate specificity; and 4) by MMP inhibitors (endogenous inhibitors, α2–macroglobulins and TIMPs) (86, 89).

Collagenase activity in GCF in periodontally healthy and diseased patients have been demonstrated by several studies. Interstitial collagenase activities in gingival tissue extracts and GCF from periodontitis sites are pathologically elevated when compared to the periodontally healthy gingival tissue and GCF whereas TIMP levels are measured only in healthy individuals or in clinically healthy sites. The initial split of gingival and

periodontal ligament collagens is a key feature of progressive and active periodontitis lesions or pockets, and this initial cleavage is carried out by the host cell-derived interstitial collagenases (90).

2.7.1. Matrix Metalloproteinase-8

MMP-8 also referred to as neutrophil collagenase and collagenase-2 is stored intra-cellularly as a latent proenzyme in the specific granules of Polymorphonuclear leukocytes **(**PMNs). PMNs play an essential role in phagocytosis and also have a high capacity to infiltrate connective tissue. MMP-8 is expressed in a latent, non-active form and it is activated by reactive oxygen species and other proteolytic enzymes such as cathepsin G, IL-1 and IL-8, TNF-α, MMP-3 and MMP-10. Active MMP-8 (aMMP-8) derived from neutrophils is known to be the main host cell-derived collagenase leading to periodontal tissue destruction as a result of the degradation of gingival and periodontal ligament collagen (91). MMP-8 is one of the main biomarkers responsible for the connective tissue breakdown in GCF, saliva and inflamed gingiva in CP and it has been correlated with the severity of periodontal disease (86, 92, 93, 94, 95).

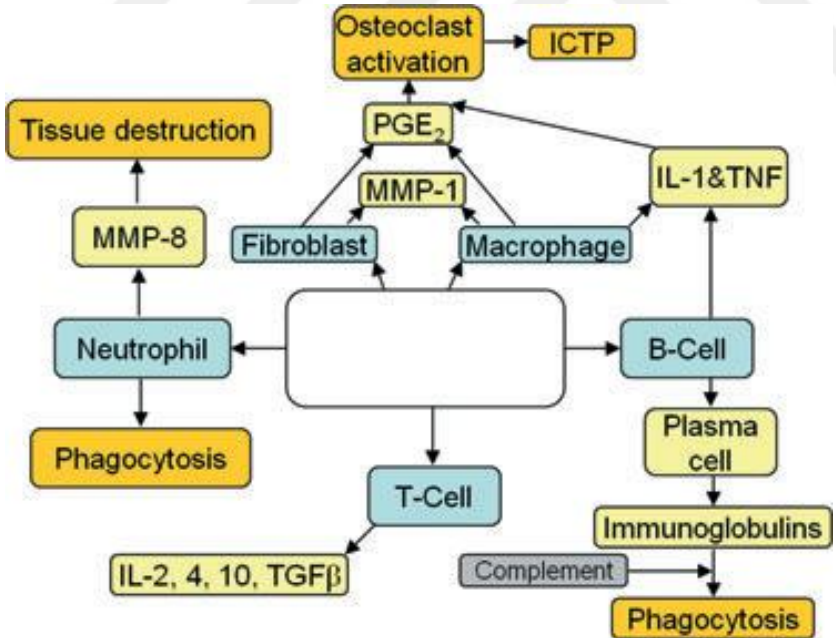

Figure 8. Biomarkers for periodontal disease and their involvement in its pathogenesis (95)

2.7.2. Tissue Inhibitor Metalloproteinase-1

Even though MMP activity can be inhibited by the plasma proteinase inhibitor α2-macroglobulin, the major group of inhibitors of the MMPs in tissues are the TIMPs. TIMPs are important modulating factors in the actions of MMPs, and tissue destruction in disease processes often correlates with an imbalance of MMPs over TIMPs (96). Four types of TIMPs (TIMP-1, TIPM-2, TIMP-3, TIMP-4) have been identified, and their expression is regulated during development and tissue remodelling. Among those, TIMP-1 and TIMP-2, which have inhibitory effects on all MMPs, are found in periodontal lesions. TIMP-2 shows strong inhibition against PMN-derived MMPs, whereas TIMP-1 shows greater inhibition against fibroblast-derived MMPs (88, 97). TIMP-1 is a 30-kDa glycoprotein that is synthesized and secreted by most connective tissue cells as well as by macrophages; it can be identified in most body fluids. Human melanoma cells secrete a 21-kDa unglycosylated protein, TIMP-2, which has similar inhibitory properties to TIMP-1, with a related but different sequence (98). TIMP-1 regulates both MMP activity and function. It inhibits MMP-1, -2, -3, -8, -9 and -13. TIMP-1 forms high affinity complexes with the active forms of MMPs. Other biological functions that are distinct from MMP inhibition are erythroid potentiating activity and antiapoptotic activity. TIMP-2 is less abundant, and has the interesting property of binding to the proform of gelatinase A and is involved in controlling its activation.The third member of the family, TIMP-3, was first isolated from chicken cells and has recently been cloned from human and mouse sources (88, 98). Although, TIMP-3 is considerably different in sequence, still is an efficient MMP inhibitor. It also has the unusual property of being found almost exclusively bound to the ECM. A sequence for TIMP-4 has recently been identified (88).

The expression of MMPs and TIMPs by cells is specific to cell type and many of them are produced by monocytes/macrophages. Their production in inflammatory situations is therefore part of the chain of events leading to tissue degradation. Under pathological conditions associated with unbalanced MMP activities, changes of TIMP levels are considered to be important because they directly affect the level of MMP activity.

Passoja et al. (91) aimed to analyse the association between MMP-8 concentration in shallow, mostly non-bleeding gingival crevices, and the extent of periodontal disease. 48 patients with CP were included in the study and PI, BoP, PD and AL were assessed clinically. MMP-8 concentrations in GCF from four shallow (PD of

>3 mm), and four diseased sites and in serum, were measured by ELISA. The mean concentration of MMP-8 in GCF from shallow crevices was 11.8±12.8 ng/ml and from diseased sites was 150.1±91.8 ng/ml. In subjects with moderate to high plaque scores, a statistically significant association was found between MMP-8 concentration from shallow crevices and the extent of 4-6 mm attachment loss. Results of this study demonstrated that, the association between MMP-8 concentration in shallow crevices and attachment loss provides a new aspect to future studies of MMP-8 as a prognostic marker for periodontal disease.

Kraft-Neumarker et al. (92) aimed to investigate if there was a relationship between clinical diagnostic parameters and the concentration of aMMP-8 in GCF in a site-level fullmouth analysis. Clinical measurements (PD, BOP, PI and GI) as well as samples of GCF, were obtained from four sites of each tooth of 9 healthy female patients with CP. The aMMP-8 concentration in GCF was quantified by ELISA using specific monoclonal antibodies. Multiple linear regression models for the single measures of aMMP-8 and PD were calculated with GI and BOP as additional variables. Mean values of between 31.5 and 88.8% were calculated for PD of ≥ 4 mm. Mean PDs ranged from 3.11 to 4.73 mm, the mean BOP values ranged from 34.0 to 96.7% and the mean full-mouth GCF aMMP-8 concentration ranged from 3.2 to 23.7 ng/mL. As a result of this study the researchers concluded; there was a broad range of intra individual and inter individual aMMP-8 values were detected within the samples of female CP patients. Although the explained variance was rather weak, a statistically significant relationship between aMMP-8 and PD was detected.

Mouzakiti et al. (99) evaluated the mRNA expression of MMP-1, -3, -8, -9 and -13 and TIMP-1 in CP before and after initial periodontal treatment. 90 gingival samples were harvested from 30 patients with CP (15 non-smokers and 15 smokers) before and after non-surgical treatment and from 30 periodontally healthy control subjects (15 non-smokers and 15 smokers). Gingival tissue samples were harvested during extraction of the periodontally hopeless tooth before the periodontal treatment, while the second sample was harvested from another area during pocket elimination surgery. Clinical parameters were assessed before and after treatment. The total RNA was isolated, and mRNA expression of MMPs and TIMP-1 was assessed by RT-PCR. Periodontal treatment significantly increased TIMP-1 expression and decreased the ratios of MMPs/TIMP-1. At the end of the study authors concluded that, post-treatment MMP-8 expression was found to be significantly higher in non-smokers with periodontitis than

in healthy non-smokers, whereas MMP-13 expression was detected higher in smokers with periodontitis than in healthy smokers.

Konopka et al. (55) investigtated the influence of SRP on amounts of IL-1β, IL-8 and MMP-8 in GCF of CP patients. The study population consisted of 30 patients with generalized advanced CP and 21 periodontally healthy subjects as control group. The amounts of IL-1β, IL-8 and MMP-8 in GCF were measured by ELISA at baseline and at week 1 and 4 after SRP. The amounts of these humoral factors were significantly lower in the control group at baseline. SRP led to improvement in all examined clinical parameters, apart from clinical attachment loss. Periodontal treatment also resulted in a significant decrease in the amounts of IL-1β, IL-8 and MMP-8 in comparison to baseline, especially 4 week after SRP. However, they were still higher than those in control group. At the end of the 4 week follow-up period, no significant correlations were found between clinical parameters and amounts of humoral factors after therapy. their observations indicated that short-term nonsurgical therapy resulted in a significant improvement in periodontal indices and in a marked decrease of IL-1 β, IL-8 and MMP-8 GCF levels.

Soder et al. (100) aimed to determine the levels of granulocyte elastase, MMP-8 and PGE_2 in GCF in smokers and non-smokers with persistent periodontitis. They took GCF samples from 70 matched sites in 29 periodontitis and 6 gingivitis sites in 34 subjects, 17 smokers, and 17 non-smokers. They also analyzed separately GCF from 28 of these subjects, 14 smokers and 14 non-smokers in 14 matched periodontitis sites. MMP-8 measurements were conducted with ELISA, functional elastase with a chromogenic substrate, and PGE_2 with radioimmunoassay (125I RIA). The significance of the findings was determined by Mann-Whitney U test. In the 29 matched periodontitis sites, smokers had significantly more functional elastase than non-smokers. In the 14 matched periodontitis sites in 14 smokers and 14 non-smokers, the former had significantly more functional elastase than the latter. A positive correlation between levels of functional elastase and MMP-8 in non-smokers were found. Granulocyte function seems to be impaired in smokers with persistent periodontitis. The cells react to the bacterial challenge by releasing serine proteases, which reflect the degradation of connective tissue. Therefore the authors conducted that the risk of progression of the disease is higher in smokers with persistent periodontitis than in non-smokers.

All these studies indicated that, elevated GCF MMP-8 concentrations shows sites at risk as well as poor response to SRP. MMPs and TIMPs expression is regulated by cytokines, particularly IL-1, hormones and GF. MMPs are produced by fibroblasts, epithelial cells, PMNs, macrophages and their production in inflammatory situations is a part of the events leading to tissue destruction. In CP patients, the imbalance between MMPs and their inhibitors lead to pathological breakdown of ECM (90, 101). Therefore MMPs are providing evidence for its involvement in tissue destruction and suggesting a role in monitoring disease activity.

To the best of our knowledge, there is no study in the literature evaluating the clinical and biochemical effectiveness of probiotic containing lozenges when compared to SDD containing tablets as an adjunct to SRP in CP patients. From this stand point, we aimed to investigate the efficacy of Probiotic and SDD on the periodontal tissue breakdown by analyzing the levels of MMP-8 and TIMP-1 levels in the 3-month follow-up period with a randomized, double blind, placebo controlled clinical trial. The null hypotesis was that neither the clinical profile nor the concentration of selected biochemical parameters in GCF would differ between the test groups and placebo groups.

3. MATERIAL AND METHODS

3.1. Patient Selection

The patient population was consisted of 45 systemically healthy CP patients aged between 35-50 years, who needed periodontal care and referred to the Yeditepe University Faculty of Dentistry Department of Periodontology.

Patient selection criteria were as follows;

1) CP patients with presence of at least 2 teeth, having one approximal site with PD of 5-7 mm and GI of ≥ 2 in each quadrant
2) Presence of radiographically detected horizontal bone loss
3) No systemic disease
4) No use of probiotic supplements
5) No pregnancy and lactating
6) No periodontal or antimicrobial treatment within 6 months
7) No smoking
8) No adverse reactions to lactose or fermented milk products
9) No allergies to tetracycline product
10) No consumption of Ca^{+2} and Zn^{+2} products

Patients in conformity with the inclusion criteria were invited to participate in the study. A written informed consent was obtained from all participants after explaining the nature, the purpose and the implications of participating in this study (Appendix 1). No changes in the trial of design were made after approval by the local Ethics Committee.

3.2. Probiotic Containing Product Under Investigation

The probiotic lozenges consisted of *L. reuteri* ($1x10^8$ cfu) for each of the strains DSM 17938 and ATCC PTA 5289. Both the probiotic and placebo lozenges could not be discriminated from each other by shape, texture or taste. The patients were asked to suck one lozenge in the morning and one at night, after tooth brushing and were instructed not to use any probiotic containing products during the course of the study.

3.3. Doxycycline Hyclate Containing Product Under Investigation

The doxycycline hyclate tablets have 20 mg of doxycyline. The patients were asked to take one tablet 1 hour before the meal with a full glass of water twice a day and also informed not to use any Al^{+2}, Ca^{+2}, $Fe^{+2/+3}$, Mg^{+2} and Zn^{+2} containing products during the course of the study.

3.4. Sample Size Collection

The sample size was calculated for the primary outcome variable, PD reduction, based on the method described by Vivekananda et al (75). According to the results of the power analysis, a sample size of seven participants for each group would yield 80% statistical power at $\beta = 0.20$ and $\alpha = 0.05$ to detect D = 0.82 with SD 0.5.

3.5. Study groups

I. Group (SRP+ Probiotic) (Test group n=15):

Lozenges containing probiotic and sucralose were used for the test group. Each subject was instructed to place one lozenge in the mouth and allow it to dissolve without chewing, twice a day for three months after tooth brushing.

II. Group (SRP+SDD) (Test group n=15):

Tablets containing doxycycline hyclate (20 mg tablet formulation of doxycycline) were used for the test group. Each subject was instructed to place one tablet in the mouth and swallow it with a full glass of water twice a day for three months after tooth brushing.

III. Group (SRP +Placebo) (Control group n=15):

Only sucralose containing lozenges were used for the control group. Each subject was instructed to place one tablet in the mouth and allow it to dissolve without chewing, twice a day for three months after tooth brushing.

The patients returned the bottles containing probiotic or placebo lozenges and the SDD tablets at day 90 visits, to check for compliance. Each time in clinical examination, the patient was inquired in relation to general health changes, use of antiinflammatory drugs, use of mouth rinses, use of probiotic products and any adverse events that the patients have noticed (e.g. gastrointestinal disturbance or dizziness). No adverse event was reported throughout the study period.

3.6. Study Design and Randomization

The study was designed as a randomized, placebo-controlled clinical trial. Patients were randomly assigned into three treatment groups according to a computer-based randomization table (www.randomizer.org / copyright© 1997-2011 by Geoffrey C. Urbaniak and Scott Plous). 15 patients received SRP + Probiotic containing Lozenges (Prodentis®), 15 patients received SRP + SDD containing tablets (Periostat®) as test groups whereas the other 15 patients received SRP+ Placebo control group.

Table 4. Randomization Table

Group I (SRP + Probiotic)														
P 1	**P 2**	**P 3**	**P 4**	**P 5**	**P 6**	**P 7**	**P 8**	**P 9**	**P 10**	**P 11**	**P 12**	**P 13**	**P 14**	**P 15**
31	15	42	34	3	25	11	7	24	41	16	12	6	33	21
Group II (SRP + SDD)														
P 1	**P 2**	**P 3**	**P 4**	**P 5**	**P 6**	**P 7**	**P 8**	**P 9**	**P 10**	**P 11**	**P 12**	**P 13**	**P 14**	**P 15**
39	32	26	22	35	18	27	8	13	45	5	43	17	28	23
Group III (SRP + Placebo)														
P 1	**P 2**	**P 3**	**P 4**	**P 5**	**P 6**	**P 7**	**P 8**	**P 9**	**P 10**	**P 11**	**P 12**	**P 13**	**P 14**	**P 15**
30	36	19	14	10	44	40	9	4	2	37	29	1	20	38

The study design was shown in Figure 9. Every patient included in the study was given OHI one week before the experimental period. Patients were instructed to brush with a medium toothbrush in a combination with flossing and/or interdental brushing twice a day. The patients were randomly divided into SRP + Probiotic, SRP + SDD and SRP + Placebo treatment groups.

At baseline, intraoral photographs were taken and GCF samples were collected by using Periopaper®[1]. After GCF sampling, clinical indices and measurements were performed including PI and GI, PD, RAL, BoP. Both SRP + Probiotic, SRP + SDD and SRP + Placebo groups received non-surgical periodontal therapy. SRP was completed under local anesthesia using ultrasonic devices[2] and Gracey curettes[3] at two appointments with one-week interval.

[1]PerioPaper® Oraflow Inc., New York, USA

[2]Piezon® OEM built in kit. EMS, SWITZERLAND

[3]Gracey SG 5/6, 11/12, 13/14, Mini-Five SAS ¾,Hu-Friedy, USA

Occlusal adjustment was performed if it is indicated. Probiotic containing lozenges, SDD containing tablets and placebo containing lozenges were administered after first SRP appointment. At day 90 intraoral photographs were taken, GCF sampling and clinical examination were again performed. Subjects were seen for checking their oral hygiene at day 90, and a complete biochemical and clinical examination were repeated.

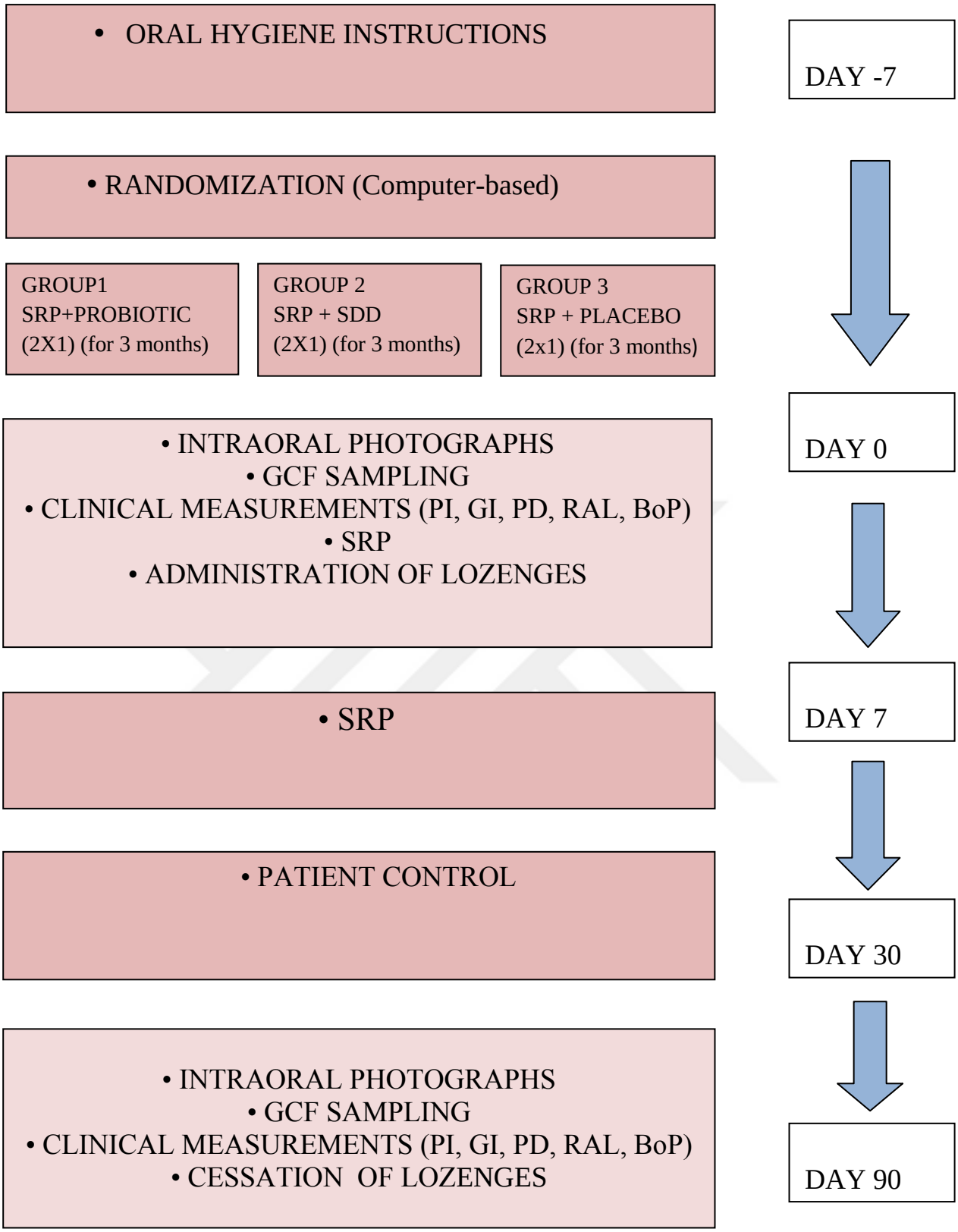

Figure 9. Study design

3.7. Clinical Indices and Measurements

All measurements were performed at baseline, and at day 90 after treatments and recorded by the same calibrated examiner using a 0.4 mm diameter 15 mm calibrated periodontal probe[4]. Individually prepared acrylic occlusal stents were used and served as the constant points in order to align the probe properly and reduce the errors associated with probe placement at different time intervals. The occlusal stent was made to cover the occlusal surfaces of all teeth and extended apically on the buccal and lingual surfaces to cover the coronal third of the teeth. Six grooves were placed on the stents so that the measurements could be made at the same position and angulation at every evaluation periods. Measurements were recorded to the data sheet (Figure 10).

The following indices and measurements were used:

3.7.1. Plaque Index

Teeth were isolated with cotton rolls and after drying by air syringe, microbial dental plaque biofilm was evaluated with the probe from 4 tooth surfaces (mesio-buccal, mid-buccal, disto-buccal and mid-lingual) and scores between 0-3 were given for each point (102).

Scoring was made as follows:

0 – No microbial dental plaque in the gingival area.

1 – A film of microbial dental plaque adhering to the free gingival margin and adjacent area of the tooth, recognized only by running a probe across the tooth surfaces.

2 – Moderate accumulation of soft deposits within the gingival pocket and on the gingival margin and/or adjacent tooth surfaces that can be seen by naked eye.

3 – Abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface.

3.7.2. Gingival Index

Periodontal probe was used to asses the bleeding potential of the tissues from 4 tooth surfaces (mesio-buccal papilla, mid-buccal margin, disto-buccal papilla and midlingual margin) and scores between 0-3 were given for each point. (103)

[4]University of North Carolina PCPUNC15, Hu Friedy Ins. Co., USA

Scoring was made as follows:

0 – Normal gingiva

1 – Mild inflammation, slight change in color, slight edema, no BoP

2 – Moderate inflammation, redness, edema, ulcerations; tendency to spontaneous bleeding.

3 – Severe inflammation, ulceration and spontaneous bleeding.

3.7.3. Probing Depth

Full mouth PD was measured by the periodontal probe from 6 tooth surfaces (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) as the distance between the gingival margin and the bottom of the gingival sulcus.

3.7.4. Relative Attachment Level

Full mouth RAL was measured by the periodontal probe from 6 tooth surfaces (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) as the distance between the occlusal stent margin and the bottom of the gingival sulcus.

3.7.5. Bleeding on Probing

BoP was assessed simultaneously to the probing measurements, and the presence or absence of bleeding up to 30s after probing was recorded from 6 tooth surfaces (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual) and scored as positive (+) or negative (-) bleeding for each point. (104)

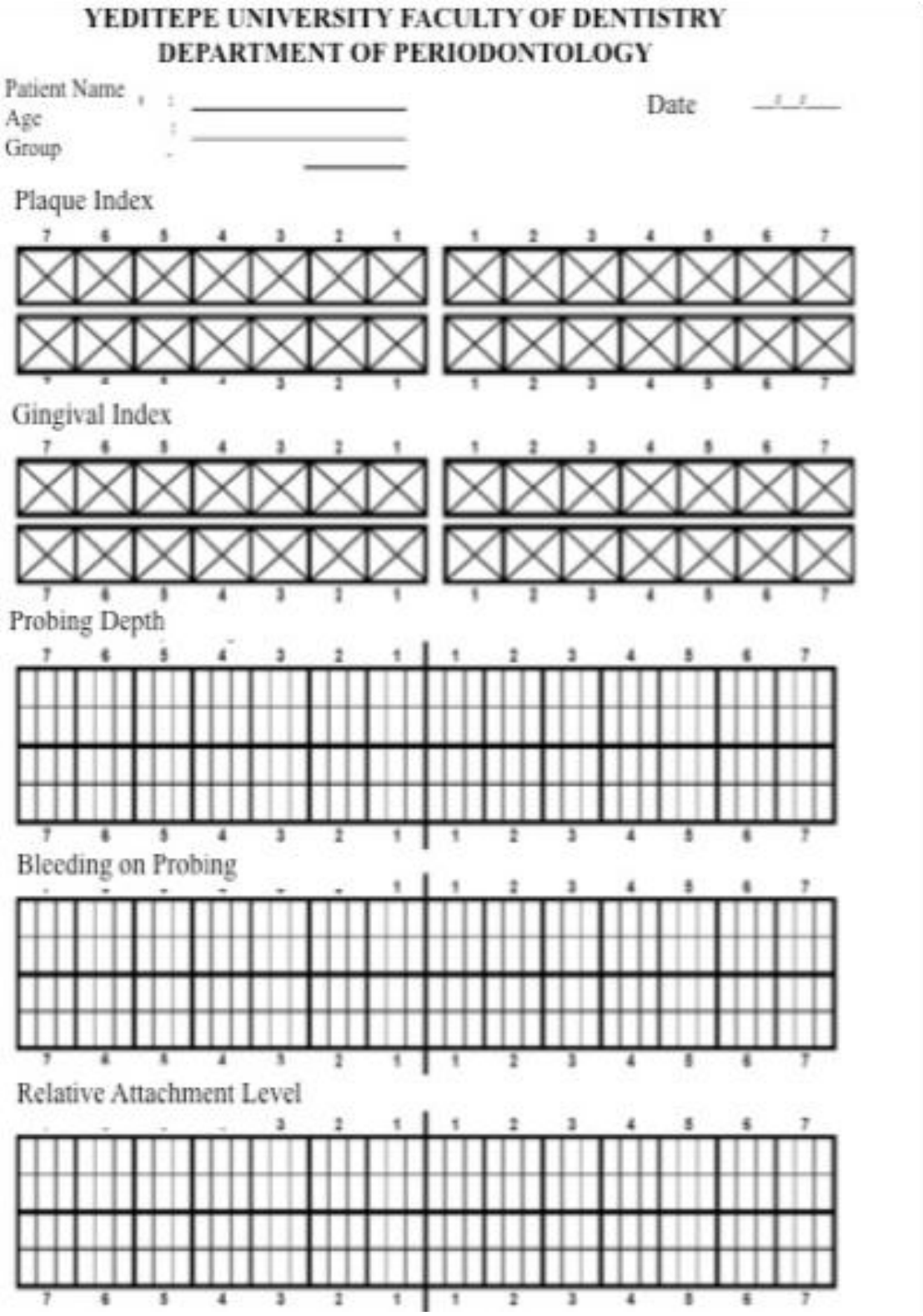

Figure 10. Data Sheet

3.8. Clinical Procedures

3.8.1. Gingival Crevicular Fluid Sampiıng

For GCF sampling, in each quadrant, two teeth with approximal PD of 5-7 mm and GI ≥2 were selected. Therefore, a total of eight sites were sampled per patient. Samples were taken from the same sites at baseline and at day 90. The samples obtained from an individual were pooled before the biochemical analysis. The GCF samples were obtained from selected periodontal sites in each participant after the PI was scored and before the measurement of other clinical parameters. After the isolation of sites with cotton rolls, saliva was removed using a high-power suction tip, supragingival plaque was removed with a periodontal probe, and GCF samples were taken by using sterile filter paper strips (Periopaper®) inserted into the crevice for 30 seconds. Strips contaminated by blood were discarded, and the sampling was repeated after 30 minutes. The volume of GCF was determined by means of a previously calibrated electronic device (Periotron[5]) and converted into an actual volume (milliliters) by reference to the standard curve. All strips with GCF were immediately and individually placed in Eppendorf vials and stored at -70° C until further analysis (105).

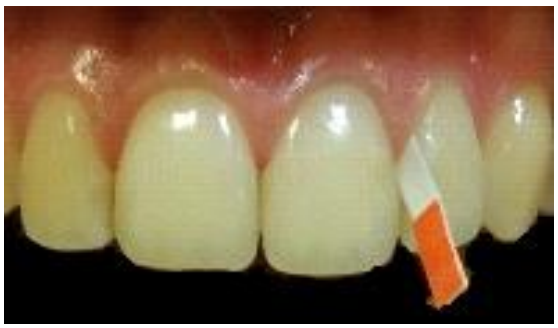

Figure 11. Collection of GCF samples

[5]Periotron 8000 Smithtown, New York, USA

3.9. Laboratory Procedures

3.9.1. Elution of GCF Samples

GCF samples were eluted from the strips by acentrifugal method (104). The GCF blotted paper strips were brought to room temperature for 30 minutes. Before proceeding with the elution process, Beckman tubes were specially prepared. At this stage, the lid of another Beckman tube drilled in the middle was placed within half portion of 500 µl Beckman tubes without being damaged (Figure 12) (104). For elution of the GCF samples, 50 µl of phosphate-buffered saline (PBS)[6] was added to each tube containing the multiple strips and centrifuged at 11,000 rpm (Figure 13). This step was repeated, the total volume of 100 µl of eluates were combined. After centrifugation, the strips were removed and the fluid remaining in the tubes were analysed for MMP-8 and TIMP-1 using the below-mentioned commercial ELISA kits (105)

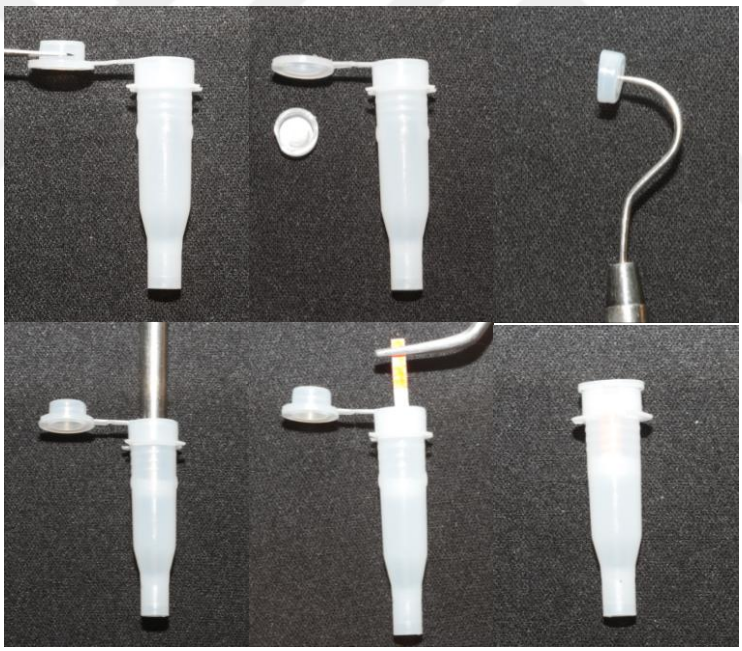

Figure 12. Preparation of the Beckman tubes for elution(104)

[6] Phosphate- buffered saline, PBS buffer, Applichem Biochemia Synthesis Services, Germany

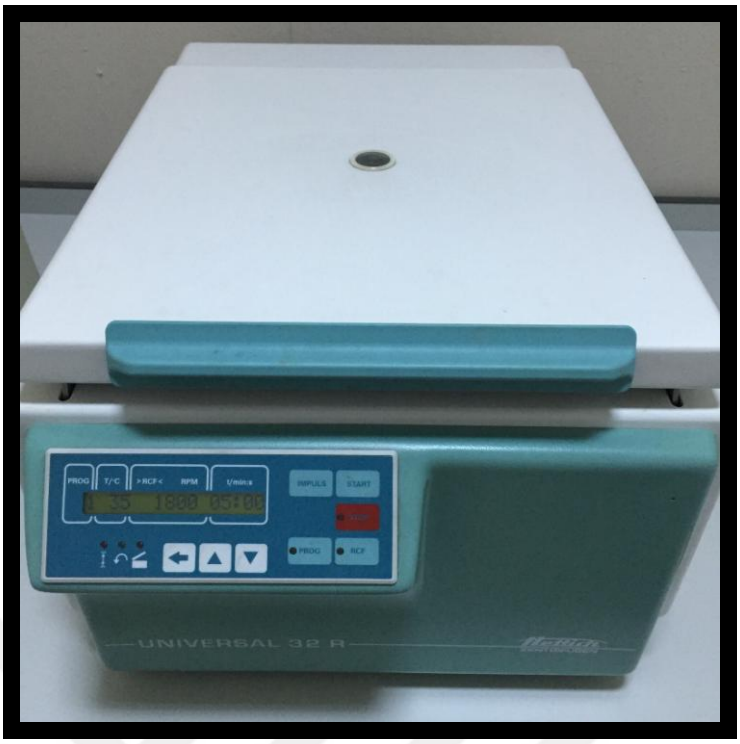

Figure 13. Centrifuge machine.

3.9.2. Analysis of MMP-8 and TIMP-1 levels

Levels of MMP-8 and TIMP-1 in GCF samples were assayed by sandwich ELISA[7] kit. All assay procedures were carried out according to the manufacturer's instructions (Figure 14). GCF samples were assayed at dilutions 1:20 for MMP-8 and 1:100 for TIMP-1, respectively.

[7] **ELISA** kit Quantikine R& D Systems Inc., Minneapolis, MN, USA

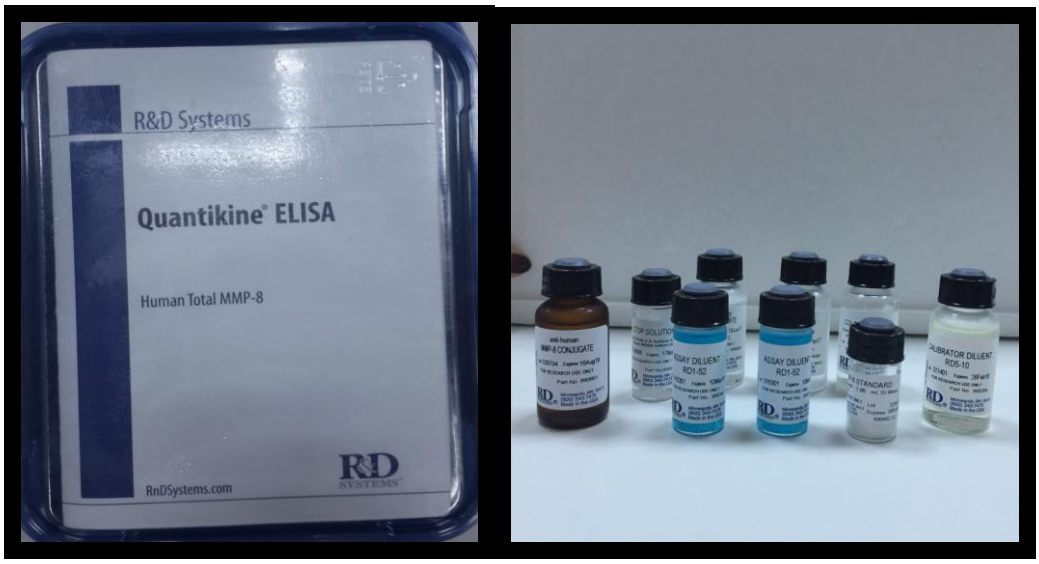

Figure 14. Specific MMP-8 sandwich ELISA kit.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for MMP-8 and TIMP-1 has been pre-coated onto a microplate. Standarts and samples are pipetted into the wells and any MMP-8 and TIMP-1 present, bound by the immobilized antibody. After washing away any unbound substances, an enzyme- linked monoclonal antibody specific for MMP-8 and TIMP-1 are added to the wells. Following a wash to remove any unbound antibody- enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of MMP-8 and TIMP-1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

ELISA kit and all GCF samples were brought to room temperature before use. Standart solutions were prepared as 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 ng/ml concentrations. 150 µl of Assay Diluent RD1- 52 was added to each well. After addition of 50 µl standart and eluated samples, microplate was covered with adhesive strip provided. Microplate was incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500±50 rpm. Later on, each well was aspirated and washed, repeating the process 3 times for a total of 4 washes with autowasher. After the last wash, any remaining Wash Buffer removed by aspirating and inverting the plate against clean paper towels. Then, 200 µl MMP-8 conjugate was added and incubated for 2 hours at room temperature on the shaker. Aspiration and washing each well were

repeated. 200 µl of Substrate Solution was added and microplate was incubated for 30 minutes at room temperature on the benchtop. Finally, 50 µl of Stop Solution were added to each well and the color in the wells changed from blue to yellow. Optical density was determined within 30 minutes, using a microplate reader set to 450 nm.

Levels of TIMP-1 in GCF samples were also analysed by using specific sandwich ELISA kit (Figure 15).

Figure 15. Specific TIMP-1 sandwich ELISA kit

All reagents, working standards, and samples were prepared as described in the previous section. 100 µl of Assay Diluent RD1- 52 was added to each well. After addition of 50 µl standart and eluated samples, microplate was covered with adhesive strip provided. Microplate was incubated for two 2 hours at room temperature on a horizontal orbital microplate shaker set at 500±50 rpm (Figure 16). Later on, each well were aspirated and washed, repeating the process 3 times for a total of 4 washes with autowasher (Figure 17). After the last wash, any remaining Wash Buffer removed by aspirating and inverting the plate against clean paper towels. Then, 200 µl TIMP-1 conjugate was added and incubated for 1 hour at room temperature on the shaker. Aspiration and washing each well were repeated. 200 µl of Substrate Solution was added and microplate was incubated for 30 minutes at room temperature on the benchtop. Finally, 50 µl of Stop Solution were added to each well and the color in the wells changed from blue to yellow (Figure 18). Optical density was determined within 30 minutes, using a microplate reader set to 450 nm (Figure 19).

Figure 16. Horizontal orbital incubator

Figure 17. Microplate washer

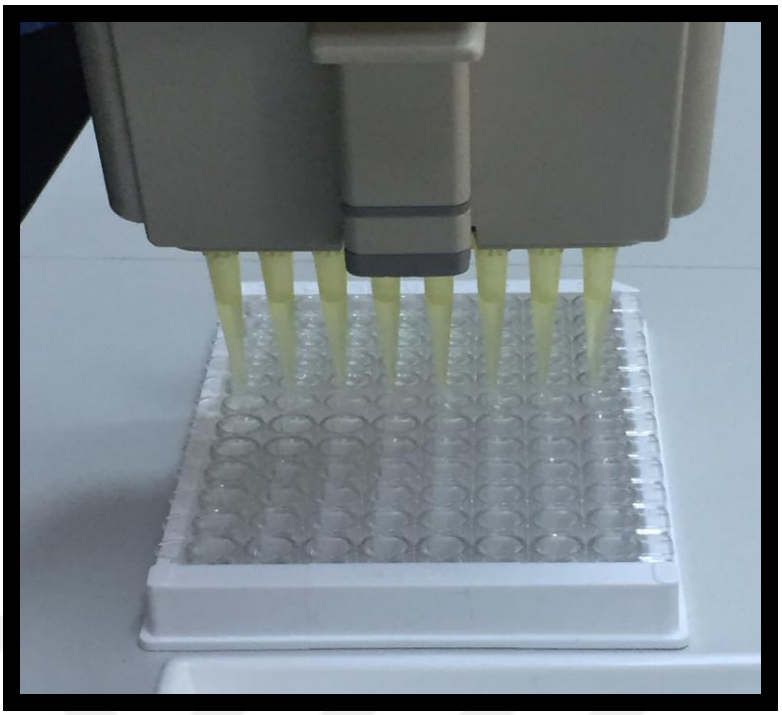

Figure 18. Addition of Stop Solution with multichannel pipettes

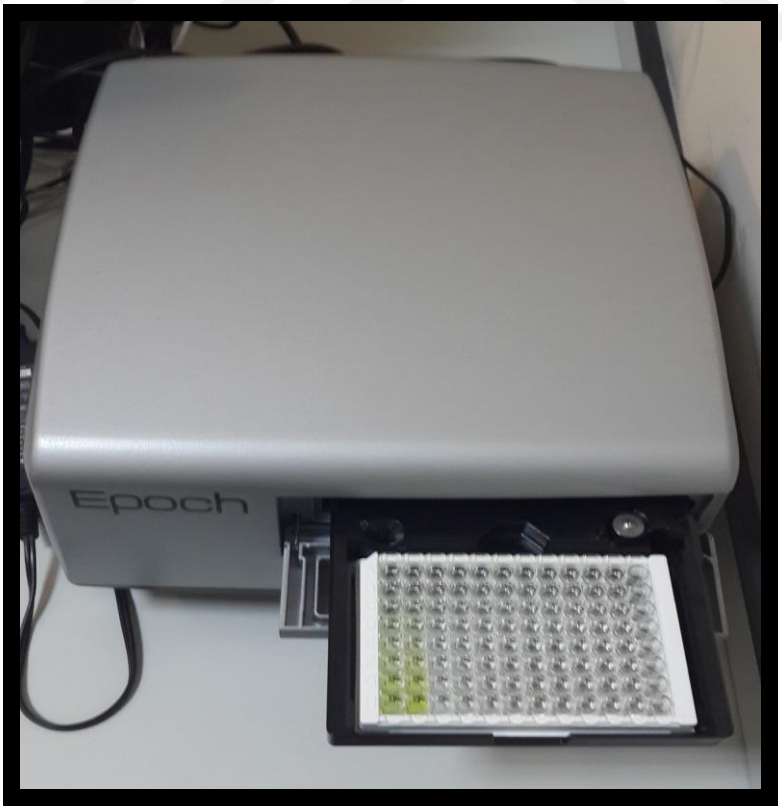

Figure 19. Absorbance measurement with microplate reader

3.10. Statistical Analysis

For all statistical evaluations, the patient was maintained as the unit of measurement. Data analysis was performed for the full mouth for PI, GI, BOP, PD, RAL, GCF volume, MMP-8 and TIMP-1 concentrations by using a statistical package. The compliance of parameters to the normal distribution was evaluated using Shapiro Wilks test. The balancing of groups by age and gender was tested by Oneway Anova and Chi-square test, respectively. Quantitative data were recorded as the mean value ± SD for all investigated parameters. Intra-group comparisons of all parameters were evaluated with Paired Sample t test while inter-group comparisons of all clinical and biochemical parameters were evaluated with Oneway Anova test. Tukey test was used for the double comparisons of the groups. The statistical significance was set at $p < 0.05$.

4.RESULTS

4.1. Demographic and Baseline Datas

45 patients (24 male, 21 female) aged between 35-50 years were included in the study. The mean years of age was 39.93±6.49 in SRP + Probiotic group, 40.27±6.33 in SRP+ SDD group, and 40.00±6.57 in SRP + Placebo group. No statistically significant differences were detected between the groups in terms of demographic and clinical baseline parameters (Table 5.1). No adverse effects were reported and none of the subjects was excluded from the study. Intra-oral photographs and radiographs of one representative case from each groups are shown in Figures 20 (1.a-1.c), (2.a-2.c), and (3.a-3.c)

Table 5.1. Demographic and baseline data of the patients

	GROUP I SRP+PROBIOTIC MEAN ± SD	**GROUP II SRP+SDD MEAN ± SD**	**GROUP III SRP+PLACEBO MEAN ± SD**	**P**
AGE^{+}	39.93 ± 6.49	40.27 ± 6.33	40.00 ± 6.57	0.989
GENDER (M/F)$^{++}$	9/6	7/8	8/7	0.765
PI^{+}	2.36 ± 0.35	2.47 ± 0.20	2.27 ± 0.30	0.507
GI^{+}	2.15 ± 0.17	2.3 ± 0.11	2.2 ± 0.38	0.574
BoP(%)$^{+}$	86.50 ± 13.5	89.01 ± 3.40	89.50 ± 3.67	0.801
PD (mm)$^{+}$	5.19 ± 0.61	5.59 ± 0.21	5.13 ± 1.05	0.486
GCF Volume^{+} **(µl)**	0.50 ± 0.26	0.54 ± 0.26	0.40 ± 0.11	0.379
MMP-8^{+} **(ng/ml)**	28.91 ± 7.76	29.67 ± 11.66	24.65 ± 0.75	0.341
TIMP-1^{+} **(ng/ml)**	9.20 ± 0.32	9.30 ± 0.23	9.41 ± 0.03	0.129

$^{+}$ *Oneway ANOVA test* $^{++}$*Chi-Square test* $p < 0.05$

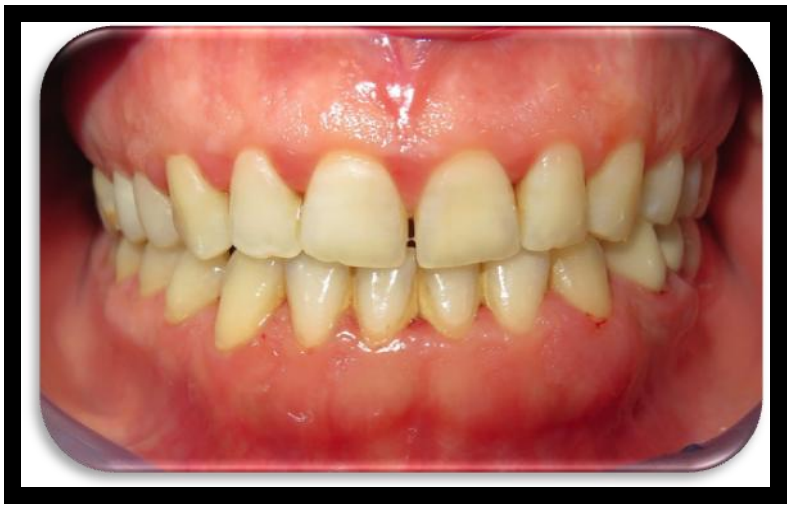

Figure 20.1.a. Clinical view of the patient in SRP + Probiotic group at day 0

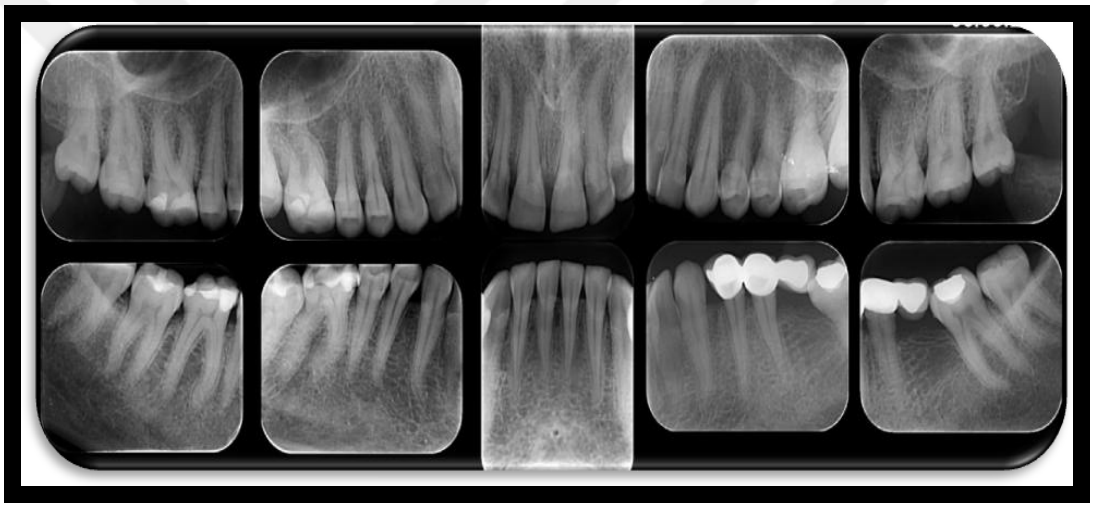

Figure 20.1.b. Radiographic view

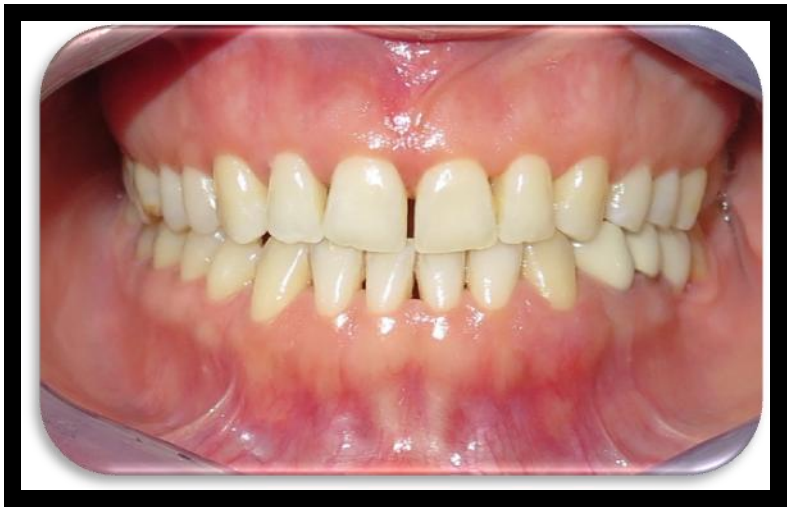

Figure 20.1.c. Clinical view of the patient in SRP + Probiotic group at day 90

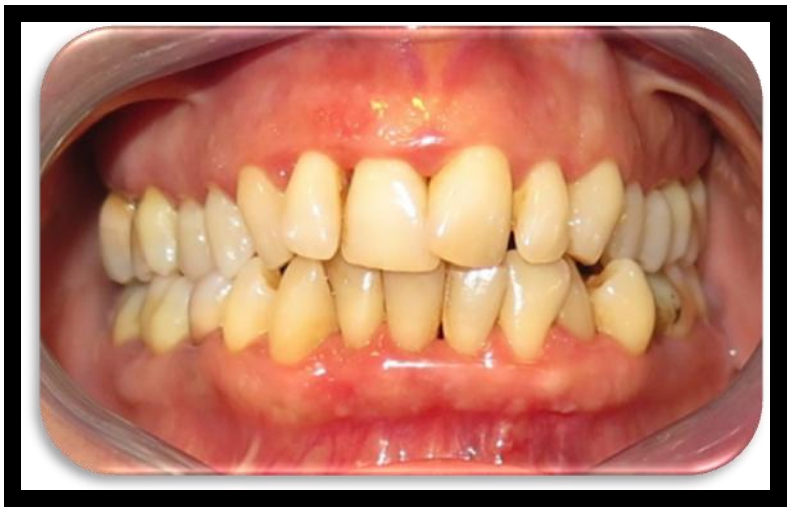

Figure20.2.a. Clinical view of the patient in SRP + SDD group at day 0

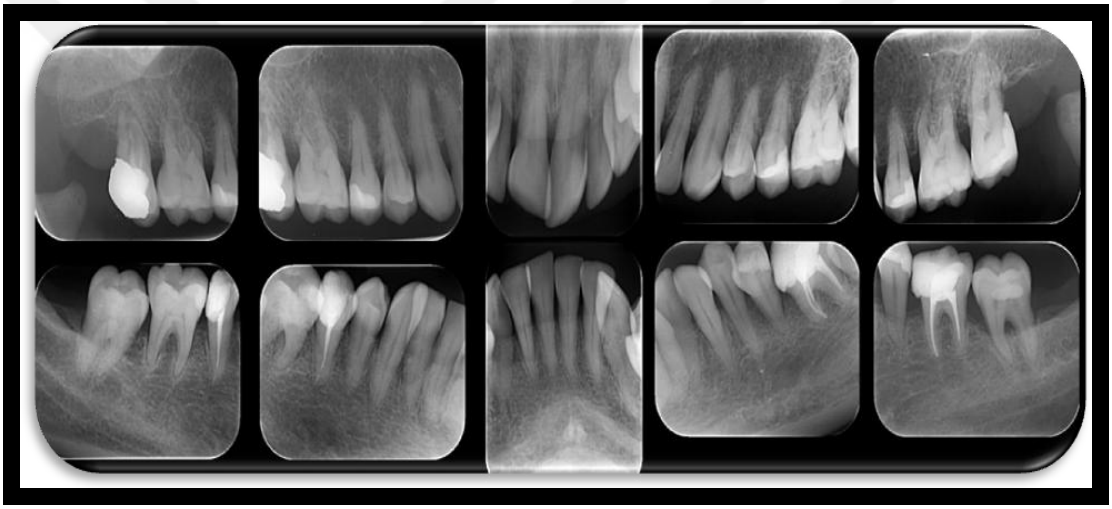

Figure 20.2.b. Radiographic view

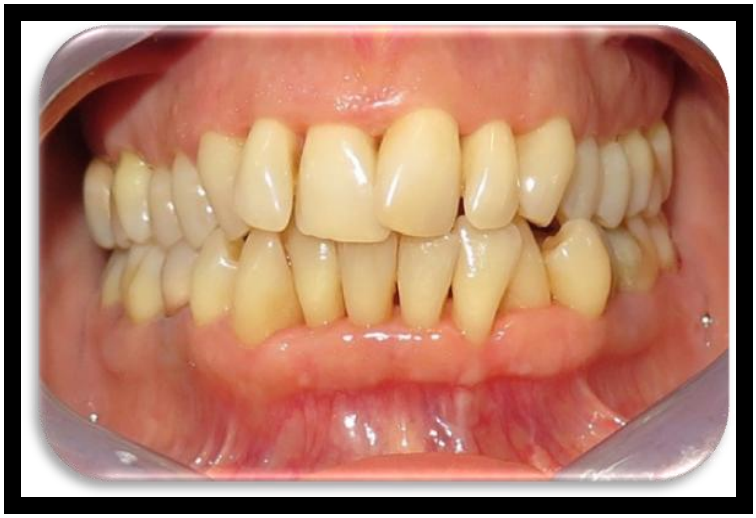

Figure20.2.c. Clinical view of the patient in SRP + SDD group at day 90

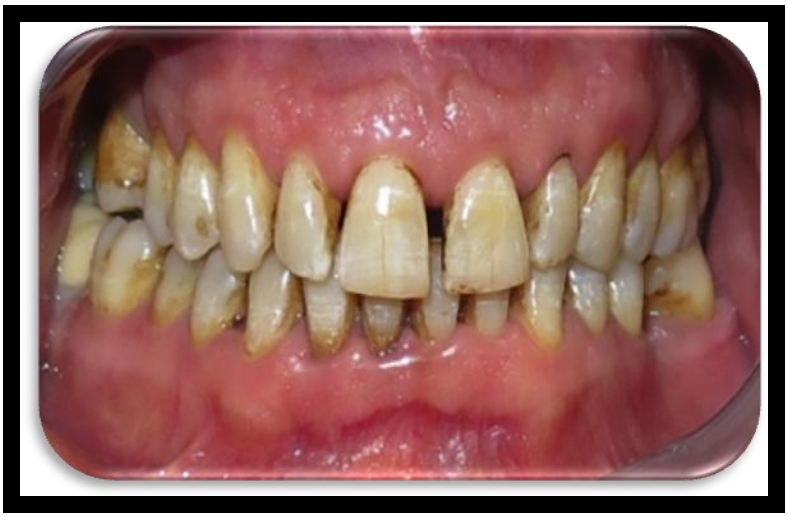

Figure 20.3.a. Clinical view of the patient in SRP + Placebo group at day 0

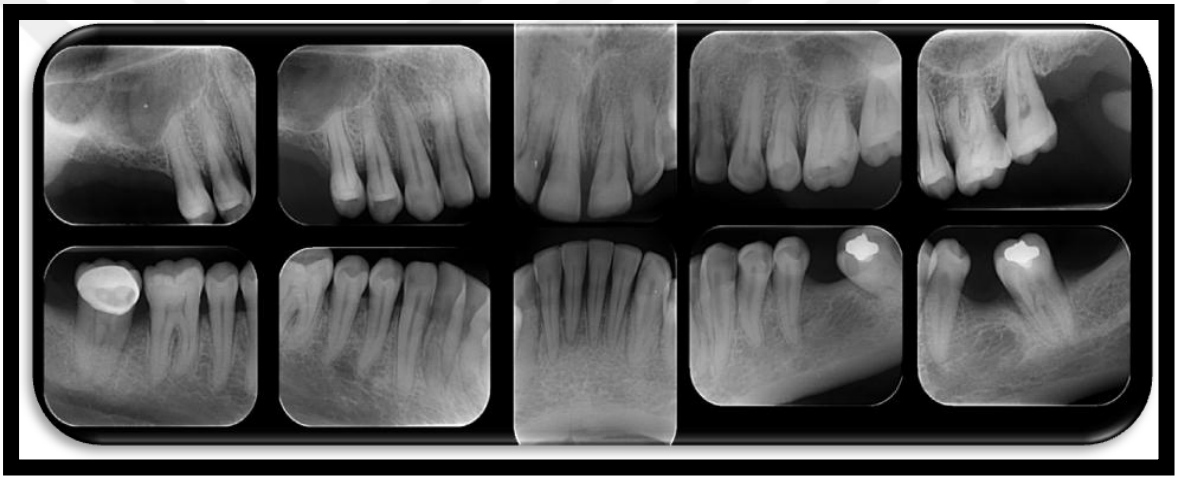

Figure 20.3.b. Radiographic view

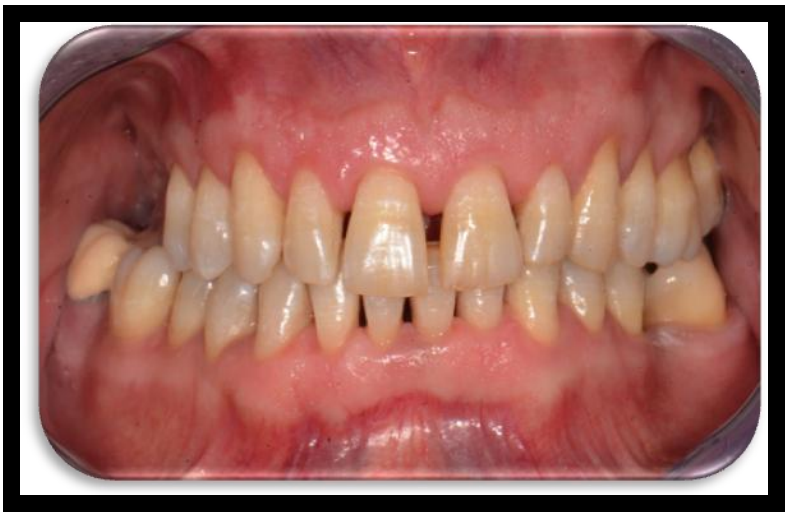

Figure 20.3.c. Clinical view of the patient in SRP+ Placebo group at day 90

4.2. Clinical Results

The mean values and standard deviations of clinical parameters in terms of PI, GI, BoP (%) and PD at baseline are presented in Table 5.1.

4.2.1. Plaque Index

PI values were detected 2.36±0.35 at day 0 and it reduced to 0.55±0.19 at day 90 in SRP + Probiotic group (p=0.001). In SRP + SDD group 2.47±0.20 at day 0 and it reduced to 0.62±0.16 at day 90 (p=0.001) and in SRP + placebo group, it was detected 2.27±0.30 at day 0 and reduced to 1.25±0.18 at day 90 (p=0.003) (Table 5.2.1).

Double comparisons of mean differences of PI values revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.004), and SRP+ SDD and SRP + placebo groups (p=0.003) between day 0 and 90. Whereas no significant differences were observed between SRP + Probiotic and SRP + SDD. (p=0.986) (Table 5.2.4).

4.2.2. Gingival Index

GI values were detected 2,15±0,17 at day 0 and it reduced to 0,60±0,23 at day 90 in SRP + Probiotic group (p=0.001). In SRP + SDD group, GI values were detected 2,30±0,11 at day 0 and it reduced to 0,69±0,12 at day 90 (p=0.001) and in SRP + placebo group it was detected 2,20±0,38 at day 0 and reduced to 1.70±0.70 (p=0.043) (Table 5.2.1).

Double comparisons of mean differences of GI values revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.001), and SRP+ SDD and SRP + placebo groups (p=0.001) between day 0 and 90. Whereas no significant differences were observed between SRP + Probiotic and SRP + SDD. (p=0.921) (Table 5.2.4).

4.2.3. Bleeding on Probing

BoP values were detected %86,50±13,50 at day 0 and it reduced to %15,67±4,27 at day 90 in SRP + Probiotic group (p=0.001). In SRP + SDD group, BoP values were detected %89,01±3,40 at day 0 and it reduced to %6,67±1,96 at day 90 (p=0.001) and in SRP + placebo group BoP values were detected %89,50±3,67 and reduced to %22,83±5,53 at day 90 (p=0.001). In all groups, intra-group comparisons of BoP scores showed statistically significant reduction between day 0 and 90. (Table5.2).

Double comparisons of mean differences of GI values revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.001), and SRP + SDD and SRP + placebo groups (p=0.001) between day 0 and 90. Whereas no

significant differences were observed between SRP + Probiotic and SRP + SDD. (p=0.938) (Table 5.2.4).

4.2.4. Probing Depth

PD values were detected 5.19±0.61 mm at day 0 and it reduced to 3,53±0.77 mm at day 90 in SRP + Probiotic group (p=0.001). In SRP + SDD group PD values were detected 5.59±0.21 mm and reduced to 3.90±0.21 mm at day 90 (p=0.001)and in SRP + Placebo group, PD values were detected 5.13±1.05 mm at day 0 and it reduced to 4.61±1.08 mm at day 90 (p=0.001) (Table 5.2.1).

In all groups, subsequent comparisons of mean values of PD scores at day 90 compared to baseline revealed statistically significant differences (p=0.001) (Table 5.2.1).

PD reduction was detected 1.66±0.22 mm in SRP + Probiotic group, 1,70±0,17 mm in SRP + SDD group and 0,52±0,16 mm in SRP + placebo (Table 5.2.3).

Double comparisons of mean differences of PD values revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.001), and SRP + SDD and SRP + placebo groups (p=0.001) between day 0 and 90. Whereas no significant differences were observed between SRP + Probiotic and SRP + SDD. (p=0.944) (Table 5.2.4).

4.2.5. Attachment Gain

Attachment gains were detected 1.25±0.15 mm in SRP + Probiotic group, 1.32±0.06 mm in SRP + SDD group, and 0.40±0.14 mm in SRP + placebo group at day 90 (Table 5.2.3).

Double comparisons of mean differences of attachment gain values revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.001), and SRP + SDD and SRP + placebo groups (p=0.001) at day 90 whereas no significant differences were observed between SRP + Probiotic and SRP + SDD (p=0.856) (Table 5.2.4).

Table 5.2.1. Inter and Intra-group comparisons of clinical parameters at day 0 and 90

		Group I SRP + Probiotic Mean±SD	Group II SRP + SDD Mean±SD	Group III SRP + Placebo Mean±SD	[1]p
PI	**0**	2.36 ± 0.35	2.47 ± 0.20	2.27 ± 0.30	0.507
	90	0.55 ± 0.19	0.62 ± 0.16	1.25 ± 0.18	**0.001**
	[2]p	**0.001**	**0.001**	**0.003**	
GI	**0**	2.15 ± 0.17	2.30 ± 0.11	2.2 ± 0.38	0.574
	90	0.60 ± 0.23	0.69 ± 0.12	1.7 ± 0.70	**0.001**
	[2]p	**0.001**	**0.001**	**0.043**	
BOP (%)	**0**	86.5 ± 13.50	89.01 ± 3.4	89.5 ± 3.67	0.801
	90	15.67 ± 4.27	6.67 ± 1.96	22.83 ± 5.53	**0.001**
	[2]p	**0.001**	**0.001**	**0.001**	
PD (mm)	**0**	5.19 ± 0.61	5.59 ± 0.21	5.13 ± 1.05	0.486
	90	3.53 ± 0.77	3.90 ± 0.21	4.61 ± 1.08	**0.080**
	[2]p	**0.001**	**0.001**	**0.001**	
RAL (mm)	**0**	10.27 ± 0.87	10.16 ± 1.07	10.03 ± 0.85	
	90	9.02 ± 0.85	8.84 ± 1.03	9.63 ± 0.87	
	[2]p	**0.001**	**0.001**	**0.001**	

[1] Oneway ANOVA test *[2] Paired Samples t Test p< 0.05*

PI; Plaque Index, GI; Gingival Index, BoP; Bleeding on Probing, PD; Probing Depth, RAL; Relative Attachment Level

Table 5.2.2. Inter- group comparisons of clinical parameters in pairs at day 0 and 90

		p	SRP+Probiotic-SRP+SDD	SRP+Probiotic-SRP+Placebo	SRP+SDD-SRP+Placebo
PI	**0**	[1]**p**	0.788	0.859	0.477
	90	[1]**p**	0.776	**0.001**	**0.001**
GI	**0**	[1]**p**	0.555	0.931	0.771
	90	[1]**p**	0.933	**0.001**	**0.003**
BOP	**0**	[1]**p**	0.861	0.809	0.994
	90	[1]**p**	**0.005**	**0.025**	**0.001**
PD	**0**	[1]**p**	0.598	0.988	0.509
	90	[1]**p**	0.690	0.070	0.283

[1] *Tukey Test p< 0.05*

Table 5.2.3. Inter-group comparisons of the mean differences in clinical parameters between days 0-90

Clinical Parameters	Group I. SRP +Probiotic (Mean±SD)	Group II. SRP + SDD (Mean±SD)	Group III. SRP + Placebo (Mean±SD)	[1]p
PI	1.81 ± 0.4	1.84 ± 0.06	1.30 ± 0.46	**0.002**
GI	1.55 ± 0.16	1.61 ± 0.05	0.5 ± 0.46	**0.001**
BOP(%)	80.55 ± 6.59	82.34 ± 3.12	66.67 ± 7.66	**0.008**
PD(mm)	1.66 ± 0.22	1.7 ± 0.17	0.52 ± 0.16	**0.001**
Attachment Gain (mm)	1.25 ± 0.15	1.32 ± 0.06	0.40 ± 0.14	**0.001**

[1] *Oneway ANOVA test p< 0.05*

Table 5.2.4. Inter-group comparisons of the mean differences of the clinical parameters in pairs at day 0-90.

Clinical Parameters	P	SRP+Probiotic-SRP+SDD	SRP+Probiotic-SRP+Placebo	SRP+SDD-SRP+Placebo
PI	[1]**p**	0.986	**0.004**	**0.003**
GI	[1]**p**	0.921	**0.001**	**0.001**
BOP	[1]**p**	0.938	**0.001**	**0.001**
PD	[1]**p**	0.944	**0.001**	**0.001**
Attachment Gain (mm)	[1]**p**	0.856	**0.001**	**0.001**

[1]*Tukey HSD Test p< 0.05*

4.3. Biochemical Results

The mean values and standard deviations of biochemical parameters in terms of GCF volume, MMP-8 and TIMP-1 concentrations at baseline and day 90 are presented in Tables 5.3.1-5.3.2.

4.3.1. GCF Volume

GCF volume values were detected 0.50±0.26 µl at day 0 and it reduced to 0.25±0.14 µl at day 90 in SRP + Probiotic group (p=0.002). In SRP + SDD group, GCF volume values were detected 0.54±0.26 µl and it reduced to 0.29±0.30 µl at day 90 (p=0.001) and in SRP + Placebo group, GCF volume was detected 0.40±0.11µl at day 0 and it reduced to 0.33±0.10 µl, at day 90 (p=0.001) (Table 5.3.1).

In all groups subsequent comparisons of mean values of GCF volume (µl) at day 90, compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001).

Mean changes of GCF volume values were detected -0.25±0.18 µl, -0.25±0.12, -0.07±0.04 µl between day 0-90; in SRP + Probiotic, SRP + SDD and SRP + placebo groups. Double comparisons of mean differences of GCF volumes revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.010), and SRP + SDD and SRP + placebo groups (p=0.009) between day 0 and 90, whereas no

significant differences were observed between SRP + Probiotic and SRP + SDD. (p=0.999) (Table 5.3.2).

4.3.2. MMP-8 Concentrations

MMP-8 concentrations were detected 28.91±7.76 ng/ml at day 0 and it reduced to 16.37±5.28 ng/ml, at day 90 in SRP + Probiotic group (p=0.001). In SRP + SDD group, MMP-8 concentrations (ng/ml) were detected 29.67±11.66 ng/ml at day 0 and it reduced to 17.32±8.71 ng/ml at day 90 (p=0.001), in SRP + placebo group, MMP-8 concentrations were detected 24.65±0.75 ng/ml at day 0 and it reduced to 21,8±0,45 ng/ml at day 90 (p=0.001) (Table 5.3.1).

Mean differences of MMP-8 concentration values were detected -12.54±4.91 ng/ml, -14.35±6.39 ng/ml and -2.84±0.66 between day 0-90 in SRP + Probiotic, SRP + SDD and SRP + placebo groups. Double comparisons of mean differences of MMP-8 concentration values revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.001), and SRP + SDD and SRP + placebo groups (p=0.001) at day 90, whereas no significant differences were observed between SRP + Probiotic and SRP + SDD. (p=0.995) (Table 5.3.2).

4.3.3. TIMP-1 Concentrations

TIMP-1 concentrations were detected 9.20±0.32 ng/ml at day 0 and it increased to 9.55±0.31 ng/ml at day 90 in SRP + Probiotic group (p=0.001). In SRP + SDD group TIMP-1 concentrations were detected 9.30±0.23 ng/ml at day 0 and it increased to 9,66±0,36 ng/ml at day 90 (p=0.003)and in SRP + Placebo group, TIMP-1 concentrations were detected 9,41±0,03 ng/ml at day 0 and it increased to 9,44±0,04 ng/ml at day 90 (p=0.008) (Table 5.3.1).

In all groups subsequent comparisons of mean values of TIMP-1 concentrations at day 90 compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001),

Mean differences of TIMP-1 concentration values were detected 0.35±0.23 ng/ml, 0.36±0.29 ng/ml and 0.03±0.03 ng/ml between day 0-90 in SRP + Probiotic, SRP + SDD and SRP + Placebo groups. Double comparisons of mean differences of TIMP-1 levels revealed significant differences between SRP + Probiotic and SRP + placebo groups (p=0.007), and SRP + SDD and SRP + placebo groups (p=0.001)between day 0 and 90. Whereas no significant differences were observed between SRP + Probiotic and SRP + SDD (p=0.997) (Table 5.3.2).

Table 5.3.1. Intra-group and inter-group comparisons of biochemical parameters at baseline and day 90 and the mean differences between days 0-90

		Group I. SRP +Probiotic (Mean±SD)	Group II. SRP + SDD (Mean±SD)	Group III. SRP + Placebo (Mean±SD)	[1]p
GCF Volume (µl)	**0**	0.50 ± 0.26	0.54 ± 0.26	0.40 ± 0.11	0.379
	90	0.25 ± 0.14	0.29 ± 0.30	0.33 ± 0.10	0.632
	0-90	-0.25 ± 0.18	-0.25 ± 0.12	-0.07 ± 0.04	**0.004**
	[2]p	**0.002**	**0.001**	**0.001**	
MMP-8 (ng/ml)	**0**	28.91 ± 7.76	29.67 ± 11.66	24.65 ± 0.75	0.341
	90	16.37 ± 5.28	15.32 ± 8.71	21.80 ± 0.45	0.107
	0-90	-12.54 ± 4.91	-14.35 ± 6.39	-2.84 ± 0.66	**0.001**
	[2]p	**0.001**	**0.001**	**0.001**	
TIMP-1 (ng/ml)	**0**	9.20 ± 0.32	9.30 ± 0.23	9.41 ± 0.03	0.129
	90	9.55 ± 0.31	9.66 ± 0.36	9.44 ± 0.04	0.229
	0-90	0.35 ± 0.23	0.36 ± 0.29	0.03 ± 0.03	**0.003**
	[2]p	**0.001**	**0.003**	**0.008**	

[1]Oneway Anova test [2] Paired sample t test p<0.05

Table. 5.3.2. Inter-group comparisons of biochemical parameters in pairs at day 0 and day 90 and intergroup comparisons of the mean differences in pairs between days 0-90

GCF Volume (µ/ml)		**SRP+Probiotic-SRP+SDD**	**SRP+Probiotic-SRP+ Placebo**	**SRP+SDD-SRP+Placebo**
Day 0	[1]p	0.904	0.610	0.363
Day 90	[1]p	0.898	**0.001**	**0.001**
Difference	[1]p	0.999	**0.010**	0.009
MMP-8 (ng/ml)	[1]p	**SRP+Probiotic-SRP+SDD**	**SRP+Probiotic-SRP+ Placebo**	**SRP+SDD-SRP+Placebo**
Day 0	[1]p	0.976	0.476	0.371
Day 90	[1]p	0.930	0.116	0.222
Difference	[1]p	0.995	**0.001**	**0.001**
TIMP-1 (ng/ml)	[1]p	**SRP+Probiotic-SRP+SDD**	**SRP+Probiotic-SRP+ Placebo**	**SRP+SDD-SRP+Placebo**
Day 0	[1]p	0.561	0.108	0.542
Day 90	[1]p	0.630	0.681	0.200
Difference	[1]p	0.997	**0.007**	**0.001**

[1] Tukey test p<0.05

5. DISCUSSION

Periodontitis is a biofilm associated inflammatory disease and develops as a result of an imbalance between dental plaque biofilm microorganisms and host defense mechanisms that is characterized by attachment and alveolar bone loss (17). The presence of pathogenic bacteria, the absence of "beneficial bacteria" and the susceptibility of the host are the main factors that develop the periodontal diseases. To suppress or eliminate the periodontal pathogens and to control their overgrowth, new treatment strategies have been developed (106).

CP is a slowly progressing disease and is the most prevalent type of periodontitis. SRP together with OHI has been considered the gold standard for the treatment of periodontal disease (2, 108, 109). Although the initial number of pathogens can be greatly reduced by SRP, periodontopathogens quickly re-colonize the treated niches in the oral cavity. This re-colonization occurs within one week. The resulting shift to a less pathogenic composition of the subgingival microbiota is only temporary, even when combined with antiseptics or antibiotics (110). Multiple approaches have been suggested for SRP in the literature however no differences have been observed in terms of their outcomes. One Stage Full Mouth Disinfection (OSFMD), Full Mouth SRP (FMSRP), Quadrant Wise SRP (QWSRP) are the main treatment modalities that are used for the SRP purpose (111, 112). The success of the non-surgical periodontal therapy remains the effectiveness of root debridement and the patient's standard of oral hygiene, rather than the treatment modality (112). The efficacy of single session of SRP depends on the experience of the clinician, inflammation status of tissues and anatomy of the root (110). After all, the treated areas are needed to be re-evaluated after single session of instrumentation. So, in the present study, SRP was performed in two sessions with one-week interval.

Non-surgical therapy alone may not be effective to control infection mostly in deep pockets, which are not easily accessed by periodontal instruments (107, 113). The other niches including tongue, tonsils in the oral cavity shelter pathogenic species and and may be sources for re-infection. In order to decrease the number of periodonto-pathogens successfully, local and systemic antibiotics as adjuncts to SRP are needed to improve the periodontal health. However, this adjunctive agents improves the outcome of the therapy temporarily (4, 114). On the other hand the administration of these antibiotics may induce the risk of developing resistant microbial species. Also the use of antimicrobial agents is associated with unsatisfactory drug concentrations in the

periodontal pockets and this limits the antimicrobial action of the drug (110, 115). Also the application of lasers and photodynamic therapy still need further investigations in the treatment of periodontal diseases. In recent years another treatment approach, HMT became popular in the field of periodontology.

Host modulation offers the potential for down-regulating destructive aspects of the host response so that, in combination with conventional treatments, it can reduce the bacterial burden, thus the balance between health and disease progression is tipped in the direction of a healing response (5). A variety of drug classes have been evaluated as host response modulators, including the NSAIDs, bisphosphonates, and tetracyclines (5). In recent years, probiotics have been drawn attention in medicine and dentistry as well. Their unwanted effects preclude their usage as adjuncts to periodontal treatment. At present SDD remains the only systemic host response modulator specificly indicated as an adjunctive treatment for periodontitis. Its recommended dosage is 20 mg administration twice daily for 3-9 months as an adjunct to root surface debridement in the treatment of periodontitis. Also probiotics have been suggested as another host modulatory agent due to its anti-inflammatory properties. Among the different probiotic strains *Lactobacilli*, are the most common species in the commercially available preparations. *L. reuteri* is a member of the beneficial and commensal microbiota in the mammalian oral cavity and gastrointestinal tract; ATCC 55730 is derived from mother's milk, while the PTA 5289 is an oral isolate. It can suppress the TNF-α production by human monocytes and macrophages. It produces reuterin which induces oxidative stress in pathogenic organisms, which accounts for its anti-pathogenic effect. It is also effective against a vast array of intestinal pathogens (116, 117). From this standpoint, we aimed to evaluate the immuno-modulatory effect of both agents as adjuncts to initial periodontal therapy in CP patients on MMP-8 and TIMP-1 levels and clinical parameters as well.

Only few studies have been performed, for the evaluation of the daily dosage of the probiotic lozenges (71). Twetman et al. (71) stated a dose-response relationship or a treshold level. However they reported that it is too early to recommend a daily dosage for the probiotic lozenges in the treatment of periodontal disease. In the present study, *L. reuteri* containing lozenges were prescribed twice a day after toothbrushing without chewing. The rationale for our choice of dosage based from an analysis of previous studies and the manufacturer's recommendation (67, 71, 75, 76).

For oral hygiene level assesment and dental plaque accumulation, PI score was used (102). This index shows the thickness of plaque along the gingival margin, which is considered the primary etiologic factor of periodontal diseases. All patients were given a brief OHI instruction one-week prior to the experimental period and they were instructed to brush their teeth by modified Bass method (118) and to use interdental brushes. Patients were seen regularly to check their oral hygiene levels. Intra-group comparisons of all groups showed statistically significant reduction regarding PI scores compared to baseline values ($p< 0.05$). PI reductions were detected as 1.81, 1.84 and 1.02 in SRP + Probiotic, SRP + SDD and SRP + Placebo groups, respectively. This results showed that all patients in the present study provided optimal oral hygiene level.

GI and BoP scores were used to evaluate the inflammatory status of the gingiva. All groups showed significant improvements at the end of the day 90 ($p< 0.05$). SRP together with a high level of oral hygiene led to a significant reduction of the bleeding tendency and inflammation of the periodontium (108, 111, 119, 120, 121). Inter-group comparison of the mean differences of GI values revealed statistically significance in favor of SRP + Probiotic and SRP + SDD group ($p< 0.05$). However no significant differences were found between the groups. The superior results of the SRP + Probiotic and SRP + SDD can be attributed to the anti-inflammatory effect of probiotic containing lozenges and SDD tablets. In the literature, similar results were reported as reductions in BoP and GI scores following probiotic and SDD administration as an adjunct to SRP in CP patients (59, 67, 71, 75). On the other hand, there are also contradictory studies, which showed no clinical benefits of probiotics (70, 122, 123). However in the aforementioned studies, SRP was not performed therefore it can be speculated that probiotics were not reflected in clinical parameters. It has been demonstrated that biofilms are difficult target for the penetration of therapeutic agents unless they are mechanically disrupted. So, the significant findings of the present study may be explained by the disruption of the biofilm before probiotic and SDD administration.

The reduction in PD is the result of both a gain in CAL and a recession of the marginal gingival tissues. The gingival recession results from the reduction in swelling of the marginal gingival tissue. The inflamed tissue with its inflammatory cell infiltrate and the increased numbers of capillaries present in the gingival connective tissue is gradually replaced by a more collagen-rich tissues. These changes are accompanied by a gradual shrinkage of the tissue in an apical direction and towards the root surface, which result with PD reduction. (124). Reduction of the PD values were found 1.66 mm, 1.70

mm and 0.52 mm at day 90, in SRP + Probiotic, SRP + SDD and SRP + placebo groups, respectively. PD reduction can be related to the reduction of inflammation, re-organisation of collagen and establishment of a long junctional epithelium, which all would influence the probe penetration (125). In the literature, PD reduction ranges from 0.50 mm to 1.30 mm in moderately deep pockets (4-6 mm) up to 12 months following SRP (124). Therefore, our findings are in accordance with the literature. In relation to the primary outcome variable, significantly higher PD reduction in were observed at day 90 ($p< 0.05$). Similarly, Vivekananda et al. (75) reported 1.31 mm PD reduction, SRP + Probiotic and 0.49mm SRP + Placebo Group when the same probiotic containing lozenges were administered twice a day for 3 weeks as an adjunct to SRP. In a similar study by Teughels et al. (76) evaluated the effects of the same probiotic containing lozenges as an adjunct to SRP in 30 CP patients. Significant PD reduction of 1.84 mm was reported in SRP + Probiotic Group in moderately deep pockets in 12-week period in CP patients.

The interface between the root surface and the former pocket epithelium is partially transformed into a long junctional epithelium. Both the presence of the long junctional epithelium and the increased content in collagen fibers in the gingival connective tissue result in the gain in CAL, i.e. an increased resistance of the tissues against the penetration of a periodontal probe. Due to these phenomena the probe that did penetrate the base of the pocket in an inflamed untreated site, does no longer reach the base of the junctional epithelium of a site treated with nonsurgical periodontal therapy (124). CAL can be measured, relative to a landmark, such as the cementoenamel junction, a restoration, occlusal surface, or stent. Since probing the attachment levels from cementoenamel junction reproduce an error, relative we prefer the guidance of individual acrylic stents in our study. Distance between the base of the pocket and the edge of the stent was measured and recorded. Negative changes in RAL values in different evaluation periods were accepted as attachment gain. In SRP + Probiotic group attachment gain was found 1,25 mm at day 90, it was detected 1,32 in SRP + SDD group, and 0,40 in SRP + placebo group. In the literature, attachment gain is reported between 0.07 mm and 1.40 mm after SRP in CP (120). At the end of the experimental period , PD reduction and attachment gain resulted in favor of the SRP + Probiotic and SRP + SDD group ($p< 0.05$). These findings could possibly be due to the mechanical effect of SRP, significant reductions in the PI and GI scores, which can be

releated to the anti-collagenase and anti-inflammatory properties of probiotic and SDD (32-36, 75, 126, 127).

The success of periodontal therapy depends on the reduction of pathogenic species in the subgingival area. Studies have shown that subgingival debridement in periodontitis patients is followed by profound shifts in the composition of the subgingival microflora which is comparable with the microflora found in healthy sites. Re-colonization of the subgingival area by motile rods and spirochetes may occur within 2–8 weeks, which seems to be dependent on the level of oral hygiene of the patient, the efficacy of the mechanical instrumentation and residual PD. (128, 129, 130) Effective supragingival plaque control is crucial for long-term control of inflammatory periodontal diseases. From this point of view, every patient was given OHI 1 week prior to the study and maintained at a good level through experimental period. Re-establishment of the epithelial attachment appears to occur within a week after SRP was performed (119). Therefore, the interval between two sessions was chosen as 1 week based on this information. The data in the literature shows the most of the healing complete at 3 months. Proye et al. (119) informed that most of the changes in PD reduction could be observed 3 weeks after SRP and no further changes could be obtained during the 3 months. Thus, our examination period was determined as day 90. In the literature it is stated that; subject-related and tooth site-related factors may compromise the healing response to periodontal therapy (131). Due to the furcation anatomy, molar sites have been reported to represent a compromised healing response. Poor accessibility for instruments to the furcation fornix, the presence of concavities and other root surface irregularities in the furcation areas may decrease the efficacy of root surface debridement (132). Also distal location of molars may have limited access for plaque control by the patient on a regular basis, which may lead to the re-colonization of a microflora similar to the pretreatment conditions. Some researches have shown that the inflammatory mediators are found intensively in PD of ≥5mm and also the studies with 2 years of follow up period after SRP indicated that, single root teeth with PD of ≥5mm show greater reduction in PD and gain in attachment level than molar teeth (130, 133, 134). Because of this reason, we preferred single rooted-teeth with PD of ≥5mm were chosen for sampling to compare different treatment approaches.

PD, CAL or BoP alone have limitations to evaluate the disease status. The GCF carries biological molecular markers aggregated from the surrounding site as it traverses from the microcirculation across inflamed periodontal tissues. Due to the ease of its

collection GCF has an important value and ability for the clinician to sample multiple sites within the oral cavity simultaneously. The use of GCF suggests potential diagnostic value to identify the severity of periodontal disease and the response of the tissues to therapy (135).

In periodontal studies, different molecules in the oral fluids such as GCF and saliva and saliva or the molecules in the blood circulation, serum or plasma have been used in order to provide a sensitive and specific marker for periodontal tissue destruction. With non-invasive methods and with any discomfort, GCF and saliva samples were easily collected and they consist both locally synthesized and systemically derived molecules, serum proteins, inflammatory mediators, host cell degradation products and microbial metabolites (136). Saliva is a complex fluid mixture derive from salivary glands, contains contributions from GCF, oral bacteria, cells and other sources to make identification of exact sites of the disease activity.

Depending on the gender, age, biorythm, the usage of different types of medication and also systemic conditions, flow rate of saliva differs within and between subjects. GCF collection with paper strips is easy, non-invasive and rapid method for assessment of inflammatory molecules. In our study, GCF samples obtained from single rooted teeth having at least on approximal site with a PD of ≥5mm and GI of ≥2 in each quadrant. All collected samples were pooled in order to increase the total amount of GCF. Since the samples from several sites of similar status in each subject were pooled, the average GCF volume per site was calculated as the total GCF volume divided by the total number of paper strips (137).

Due to their effects on the physiological and inflammatory response; systemic disorders and hormonal status especially pregnancy have the potential to modify the plaque microbiota, disease progression and response to treatment. Therefore, study population was consisted of systemically healthy, 45 non-smoker patients. Smoking effects periodontal status and a risk factor for periodontitis (138). Smokers display higher levels of plaque, reduced inflammatory reaction, significantly less favourable clinical outcome and have an altered host antibody response to antigenic challenge than non-smokers. Söder et al. investigated the levels of granulocyte elastase, MMP-8 and PGE_2 in GCF of smokers and non-smokers. They resulted granulocyte function seemed to be impaired in smokers with persistent periodontitis. The cells of smokers react to the bacterial challenge by releasing increased levels of serine proteases, elastase and MMP-8, which reflect the degradation of connective tissue. Therefore, the risk of progression

of the disease was found to be higher in smokers with persistent periodontitis than in non-smokers (100).

In different studies, GCF sampling time ranges from 3 seconds, 5 sec, 15 sec, 20 sec, 25 sec, 30 sec, 60 sec, 90 sec, 120sec and 180 sec (139). Short sampling time periods are relevant to decreased volume of GCF sample for subsequent laboratory analysis, on the other hand prolonged collection times effeccs the nature of the GCF sample collected. While protein concentration of the initial GCF collected is comparable to interstitial fluid, prolonged sampling time at the site results in protein concentrations approaching to serum levels (139). Gürkan et al. (35) used Periopaper with a non-traumatic method in their study and they inserted the paper strip into the crevice until mildresistance was felt and left there for 30s.

Staining techniques, weighing of strips before and after sample collection and an electronic measuring device, The Periotron® has been proposed to estimate the GCF volume collected (139). Since evaporative losses because of delays in determining the volume in staining technique and very sensitive balance required in weighing the accumulated fluid, In our study we used Periotron 8000®, which allowed accurate determination of the GCF volume and subsequent laboratory investigation of the sample composition. Periotron® quantifies the volume of GCF or saliva collected on filter papers by measuring the electrical capacitance of a wet paper strip. Evaporation is an important factor that varies in Periotron® readouts, because of this reason, samples were immediately measured by the device and than stored at – 70°C until analysis. The current data indicate that GCF resting volumes has a disease-related spectrum of values. The amount of GCF produced significantly increases with the severity of inflammation. Shallow sulcus in healthy subjects are reported to have resting GCF volumes in the order of 0.06 µl. However, pockets with periodontal disease have resting volumes from 0.4 to 1.5 µl (18). In the present study, in SRP + Probiotic group, GCF volume decreased from 0,50 µl to 0,25 µl at day 90. In SRP + Periostat group, GCF volume decreases from 0.54 µl to 0.29 µl at day 90. In SRP + Placebo group, GCF volume decreased from 0,40 µl to 0,33 µl at day 90. In all three groups, treatment resulted in significant reductions of GCF volume values when compared to baseline. SRP + Probiotic and SRP + SDD group showed significantly more reductions in GCF volume than SRP + Placebo group at day 90 ($p < 0.05$). Slawik et al. (120) reported similar results after 2 weeks consumption of the probiotic milk drink. Also Twetman et al. (71) aimed to investigate the effect of a chewing gum containing *L.reuteri* on gingival

inflammation and the levels of selected inflammatory mediators in GCF. 42 healthy adults with moderate gingival inflammation chewed gums either containing probiotic or placebo twice a day for a period of 2 weeks. At the end of the experimental period, GCF volume reduction was found to be significant only in the probiotic group.

There are no data available to explain the molecular biological fundamentals for the clinical effects of probiotics in the oral cavity. Several mechanisms are considered to be responsible for the beneficial clinical effects of probiotics, including an interaction with pathogenic bacteria (140), modulation of the host immune response (141) and production of antimicrobial substances (142). The observed clinical effects are very likely a combination of a 'direct competition' between pathogenic bacteria and probiotics as well as various beneficial effects on the hosts' immune response (143). Probiotic bacteria and their metabolites can be recognized by epithelial and immune cells of the host (60). Several publications reported that, *S. cristatus*, *S. salivarius*, *S. mitis* and *S. sanguinis* are able to reduce the IL-8 response triggered by periodontopathogens as *F. nucleatum* and *A.a* on epithelial cells (141). Köll et al. (144) demonstrated that several human oral *lactobacilli* possess good functional probiotic properties like antimicrobial activity against oral pathogens as well as high tolerance of environmental stress factors, which make them suitable for using as potential probiotics for oral health.

Periodonto-pathogens in microbial dental plaque biofilm can stimulate host cells to increase their MMP release, which is considered as one of the indirect mechanisms of tissue destruction occurring in periodontitis (145, 146). MMPs form the most important group of proteinases responsible for the degradation of matrix proteins during periodontitis, and any imbalance between MMPs and their inhibitors may trigger the degradation of ECM, basement membrane, and alveolar bone (147). The extent of degradative activity in periodontal tissues is largely a function of the balance between the levels of MMPs and TIMPs. It has been suggested that tissue degradation only occurs at locations where the levels of TIMPs are low (88). Fibroblasts and macrophages are the major sources of TIMPs as well as MMPs, and the nature of the message received by these cells is a major determinant of outcome (49).

Cytokines and MMPs are responsible for the most of the periodontal tissue break-down in CP, leading to clinical signs of disease (148). Among these, MMP-8 in GCF has been reported to have some value as a diagnostic marker for periodontitis (144). GCF MMP-8 levels in shallow crevices have been found to be associated with

attachment loss therefore, it was suggested as a prognostic marker (91). The total collagenase activity is mainly derived from PMN, and the enzyme collagenase-2, belongs to the class of MMPs and is classified as MMP-8, which is specifically abundant in GCF. Mancini et al. (149) have proposed using MMP-8 levels in GCF as a screening test for active periodontal destruction. In their study, MMP-8 was 18-fold higher in progressing periodontitis vs. stable periodontitis. Based on a longitudinal investigation in subjects with gingivitis, stable periodontitis and progressive periodontitis, Lee et al. (38) estimated that the total collagenase activity (most likely mainly MMP-8 activity) was 50% higher in the group with progressive lesions. Thus, MMP-8 levels give a clue about the progression of the periodontal disease. Several components in oral fluids have been identified as possible biomarkers for CP, however most of them reflect inflammation rather than periodontal attachment loss (139, 145). MMP-8 in GCF and saliva associates with the initiation and progression of periodontitis and reflects its severity (94, 145, 147, 150) and it has been shown that the active form of MMP-8 is mainly found in sites with active periodontitis, while a latent MMP-8 associates with gingivitis (81, 90, 145, 146, 151). TIMPs, on the other hand, regulate the activities of MMPs (146). In the present study, In SRP + Probiotic group, MMP-8 concentration decreased from 28,91 ng/ml to 16,37 ng/ml at day 90. In SRP + SDD group MMP-8 concentration decreased from 29,67 ng/ml to 17,32 ng/ml whereas in SRP + Placebo group MMP-8 concentration was 24,65 ng/ml at baseline and decreased to 21,8 ng/ml at day 90. Conversely, in SRP + Probiotic group; TIMP-1 concentration increased from 9,2 ng/ml to 9,55 ng/ml at day 90. In SRP + SDD group TIMP-1 concentration increased from 9,3 ng/ml to 9,66 ng/ml at day 90 whereas in SRP + Placebo group, TIMP-1 concentration increased from 9,41 ng/ml to 9,44 ng/ml at day 90.

A survey of literature reveals only two studies performed analyzing the effects of probiotics on MMP-8 levels in saliva and GCF (59, 76). In the study by Ince (76) et al. 3 week usage of probiotics twice a day caused a decrease in the number of MMP-8 levels until 6 months and an increase in the number of TIMP-1 levels along with the favorable clinical parameters. In the present study, in all groups, treatment resulted in significant reductions in MMP-8 concentrations together with significant increase in TIMP-1 concentrations when compared to baseline ($p<0.05$). We can speculate that the significant reductions in MMP-8 concentrations in favor of the SRP + Probiotic group are due to the suppressive effect of probiotics on MMPs with host immunomodulation,

similar to the results of Della Riccia et al. (59). Increased TIMP-1 concentrations after SRP have been reported by previous studies (101). However in the periodontal literature the samples of periodontitis and the healthy subjects of GCF TIMP-1 levels revealed controversial results. TIMP-1 levels increased and decreased in periodontally diseased sites in different periodontal studies. Alpagot et al. (152) stated that sites with high GCF levels of TIMP-1 are at significantly greater risk for progression of periodontitis. In their study, mean TIMP-1 value was higher in periodontitis sites than in healthy sites. They attributed their findings to increased MMP expression by host cells in response to bacterial stimuli may increase TIMP-1 levels in an attempt to prevent the destruction of host tissue cells. On the other hand, although the periodontal treatment yields to a reduction in MMPs, which would bind to free TIMPs, the regulation of TIMP-1 may not be solely dependent on the MMPs (101). Increased levels of TIMP-1 may reflect its involvement in the healing process. On the other hand we may explain the significant reductions in MMP-8 concentration in favor of group SRP + SDD with its inhibitory mechanism on oxidative activation of MMPs and down-regulation of expression of key inlammatory cytokines. Emingil et al. (33) in their study stated that 3 month usage of SDD as an adjunct to SRP can result in lower GCF MMP-8 level when compared to placebo for 6 months. The attributed their findings, were dependent on the host modulatory properties of SDD and also the properties of SSD binding to the calcified tooth surfaces and release in an active form might help to maintain low levels of GCF MMP-8 in a 6 month of period.

Studies investigating the adjunctive benefits of antimicrobials have shown that the biggest changes occur within the first 3 months after treatment. Also, it has been stated that follow-up after 6 months would be likely irrelevant to the adjunctive action, and changes would be independent of the antimicrobial action. Treatment of chronic periodontitis consisting of a reduction of dental plaque with concurrent modulation of the host response is clinically seems effective and can be used in cases of chronic periodontitis.

In conclusion, this study showed that under the given conditions the adjunctive use of *L. reuteri* containing lozenges and SDD tablets resulted in significant additional clinical and biochemical improvements in moderately deep pockets when compared to SRP alone up to day 90. The present study serves as a pioneer in the literature reporting the adjunctive immunomodulatory effects of probiotics and SDD to SRP in CP.

However, further in vitro studies are warranted in order to identify the exact molecular mechanisms of action of these immunomodulatory effect.

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7.APPENDIX

Appendix 1. Informed consent form

YEDİTEPE ÜNİVERSİTESİ HASTANESİ	**YEDITEPE ÜNIVERSITESI TIP FAKÜLTESI KLINIK ARASTIRMALAR DEGERLENDIRME KOMITESI BILGILENDIRILMIS GÖNÜLLÜ OLUR FORMU**

Araştırmanın Adı / Protokol Numarası:

Araştırmanın Konusu:
Kronik Periodontitisli Hastalarda Başlangıç Periodontal Tedaviye Ek Olarak Probiyotik İçeren Strip (Prodentis®) ve Sub-Antimikrobial Doz Doksisiklin Hiklat (Periostat®) 3 aylık Kullanımının Klinik ve Biyokimyasal Olarak Etkinliğinin Değerlendirilmesi"

Araştırmanın Amacı:

Erişkinlerde dişleri çevreleyen çene kemiğinin yatay ve dikey olarak erimesi ve periodontal cep oluşması ile karakterize kronik periodontitisli hastaların tedavisinde başlangıç periodontal tedaviye ek olarak üretici firmanın önerisi doğrultusunda kullanılacak olan probiyotik striplerin ve doksisiklin hiklat içeren tabletlerin 3 aylık kullanımının klinik ve biyokimyasal olarak değerlendirilmesidir.

Araştırmanın Süresi: 6 ay tarihleri arasında yapılacaktır.

Araştırmaya Katılan Gönüllü Sayısı:45

Araştırmada İzlenecek Yöntem:

Araştırma Yeditepe Üniversitesi Dişhekimliği Fakültesi Periodontoloji Anabilimdalı'na dişeti hastalığı şikayeti ile başvuran 30–60 yaş arasında klinik ve radyografik bulgulara göre kronik periodontitis tanısı konulacak her bir yarım çenesinde en az 3 tek köklü sondalanabilir cep derinliği ≥5, gingival indeks ≥ 2 olan dişe sahip 45 hasta seçilerek yapılacaktır.

Çalışmaya dahil edilecek hastaların seçiminde ;

Yeditepe Üniversitesi Dişhekimliği Fakültesi Periodontoloji Anabilimdalı'na başvuran hastalar arasında aşağıdaki kriterleri sağlayanlar dahil edilecektir.

1) Hastaların sistemik olarak sağlıklı olanlar,

2) Çalışmadan 6 ay öncesine kadar periodontal tedavi görmemiş ve periodonsiyumu etkileyecek ilaç kullanmamış olanlar

3) Araştırmaya dahil edilen dişlerde protetik restorasyon bulunmaması

4) Bayan hastaların hamile veya emziren anne olmaması

5) Sigara kullanmamaları

6) Laktoz ve fermente süt ürünlerine alerjik reaksiyon bulunmaması

7) Probiyotik destek ürünü kullanmıyor olmaları.

8) Her yarım çenede, radyografik olarak kemik yıkımının gözlendiği, en az bir periodontal bölgede sondalanabilir cep derinliği (SCD) ≥ 5 mm ve gingival indeks (GI; Löe & Sillness 1963) ≥ 2 değerlerine sahip olan en az 3 adet tek köklü dişin bulunması

9) Doksisiklin hiklat ürünlerine alerjik reaksiyonlarının bulunmaması

10) Ca^{+2} ve Zn içeren ürün kullanmıyor olması

Araştırmanın Planı ve Hasta Grubu

Çalışmaya dahil edilecek hastalara herhangi bir işlem yapılmadan önce periodontal hastalıklar, periodontal hastalığın nedeni olan mikrobiyal dental plak, mikrobiyal dental plaktan korunma yöntemleri, yapılacak periodontal tedaviler ve hastalardan alınacak olan biyokimyasal örnekler, probiyotikler ve kullanılacak striplerle ilgili detaylı bilgiler verilerek sözlü ve yazılı onamları alınacaktır. Onamları alınan hastalara ağız hijyen eğitimi, uygun diş fırçası seçimi, diş ipi ve/ veya arayüz fırçası seçimi ve kullanımı öğretilecektir. Diş fırçalarken Modifiye Bass tekniğinin kullanımı anlatılacak ve günde iki kez, sabah ve akşam olmak üzere dişlerin bu teknikte fırçalanmasını takiben arayüz temizliği yapılması istenecektir.

Araştırmaya dahil edilen hastaların periodontal tedavileri tek bir hekim tarafından yapılacaktır. Başlangıç tedavisinden önce ağız hijyen eğitimi verilen hastalar 1 hafta sonra kontrole çağırılacak ve yeterli düzeyde ağız hijyenini sağlayan hastalar rastgele 7'er kişilik 3 gruba ayrılacaktır. Çalışmaya başlamadan 1 hafta önce hastalardan stent hazırlanması için aljinat ile ölçü alınacak, model hazırlanacak ve seri radyografiler hazırlanacaktır. Çalışmaya dahil edilen tüm hastalardan daha önce tespit edilmiş sondalanabilir cep derinliği ≥5 mm ve gingival indeks ≥2 olan dişe sahip iki bölgeden steril paper pointlerle diş eti oluğu sıvısı örnekleri alınacak ve tüm ağız plak indeksi, gingival indeks, sondalanabilir cep derinliği ve

rölatif ataşman seviyesi değerlerini içeren klinik indeks ve ölçümler yapılıp ağız içi fotoğrafları çekilecektir.

Tüm tedavi gruplarında diş eti oluğu sıvısı alındıktan ve klinik ölçümleri yapıldıktan sonra diş yüzeyi temizliği ve kök yüzeyi düzleştirmesi işlemi 1 hafta arayla toplam 2 seans olarak uygulanacaktır. Bu işlemler ultrasonik cihazlarla (piezon® OEM Built- in Kit, EMS, Switzerland) ve Gracey küretlerle (Gracey, SG 3/4, 5/6, 7/8, 11/12, 13 / 14 minifive, SAS $^{3}/_{4}$,Hu – Friedy, USA) gerçekleştirilecektir. Tur ucuna takılan kıl fırça, lastik kon ve temizleme patları ile dişler cilalanacaktır. Bu dönemde hastaların öğretilen mikrobiyal dental plak uzaklaştıma yöntemleri doğru uygulayıp uygulamadıkları da kontrol edilerek gerekli düzeltmeler yapılacaktır. Başlangıç periodontal tedavi dahilinde, oklüzal travmaya neden olacak erken temas noktaları saptanıp, bu alanlar ortadan kaldırılacaktır, çürük dişler mevcutsa, tedavileri gerçekleştiricilecektir. Ayrıca endodontik konsültasyon sonrasında tespit edilen devital dişler tedavi edilecektir. Çekim yapılacak dişler araştırmaya dahil edilmeyecektir.

1. gruba diş yüzeyi temizliği ve kök yüzeyi düzleştirmesi ile beraber *Lactobacillus reuteri* (Prodentis®) içeren probiyotik strip 3 ay boyunca sabah ve akşam birer tane olmak üzere günde 2 kez kullandırılacaktır. 2. Gruba yüzeyi temizliği ve kök yüzeyi düzleştirmesi ile beraber antimikrobial etkinliğin altındaki doz doksisiklin hiklat (Periostat®) tablet 3 ay boyunca sabah ve akşam birer tane olmak üzere günde 2 kez kullandırılacaktır. 3. gruba diş yüzeyi temizliği ve kök yüzeyi düzleştirmesi ile beraber plasebo (etken madde içermeyen) strip 3 ay boyunca sabah ve akşam birer tane olmak üzere günde 2 kez kullandırılacaktır. 3.ayda klinik ve biyokimyasal örneklemeler tekrarlanacaktır.

Araştırmada Kullanılacak Klinik İndeks ve Ölçümler

Araştırmada kullanılacak indeks ve ölçümlerin birbirini olumsuz yönde etkilememeleri için belirli bir düzen içinde yapılacaktır. Klinik ölçümler, uygulanacak tedavinin içeriği hakkında bilgisi olmayan bir hekim tarafından 0. Gün 3 ve 6. ayda yapılacaktır. Bu işlemler sırasında, muayene sondu ve 0.4 mm çapında 15 mm'lik periodontal sonda (*Universitiy of North Carolina PCPUNC15, Hu-Friedy Ins. Co., ABD*) kullanılacaktır. Periodontal sondanın doğru yerleştirilebilmesi ve tüm ölçüm dönemlerinde hataların en aza indirgenmesi amacıyla sabit rehber noktaları bulunan hastaya özel akrilik stentler yapılacaktır. Bu stentler üst ve altçene için ayrı ayrı dişlerin oklüzal yüzlerini ve kuronal 1/3 ünü kaplayacak şekilde hazırlatılacaktır.

Plak indeksine göre;

0- Gözle bakıldığında ve sondla muayene edildiğinde dişeti kenarında mikrobiyal dental plak yoktur.

1- Dişeti kenarında mikrobiyal dental plak gözle zor seçilirken sadece sonda ile muayenede sondanın ucunda mikrobiyal dental plak gözlemlenmektedir.

2- Dişeti bölgesinde gözle görülebilen ince ve orta düzeyde mikrobiyal dental plak vardır, interdental bölge tamamen dolmamıştır.
3- Dişeti kenarında, dişeti oluğu içerisinde ve komşu diş yüzeyinde fazla miktarda mikrobiyal dental plak vardır, interdental bölge tamamen dolmuştur.

Gingival indeks:

Her dişin meziyo-bukkal, distobukkal ve mid-lingual olmak üzere 4 yüzünde dişetinin renk, ödem, kıvam ve kanama durumuna göre 0-3 arasında değer verilecektir. Bu indekse göre:

0- Normal dişeti
1- Dişetinde hafif iltihap gözlenmektedir, hafif renk değişimleri ve ödem vardır, ancak sondalamada kanama yoktur.
2- Orta derecede iltihap görülür, dişetinde kırmızılık, ödem ve parlaklık vardır, sondalamada kanama mevcuttur.
3- Şiddetli iltihap, belirgin kırmızılık ve ödem vardır, ülserasyon olabilir. Spontan kanamaya eğilim söz konusudur.

Sondalamada kanama:

Sondalanabilir cep derinliği ölçüldükten sonra dişlerin çevresindeki 4 noktasından (meziyo-bukkal, mid-bukkal, mid-lingual, distobukkal) kanama var (+) ya da yok (-) şeklinde kaydedilecektir.

Sondalanabilir cep derinliği:

Akrilik oklüzal stentler ve üzerinde frezle açılan oluklar rehberliğinde, periodontal sonda cep içerisine yerleştirilecektir. Cep tabanı ile dişeti kenarı arasındaki mesafe ölçülecektir. Her dişin bukkal, oral, hem bukkal hem de oral tarafından meziyal ve distal köşe açıları olmak üzere toplam 6 noktasından ölçüm yapılacaktır.

Sondalamada kanama:

Sondalanabilir cep derinliği ölçüldükten sonra dişlerin çevresindeki 4 noktasından (meziyo-bukkal, mid-bukkal, mid-lingual, distobukkal) kanama var (+) ya da yok (-) şeklinde kaydedilecektir.

Rölatif Ataşman Seviyesi

Oklüzal stentler üzerinde sondalanabilir cep derinliği ölçümlerinin yapıldığı noktalardan, stent apikal kenarı sabit rehber noktası alınarak cep tabanı ile stent kenarı arasındaki mesafe kaydedilecektir. Her dişin bukkal, oral, hem bukkal hem de oral taraftan olmak üzere toplam 6 noktadan ölçüm yapılacaktır.

Dişeti Oluğu Sıvısı Toplanması

DOS örnekleri; tedavi öncesi 0. günde ve tedavi sonrası 3 ve 6. ayda her hastanın dört farklı bölgesinden olmak üzere 2farklı zamanda toplanacaktır. DOS örnekleri, Pİ ölçümleri yapıldıktan sonra, dokuları irrite etmemek ve kanamaya yol açmamak için diğer herhangi bir klinik ölçüm yapılmadan önce alınacaktır. DOS toplanılacak olan bölge gaz tampon yardımı ile izole edilip, supragingival plak periodontal sonda ile dikkatle temizlendikten sonra örnekler toplanmaya başlanacaktır. Örnek toplama işlemlerinde periopaper® kullanılacaktır. Periopaper®'lar özel olarak hazırlanmış 250µl'lik tüplere yerleştirilip kodlanacaktır. DOS toplama işleminden hemen önce tartılarak ağırlıkları belirlenecektir. Periopaper® 'lar, defekt bölgelerinde dişeti oluğunun içine en fazla 1mm girecek şekilde yavaşça konumlandırılacak ve 30 sn kadar beklenilecektir. Daha sonra nazikçe oluktan çıkarılan Periopaper®'lar özel olarak hazırlanmış mikrosantrifüj tüplerine aktarılacaktır. Toplanmış olan DOS miktarları hassas terazide tartılacaktır. DOS toplama sonrası ağırlıktan, DOS toplama öncesi ağırlık çıkartılarak DOS miktarı hesaplanacaktır. Hesaplama sonrası örnekler -78°C 'de deney gününe kadar saklanılacaktır. Gözle görülür bir şekilde kan ve tükürük ile kontamine olan örnekler çalışmaya dahil edilmeyecektir. Toplanan DOS örneklerinin yoğunlukları 1 kabul edilerek, sıvının ağırlığı hacime dönüştürülecektir(µg→µl).

MMP-8 Seviyelerinin ELISA ile Tespiti

MMP–8 ve TIMP-1 analizinde 2 adet R&D ELISA kiti kullanılacaktır. Bu deneyde quantitative sandwich enzyme immunoassay tekniği ile çalışılacaktır. Tek kolonda MMP–8 ve TIMP–1 antikoru microplate üzerine önceden yerleştirirlerek standartlar oluşturulacaktır. Standartlar ve örnekler özel pipetlerle kuyucuklara yerleştirilerek MMP–8 veya TIMP–1 varlığında immobilize antikor ile bağlanması sağlanacaktır. Tekrar yıkama işlemi sonrası bağlanmamış antikor enzim ayıraçları temizlenip, substrat solusyonu kuyucuklara eklenip renkleşme olan yerlerde MMP- 8 ve TIMP–1 varlığı tespit edilecektir.

Tüm deney malzemeleri ve örnekler kullanılmadan önce oda sıcaklığına gelene kadar

bekletilecektir. İlk olarak 150µl, "assay diluent RD1–52'', microplate üzerindeki her kuyucuğa eklenecektir. Sonra 50µl standart veya örnek, kuyucuklara yerleştirildikten sonra inku□basyon için 2 saat boyunca oda sıcaklığında yatay karıştırıcıda (0.12''orbit) 500±50 rpm hızında karıştırılarak bekletilecektir. Microplate üzerindeki her kuyucuk "wash buffer'' (400 µl) ile çok kanallı pipet kullanılarak yıkanarak aspire edilecek ve bu işlem her defasında 3 kez olmak üzere toplam 4 defa yıkama yapılmak suretiyle tekrar edilecektir. Son yıkama sonrası kalan artık ''washbuffer'' aspire edilip, plaka bir kağıt havlu üzerine ters çevrilip, sertçe vurularak kuyucukların içerisindeki solüsyonun tamamen boşalması sağlanacaktır. Sonraki adımda 200 µl "MMP-8 conjugate'' her kuyucuğa eklenerek karıştırıcıda 2 saat boyunca oda sıcaklığında inku□basyon için bekletilecektir. Aynı aspirasyon ve yıkama işlemi tekrarlanacaktır. 200 µl ''substrate'' solusyonu her kuyucuğa eklendikten sonra karanlıkta oda sıcaklığında 30 dakika boyunca bekletilecektir. Son olarak 50 µl "stop solution'' her kuyucuğa eklenecektir. Böylece renk değiştirme reaksiyonu durdurulacaktır. Absorbans ölçümü 450nm ayarlı microplate okuyucusu ile 30 dakika içinde yapılıp ve bulgular çıktı olarak alınacaktır. İkinci absorbans ölçümü için microplate okuyucusu 570nm ayarlanıp okuma tekrarlanacaktır. Bu okuma oluşabilecek yansıma hatalarını önlemek için yapılacaktır.

TIMP -1 Seviyelerinin ELISA ile Tespiti

İlk olarak 100µl ''assay diluent RD1X'', microplate üzerindeki her kuyucuğa eklenilecektir. Sonra 50µl ''standart'' veya örnek, kuyucuklara yerleştirildikten sonra inkübasyon için 2 saat boyunca oda sıcaklığında yatay karıştırıcıda (0.12''orbit) 500±50 rpm hızında karıştırılarak bekletilecektir. Microplate üzerindeki her kuyucuk ''wash buffer'' (400 µl) ile çok kanallı pipet kullanılarak yıkanarak aspire edilip ve bu işlem her defasında 3 kez olmak üzere toplam 4 defa yıkama yapılmak suretiyle tekrar edilecektir. Son yıkama sonrası kalan artık ''wash buffer'' aspire edilip, plaka bir kağıt havlu üzerine ters çevrilip, sertçe vurularak kuyucukların içerisindeki solüsyonun tamamen boşalması sağlanacaktır. Sonraki adımda 200 µl 'TIMP-1 conjugate'' her kuyucuğa eklenerek karıştırıcıda oda sıcaklığında 2 saat boyunca inku□basyon için bekletilecektir. Aynı aspirasyon ve yıkama işlemi tekrarlanacaktır. 200 µl ''substrate'' solüsyonu her kuyucuğa eklendikten sonra karanlıkta oda sıcaklığında 30 dakika boyunca bekletilecektir. Son olarak 50 µl ''stop solution'' her kuyucuğa eklenilecektir. Absorbans ölçümü 450nm ayarlı microplate okuyucusu ile 30dk içinde yapılıp ve bulgular çıktı olarak alınılacaktır. İkinci absorbans ölçümü için microplate okuyucusu 570nm ayarlanıp ve okuma tekrarlanacaktır. Bu okuma oluşabilecek yansıma hatalarını önlemek için yapılacaktır.

1.

Alternatif Tedavi veya Girişimler:

Araştırma Sırasında Karşılaşılabilecek Riskler: Literatürde uygulanacak yöntem ile ilgili herhangi bir riskli durum tespit edilmemiştir.

Araştırma İlacının Olası Yan Etkileri: Araştırmada ilaç kullanımı yoktur.

Araştırma Süresince 24 Saat Ulaşılabilecek Kişi Adı / Soyadı / Telefonu:

Dt. Sadberg Cihangir Hamud 05057157115

Bilgilendirilmiş Gönüllü Olur Formundaki tüm açıklamaları okudum. Bana, yukarıda konusu ve amacı belirtilen araştırma ile ilgili yazılı ve sözlü açıklama aşağıda adı belirtilen hekim tarafından yapıldı. Araştırmaya gönüllü olarak katıldığımı, istediğim zaman gerekçeli veya gerekçesiz olarak araştırmadan ayrılabileceğimi ve kendi isteğime bakılmaksızın araştırmacı tarafından araştırma dışı bırakılabileceğimi biliyorum.

Söz konusu araştırmaya, hiçbir baskı ve zorlama olmaksızın kendi rızamla katılmayı kabul ediyorum.

Gönüllünün Adı / Soyadı / İmzası / Tarih

Açıklamaları Yapan Kişinin Adı / Soyadı / İmzası / Tarih

Gerekiyorsa Olur İşlemine Tanık Olan Kişinin Adı / Soyadı / İmzası / Tarih

Gerekiyorsa Yasal Temsilcinin Adı / Soyadı / İmzası / Tarih

8. CURRICULUM VITAE

She was born in Istanbul, in 1987. She started to her education at 1992 in Bursa Özel İnal Ertekin Elementary School. Between 2001- 2005 she went to Bursa Anatolian High School. After passing university exam she went to Minnesota, USA with AFS a student exchange programme. She graduated from Friedly High School in 2006.

She was graduated from Marmara University, Faculty of Dentistry in 2011 and started to the PhD programme in Yeditepe University, Department of Perioodontology.