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CLINICAL AND BIOCHEMICAL EVALUATION OF *LACTOBACILLUS REUTERI* CONTAINING LOZENGES AS AN ADJUNCT TO NON-SURGICAL PERIODONTAL THERAPY IN CHRONIC PERIODONTITIS

PHD THESIS

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ISTANBUL- 2014

Doktora öğrencisi Dt. Gizem İNCE'nin çalışması jürimiz tarafından Periodontoloji Anabilim Dalı doktora tezi olarak uygun görülmüştür.

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ONAY

Yukarıdaki jüri kararı Enstitü Yönetim Kurulu'nun 23/10/2014 tarih ve ....24-4.... sayılı kararı ile onaylanmıştır.

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I. SUMMARY

The effect of probiotics as an adjunct to scaling and root planning (SRP) on inflammatory parameters in chronic periodontitis patients (CP) warrants further investigation because of the controversial data. Therefore, the aim of the present study was to evaluate the clinical and biochemical efficacy of *Lactobacillus reuteri* containing lozenges (ProDentis®) as an adjunct to SRP in CP.

A total of 40 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 2 groups. Group I received SRP + ProDentis® lozenges, whereas Group II received SRP + Placebo. Plaque index (PI), GI, PD, Bleeding on Probing (BoP) were measured and gingival crevicular fluid (GCF) sampling was performed at baseline and days 21, 90, 180 and 360.

At the end of the observation period, statistically significant improvements in all evaluated parameters were observed within each group. Intergroup comparisons of PI, GI, BoP, PD and GCF matrix metalloproteinase-8 (MMP-8) and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels revealed significance in favour of Group-I at all time intervals ($p < 0.05$) except day 360 ($p > 0.05$) At day 360, the significance on the clinical parameters continued to exist ($p<0.05$), however no difference was observed in terms of biochemical parameters ($p>0.05$).

Within the limits of the study, it was concluded that, the adjunctive use of *L.reuteri* containing lozenges (ProDentis®) significantly reduced inflammation in terms of clinical and biochemical parameters compared to SRP alone.

Key Words: SRP, Chronic Periodontitis, Probiotics

II. ACKNOWLEDGEMENTS

Foremost, I would like to thank my supervisor and programme director Prof. Dr. Selçuk Yılmaz for his guidance throughout my postgraduate and PhD doctoral education, sharing his expertise in the field of Periodontology.

My sincere thank to Prof. Dr. Bahar Kuru for her remarkable contributions and support throughout this research.

I have been grateful to Prof. Dr. Ülkü Noyan for offering me her clinical experience and advices in my postgraduate education.

I would like to express my deep gratitude to Prof. Dr. Leyla Kuru for her advice and assistance in keeping my progress on schedule.

I wish to acknowledge the sacrifices made by Assoc. Prof. Dr. Hare Gürsoy, whose help and contributions made this research possible.

I would also like to thank Assoc. Prof. Dr. Ebru Işık Alturfan and her staff, for their guidance and help during the biochemical analysis.

I extend my thanks to Assoc. Prof. Dr. Şebnem Dirikan İpçi, Assoc. Prof. Dr. Gökser Çakar, Dr. Ebru Özkan, Dr. Ogül Leman Tunar and Dr. Can Yenigün for their concern and support during my PhD education.

I want to express my thanks to Arife Çelik for providing me the suitable conditions during the preparation of the present thesis.

Special thanks to my collegues from our department for being helpful every time and for the great times we had together. Also I would like to thank especially to Samed Kuka, who kept a sense of humour during the research when I had lost mine.

Last but not the least, I would like to thank my family: to my dear father Coşkun İnce for his endless support and being the role model of my life, to my mother Nejla İnce for her patience and optimisim, and my only sister Hazal Ezgi İnce for her love and energy.

III. TABLE of CONTENTS

IV. ABBREVIATIONS

AL: Attachment Loss

BoP: Bleeding on Probing

CFU: Colony Forming Unit

CP: Chronic Periodontitis

ECM: Extra Cellular Matrix

ELISA: Enzyme Linked Immunosorbent Assay

FAO: Food and Agriculture Organization

GCF: Gingival Crevicular Fluid

GI: Gingival Index

IFMA: Immunofluorometric Assay

IFN- γ: Interferon gamma

IL: Interleukin

kDa: The dalton

LPS: Lipopolysaccharide

ml: milliliter

mm: millimeter

MMP: Matrix Metalloproteinase

MPO: Myeloperoxidase

mRNA: Messenger Ribonucleic acid

ng: Nanogram

nm: nanometer

OSFMD: One-stage, full-mouth disinfection

PBS: Phosphate Buffered Saline

PD: Probing Depth

PGE2: Prostaglandin E2

PI: Plaque Index

PMN: Polymorphonuclear leukocytes

RAL: Relative Attachment Level

rpm: Revolutions per minute

rRNA: Ribosomal Ribonucleic Acid

RT-PCR: Reverse Transcription Polymerase Chain Reaction

s: Second

SRP: Scaling and Root Planing

TGF-β: Transforming Growth Factor beta

TIMP: Tissue Inhibitors of Metalloproteinase

TNF- α: Tumor Necrosis Factor alpha

WHO: World Health Organisation

VSC: Volatile Sulphur Compounds

µl: microliter

V. FIGURE and TABLE LIST

1. INTRODUCTION and AIM of the STUDY

Periodontal disease results from a complex interplay between the subgingival biofilm and the host immune-inflammatory events that develop in the periodontal tissues and characterized by attachment and alveolar bone loss (1, 2).

Periodontopathogens in microbial dental plaque biofilm are capable of stimulating host cells to increase their matrix metalloproteinase (MMP) release, which is considered as one of the indirect mechanisms of tissue destruction in periodontitis (3). MMPs play an important role both in physiological events and pathological processes in periodontitis and they are produced by infiltrating and resident cells of the periodontium (4). 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 extra cellular matrix (ECM), basement membrane, and alveolar bone (4, 5, 6). One prominent member of matrix metalloproteinase family, MMP-8 is produced and released mainly by polymorphonuclear neutrophils, and is the main type of collagenase in gingival crevicular fluid (GCF) from Chronic Periodontitis (CP) patients (6).

The main aim of the treatment for CP includes reductions of the proportions of periodontal pathogens and increasing the proportions of beneficial species in periodontal pocket (7). Non-surgical periodontal therapy is directed toward removal of the microbial biofilm from the root surface and subgingival area without the surgical reflection of the soft tissues surrounding the teeth, which results a reduction of the number of biofilm microorganisms, and a disturbance of the ecology of the microbial biofilm. As a consequence, host tissues can better cope with the remaining microorganisms, reducing the inflammatory changes of the soft tissues and producing a varying degree of closure of the subgingival pocket (7, 8, 9). Non-surgical periodontal therapy results in reductions in the total microbiota however, the efficacy varies in different situations and the re-colonization of the pretreatment microbiota by periodontopathogens occurs within weeks to months (7-14).

Many adjunctive treatment approaches to non-surgical periodontal therapy have been proposed to reduce/ delay recolonization process. The combined use of local/systemic antimicrobial agents have been associated with the increasing levels of bacterial resistance as well as many side effects (15, 16) whereas lasers and photodynamic therapy still need improvements in terms of clinical efficacy. (13, 15). Recently prominence has been given to the use of probiotics as adjunctives to periodontal treatment .

According to World Health Organisation (WHO), probiotics are defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (17). The possible mechanisms of action of probiotics are; inhibition of pathogens, the promotion of beneficial species and modulation of host immune response (15). The optimistic results obtained from probiotic studies in different fields of healthcare have resulted recently in the introduction of probiotics for oral healthcare (18, 19). Studies reporting the anti- cariogenic effects of probiotics (20-28), their use in the treatment of periodontal disease (29-42), as well as their use in halitosis (43-46), and *Candida albicans* (47, 48) were identified. However, limited number of studies conducted about the usage of probiotics as an adjunct to mechanical subgingival debridement in CP and further evaluations are needed on their periodontal effects in terms of immunomodulation. ProDentis® lozenges consisting of a minimum of 200 million live *L.reuteri* Prodentis (*Lactobacillus reuteri* DSM 17938 and *Lactobacillus reuteri* ATCC PTA 5289) used specifically for the treatment of oral diseases.

In the literature, since there is no study evaluating the adjunctive immunomodulatory effect of probiotic containing lozenges to SRP in CP, therefore the aim of the present study was to investigate the MMP-8 and TIMP-1 levels together with clinical parameters in 12 months follow up period.

2. LITERATURE REVIEW

2.1. Periodontal Diseases and Pathogenesis

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 (49, 50).

The microflora associated with periodontal health remain stable over time and exists in a dynamic equilibrium. In this level, host is able to control the subgingival plaque by 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) (51, 52). Therefore, periodontal disease initiation and progression occurs as a consequence of the host immune inflammatory response to oral pathogens. Periodontal pathogens produce harmful by-products and enzymes (e.g. hyaluronidases, collagenases, proteases) that break down extracellular matrices, such as collagen, as well as host cell membranes, in order to produce nutrients for their growth and possibly subsequent tissue invasion. Many of the microbial surface protein and lipopolysaccharide (LPS) molecules are responsible for eliciting a host immune response, resulting in local tissue inflammation. *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and other periodontal pathogens possess multiple virulence factors, such as cytoplasmic membranes, peptidoglycans, outer membrane proteins, LPS, capsules and cell-surface fimbriae (*P. gingivalis*) (52).

Once immune and inflammatory processes are initiated, various inflammatory molecules, such as matrix metalloproteinases, other host enzymes, cytokines and prostaglandins are released from leukocytes, fibroblasts or other tissue-derived cells

(53, 54). Proteases can degrade the collagen structure of periodontal tissues and thus create inroads for further leukocyte infiltration. Although the production of collagenase from infiltrating neutrophils and resident periodontal tissue cells is part of the natural host response to infection, in periodontal disease and other chronic inflammatory diseases, there is an imbalance between the level of activated tissue-destroying matrix metalloproteinases and their endogenous inhibitors (55).

Acquired and environmental risk factors, such as diabetes mellitus, cigarette smoking and stress, as well as genetically transmitted traits, such as interleukin-1 (IL-1) gene polymorphisms, may accentuate the host inflammatory response to the bacterial challenge and, eventually, the susceptibility to the disease (56, 57, 58). These factors are equally as important as determinants of disease occurrence and severity of outcome (59). The clinical picture observed is a result of the sum of these events (Figure 1).

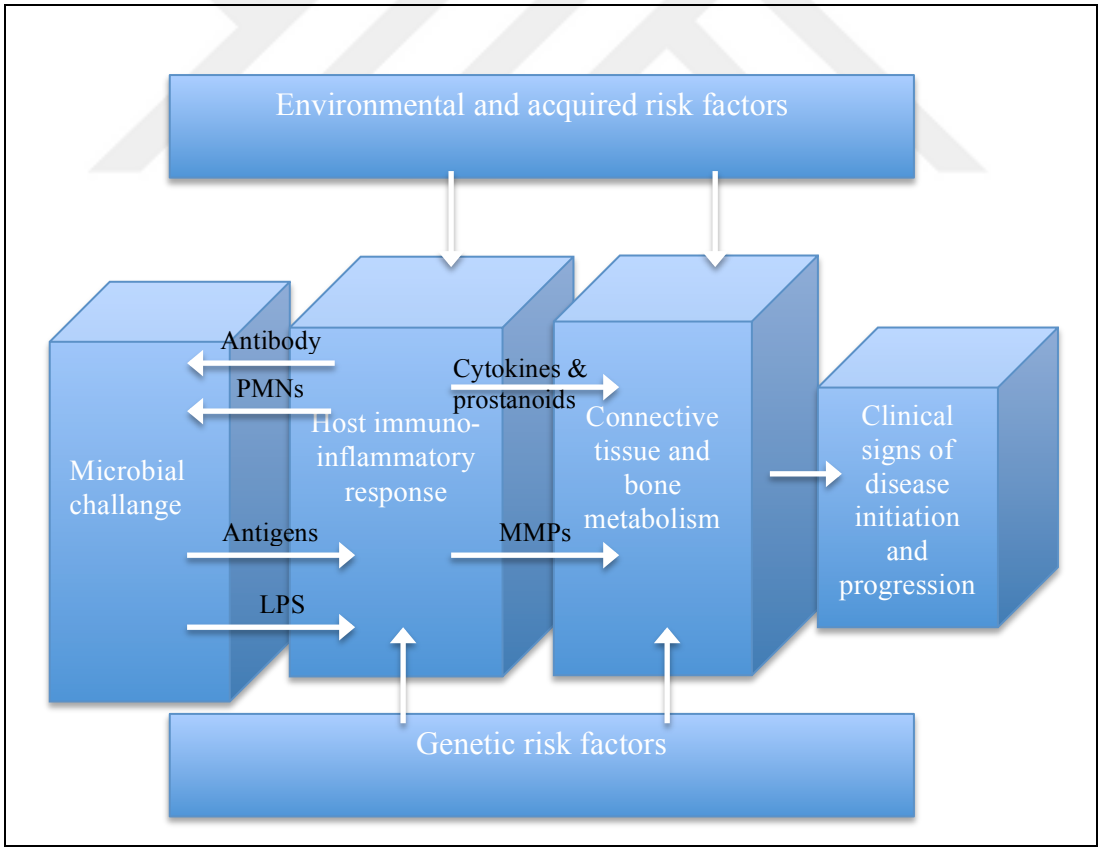

Figure 1. Pathogenesis of human periodontitis (59).

2.2. Chronic Periodontitis

According to the American Academy of Periodontology's classification in 1999, Chronic Periodontitis (CP) is associated with the accumulation of plaque and calculus (60) and is the most commonly occurring form of periodontal disease (61). General clinical findings in CP patients include supragingival and subgingival plaque accumulation associated with calculus formation, gingival inflammation, pocket formation, loss of periodontal attachment and loss of alveolar bone (62). CP generally has a slow to moderate rate of progression, but periods of more rapid destruction may be observed. Increases in the rate of disease progression may be caused by the impact of local, systemic or environmental factors that may influence the normal host-bacteria interaction (60, 63, 64). Overall characteristics of CP are (59, 61, 65):

- Even CP is initiated and perpetuated by a small group of predominantly gram-negative, anaerobic or microaerophilic bacteria that colonize the subgingival area, they are insufficient for the disease to occur. Host factors such as inheritance, tobacco smoking and various other risk factors may even outweigh the bacteria as determinants of whether the disease occurs and of the severity of clinical outcome.
- CP is prevalent in adults but may occur in children.
- The amount of destruction of the periodontal tissues seen in a given patient is commensurate with oral hygiene and plaque levels, local predisposing factors, smoking, stress, and systemic risk factors.
- The subgingival biofilm harbors variety of bacterial species, the composition of the biofilm may vary between subjects and the sites.
- CP is classified as localized when < 30% of sites are affected and generalized when this level is exceeded.
- Severity of CP at the site level may be classified based on the degree of attachment loss as mild (1-2 mm), moderate (3-4mm) an severe (≥ 5 mm).
- Tissue destruction in CP does not effect all teeth equally, has a site specific nature.

2.3. Extracellular Matrix

The connective tissues of the periodontium are composed of fibrous elements including proteins such as collagen and elastin and non-fibrous components that include a variety of glycoproteins, (laminin, fibronectin, proteoglycans) as well as minerals, lipids, water and tissue-bound growth factors (66). The extracellular matrix (ECM) of the periodontium is composed of a diverse number of macromolecules; the predominant one is collagen and the other components include proteoglycan and non-collagen proteins (elastin, fibronectin, laminin, osteocalcin, osteopontin, bone sialoprotein, osteonectin and tenascin). The breakdown of collagen occurs during inflammation, tissue breakdown, 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 chronic periodontitis.

Periodontitis involves the destruction of bone and connective tissues, including collagens, proteoglycans, and other components of the extracellular matrix being adjusted by the host-bacterial interactions (67). Microbial biofilms initiate a host-inflammatory process whereby monocytes, macrophages and neutrophils release enzymes and cytokines that activate osteoclasts and osteoblasts to stimulate bone degradation (68). 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 gingival crevice fluid (Figure 2).

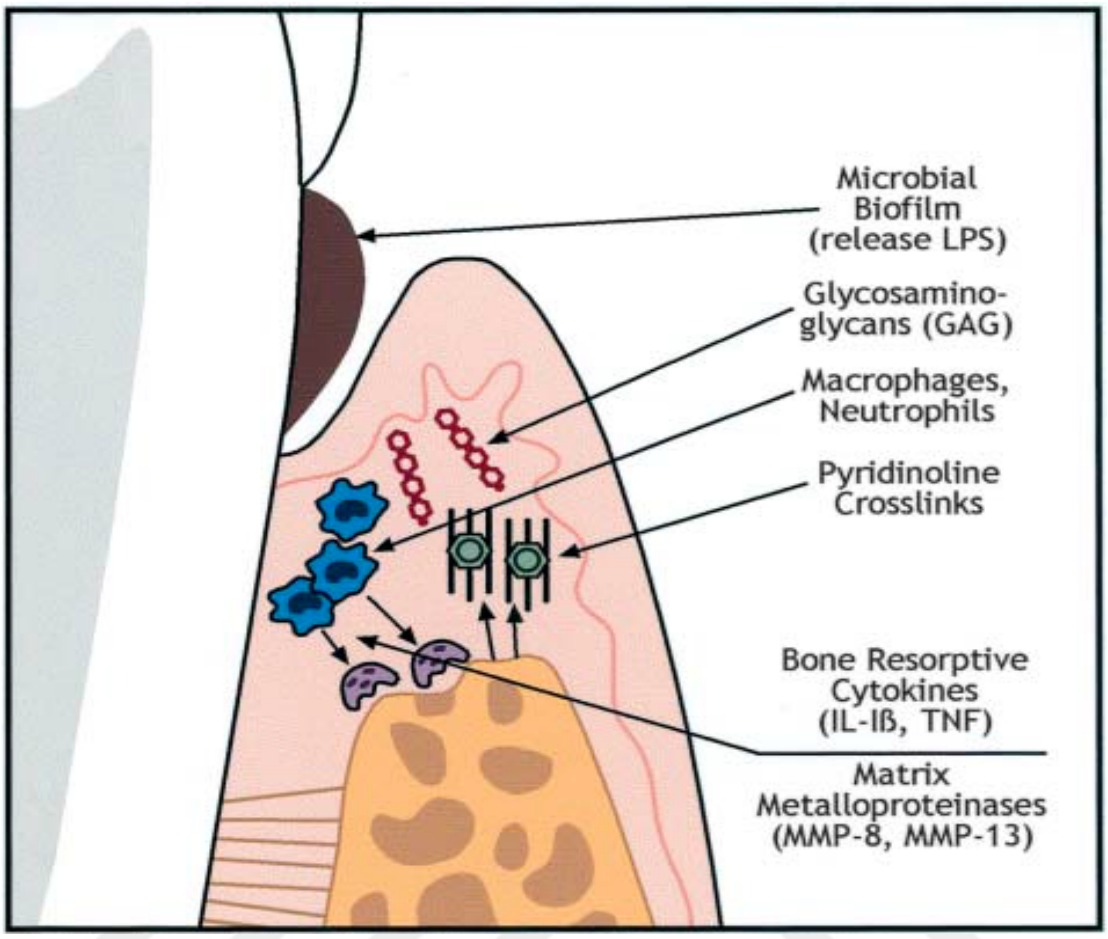

Figure 2. Release of mediators of alveolar bone resorption during active periodontitis (68).

This degradation process can occur by both intracellular and extracellular pathways (69, 70). Degradation of the extracellular matrix involves four distinct pathways. Matrix components may be dissolved by extracellular matrix metalloproteinase (MMP) dependent or plasmin dependent cleavage reactions, on the other hand, larger fragments of matrix may be disposed of by a phagocytic pathway. Mineralized matrices are degraded by osteoclastic pathway, which relies on degradation by lysosomal proteinases in an extracellular compartment (69).

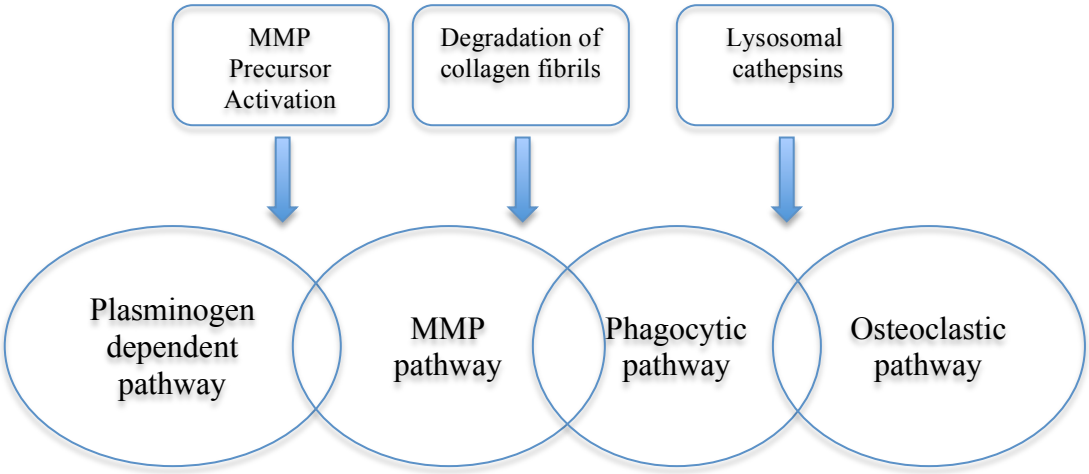

Figure 3. Pathways for degradation of the ECM (69).

2.4. Gingival Crevicular Fluid

Gingival crevice fluid (GCF) is an inflammatory exudate around teeth with inflamed gingiva harbours 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 (70).

Several techniques have been proposed for the collection of GCF and the technique chosen depend upon the objectives of the study (71).

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 may be broadly divided into the intracrevicular and the extracrevicular techniques. The intracrevicular method is the most frequently used method and can be further subdivided 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 its quickness, applicability to individual sites, ease of use and possibly, is the least traumatic when correctly used.

Healthy periodontal sites are characterized by the presence of a microbial plaque, composed of mainly gram-positive microorganisms. In this situation, GCF represents a serum exudate, flowing from the gingival tissues into the gingival crevice. Periodontal diseases are caused by a local inflammatory reaction to a bacterial infection of the teeth, and are manifested by an alteration of the integrity of the tissues supporting the teeth. Gingivitis is characterized by a change in the composition of the microbial plaque, with an increased presence of gram-negative microorganisms (72). These gram-negative bacteria trigger a localized host response, that produces gingival erythema, edema, loss of stippling, pocketing, and bleeding on probing. Histologically, vascular changes with increased vasopermeability and vasodilatation, and the presence of an exudate of polymorphonuclear neutrophils (PMNs), migrating from the tissue into the gingival crevice are observed (73). In periodontitis, the gram-negative subgingival microbial plaque evolves and colonizes deeply into the gingival crevice and triggers a chronic inflammatory response. As the plaque matures, becoming more pathogenic, in parallel,

the host inflammatory response evolves from an acute to a chronic one, which result in increase of GCF volume. The amount of the GCF volume is directly related to the increased vascular permeability and ulceration of the pocket epithelium at inflamed sites and has been found to increase significantly with the severity of gingival inflammation (51, 70, 71, 74, 75, 76). The flow of fluid into the gingival crevice has been reported to arise a few days before other signs of inflammation are detectable (76). Tissue destruction during periodontal inflammation results in production of tissue fragments and growth factors released from periodontal tissues. All these substances reflect the periodontal disease process and can be potentially used as indicators of periodontal condition (77). By the progression of periodontal disease, proteolytic activity of inflamed tissues have been identified in GCF at levels that correlate with disease activity (78, 79, 80).

2.5. Matrix Metalloproteinases

Matrix metalloproteinases 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 (70, 81). These potent enzymes are made in a proenzyme form and activated extracellularly. They play an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction and tissue remodeling (70, 82). They also participate in many pathological processes such as arthritis, cancer and cardiovascular disease (83).

Matrix metalloproteinases are classified into six groups based on the substrate specificity, sequence similarity, and domain organization (84):

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 (also called 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

While the amounts of newly synthesized MMPs are regulated mainly at the levels of transcription, the proteolytic activities of existing MMPs are controlled through both the activation of proenzymes and the inhibition of active enzymes by endogenous inhibitors, α_2–macroglobulins and tissue inhibitors of metalloproteinases (TIMPs).

Elevated collagenase levels in inflamed gingival tissues and in GCF from periodontal pockets have been demonstrated by several studies (85-92). Collagenase activity has been identified in gingival tissues and GCF of patients with periodontitis in much larger amounts than in control subjects (87, 88, 89, 92, 93) whereas tissue inhibitors of metalloproteinase levels could be measured only in healthy individuals or in clinically healthy sites (93).

2.5.1. Matrix Metalloproteinase-8

Matrix metalloproteinase-8 is also referred to as neutrophil collagenase and collagenase-2 is stored intracellularly as a latent proenzyme in the specific granules of PMNs. PMNs play an essential role in phagocytosis and also have a high capacity to infiltrate connective tissue (69, 83, 94). Various agents, such as reactive oxygen species, IL-1 and Interleukin-8 (IL-8), Tumor necrosis factor alpha (TNF-α), cathepsin G, MMP-3 and MMP-8 stimulate the release of active MMP-8 from neutrophils, a key enzyme initiating the breakdown of the ECM, especially during pathological processes such as inflammation, rheumatoid arthritis or osteoarthritis (94, 95).

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 (54). MMP-8 is one of the main biomarkers responsible for the connective tissue breakdown in GCF, saliva and inflamed gingiva in CP (78, 96, 97, 98, 99) and it has been correlated with the severity of periodontal disease (99).

2.6. Tissue Inhibitor Metalloproteinase-1

Although MMPs can be inhibited by the plasma proteinase inhibitor α_2-macroglobulin, the major group of inhibitors of the MMPs in tissues is the TIMPs. TIMPs are modulating factors of MMPs activity, and four members of TIMPs have been reported. Among those, TIMP-1 and TIMP-2, which have inhibitory effects on all MMPs, are found in periodontal lesions (85). TIMP-2 shows strong inhibition against PMN-derived MMPs, whereas TIMP-1 shows greater inhibition against fibroblast-derived MMPs (86). 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 (100). The third member of the family, TIMP-3, was first isolated from chicken cells

and has recently been cloned from human and mouse sources (84, 100). Although, TIMP-3 is considerably different in sequence, still is an efficient metalloproteinase 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 (100).

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 (101).

Chen et al. (91) assessed the periodontal diagnostic potential of immunoreactive MMP-8 in GCF by comparison with elastase activity. GCF was collected from molar and premolar sites of 16 CP patients before treatment and 2 weeks after SRP. Samples were analysed for MMP-8 by immunofluorometric assay (IFMA) and for elastase activity with a fluorogenic substrate. Mean patient clinical parameters and total GCF enzyme levels both decreased significantly after treatment. After SRP, PD reduced from 4.33 mm to 3.58 mm, GI decreased from 1.86 to 0.93 and PI reduced from 1.29 to 0.64. In accordance with clinical parameters, MMP-8 showed a significant decrease, total MMP-8 levels decreased from 19.6 ng to 2.2 ng, while MMP-8 concentration in GCF decreased from 47.9 ng/µl to 16.5 ng/µl. Total MMP-8 levels and elastase activities generally correlated significantly with gingival and bleeding indices. Amounts of these enzymes correlated significantly with each other. Authors concluded that, the immunoassay for MMP-8 is more specific and convenient than functional collagenase assays, and might be suitable for monitoring the periodontal condition.

Mantyla et al. (102) aimed to evaluate the efficacy of MMP-8 specific chair-side dip-stick test in longitudinally monitoring the periodontal status of smoking and non-smoking patients with CP, using their GCF MMP-8 concentrations. Clinical parameters, MMP-8 test results and concentrations were monitored in 15 patients before and after SRP, every other month, over a 12-month time period. Progressing and stable sites, and sites with exceptionally high MMP-8 concentrations, were analysed in smokers and

non-smokers. GCF was collected and analysed for MMP-8 concentration using the MMP-8 specific periodontal chair-side dipstick test as well as by IFMA. An increase of periodontal attachment loss of ≥ 2 mm during the maintenance phase (post-SRP to 12 months) was regarded as periodontal disease progression. SRP reduced the mean GCF MMP-8 levels, test scores, PD, attachment loss and BoP. In sites of periodontal disease progression, the distribution of MMP-8 concentrations was broader than in stable sites, indicating a tendency for elevated concentrations in patients with periodontal disease. The mean MMP-8 concentrations in smokers were lower than in non-smokers, but in smokers' and non-smokers' sites with progressive disease, MMP-8 concentrations were similar. Sites with exceptionally elevated MMP-8 concentrations were clustered in smokers who also showed a poor response to SRP. In these sites, the MMP-8 concentration did not decrease with SRP and these sites were easily identified by the MMP-8 test. Therefore, authors concluded that, persistently elevated GCF MMP-8 concentrations may indicate sites at risk, as well as patients with poor response to conventional periodontal treatment. Also, they suggested that, MMP-8 testing may be useful in order to reflect sites in risk of progression of periodontitis.

Results of these studies showed that, elevated GCF MMP-8 concentrations indicate sites at risk, as well as patients with poor response to SRP. MMP-8 testing may be a useful adjunct to periodontal diagnostic methods during the maintenance.

Passoja et al. (78) analysed the association between MMP-8 concentration in shallow, non-bleeding gingival crevices, and the extent of periodontal disease in 48 patients with CP. MMP-8 concentrations in GCF from four shallow (PD≤ 3 mm), and four diseased sites and in serum, were evaluated by enzyme linked immunosorbent assay (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 attachment loss between 4-6 mm. Authors concluded that, association between MMP-8 levels in shallow crevices and attachment loss provides a new aspect to future studies of MMP-8 as a prognostic marker for periodontal disease.

Kiili et al. (87) aimed to determine the cellular and molecular forms of MMP-8 (collagenase-2) and MMP-13 (collagenase-3) associated with CP patients in GCF and enzyme distribution in gingival tissue. GCF samples were collected directly from the periodontal pockets of 12 untreated patients and the samples were examined by Western immunoblotting with polyclonal antibodies for MMP-8 and MMP-13 and quantification by scanning image analysis. Gingival tissue biopsies from 6 patients was fixed in formalin and embedded in paraffin wax. MMP-8 and MMP-13 were localized using the same antibodies and an avidin-biotin-peroxidase detecting system. As a result, the majority of MMP-8 staining in pre-treatment GCF was present in 80, 75 and 60 kDa bands corresponding to prepro-, pro- and active forms of PMN-type enzyme. 43 and 38 kDa bands evidently represented active, fibroblast type MMP-8. MMP-13 was seen mainly as 60 kDa proenzyme with some 40 kDa active enzyme and a small proportion of 100 kDa complex. The percentages of MMP-8 PMN-type enzyme and MMP-13 proenzyme bands correlated significantly with gingival and bleeding indices. Immunohistochemistry demonstrated MMP-8 in PMNs, sulcular epithelial and also plasma cells in inflamed gingival connective tissue. MMP-13 immunoreactivity was detected in the sulcular epithelium and in macrophage-like cells. Results of this study demonstrated that; multiple species and elevated levels of both MMP-8 and MMP-13 from many rather than single cellular sources in the diseased periodontium are identified in untreated periodontitis GCF and active forms contribute to GCF collagenase activity.

Mouzakiti et al. (103) compared the mRNA expression of MMP-1, -3, -8, -9 and -13 and TIMP-1 in CP before and after initial periodontal treatment. Ninety 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 reverse transcription polymerase chain reaction (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. (80) evaluated 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 1 and 4 week after SRP. The amounts of these humoral factors were significantly lower in the control group at baseline. In test group, SRP resulted in a significant decrease in the amounts of IL-1β, IL-8 and MMP-8 in comparison to baseline, 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.

Kraft-Neumårker et al. (104) investigated the relationship between diagnostic clinical parameters and the concentration of active MMP-8 (aMMP-8) in GCF in a site-level full mouth analysis. PD, BOP, PI and GI, as well as samples of GCF, were obtained from four sites of each tooth of nine systemically healthy female patients with generalized 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 PD 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. In those samples of female CP patients, a broad range of intra individual and inter individual aMMP-8 values were detected. Although the explained variance was rather weak, a statistically significant relationship between aMMP-8 and pocket depth was found.

Romero et al. (92) aimed to determine the levels of MMP-3 and MMP-8 by using ELISA test in GCF, before and after non-surgical periodontal treatment in CP patients. Significant differences in terms of clinical parameters and levels of MMP-3 and MMP-8 were observed between patients with CP and control groups before treatment. Levels of MMP-3 and MMP-8 declined significantly after treatment in CP group showing no difference with controls. Therefore, authors concluded that, the difference between the levels in healthy individuals and patients suggests the important participation of these MMPs in tissue destruction in CP.

The expression of MMPs and TIMPs by cells is regulated by many cytokines, particularly IL-1, growth factors and hormones such as transforming growth factor beta (TGF-β). Many of these factors are products of monocytes/macrophages and their production in inflammatory situations is a part of the events leading to tissue degradation (191). An imbalance between activated MMPs and their endogenous inhibitors leads to pathologic breakdown of the ECM during CP (67, 79, 89).

Alpagot et al. (105) investigated whether MMP-3 and TIMP-1 in gingival GCF could serve as prognostic factors for the progression of periodontitis. They analyzed GCF MMP-3 and TIMP-1 and periodontal status of selected sites in 40 medically healthy subjects over a 6-month period. Clinical measurements including GI, PI, BOP, suppuration, PD, AL, and GCF samples were taken from 2 healthy sites (including sites with gingival recession, GI=0; PD≤ 3 mm; AL≤ 2 mm) and 2 periodontitis sites (GI≥ 1; PD≥ 5 mm; AL≥ 3 mm) of each patient at baseline, 3-month and 6-month visits by paper strips. GCF levels of MMP-3 and TIMP-1 were determined by sandwich ELISA assays. The mean amounts of MMP-3 and TIMP-1 in diseased sites were found significantly higher than in healthy sites. Significantly higher GCF levels of MMP-3 and TIMP-1 were found at progressing sites than in non-progressing periodontitis sites. A progressing site was defined as a site, which had ≥ 2 mm loss of attachment during 6-month study period. GCF levels of MMP-3 were highly correlated with clinical measurements taken at baseline, 3-month and 6-month visits. TIMP-1 levels were only moderately correlated with PD and AL. The regression model for the prediction of PD increase included MMP-3, smoking pack-years, TIMP-1 and accounted for 53% of the

variability. The best model for the prediction of AL increase included MMP-3, smoking pack-years, age TIMP-1 and explained 59% of the variability. These data indicated that sites with high GCF levels of MMP-3 and TIMP-1 are at significantly greater risk for progression of periodontitis.

Tüter et al. (67) evaluated the effect of non-surgical periodontal therapy on GCF MMP-3 and TIMP-1 levels in GCF in 6-week follow-up period. At baseline, clinical parameters including PI, GI, PD, CAL and GCF sampling were performed in 20 CP and 20 periodontally healthy controls. CP patients received SRP and all clinical parameters were recorded and GCF sampling was repeated after treatment. Analysis was performed with ELISA method. At the end of the study, baseline GCF levels of MMP-3 were found to be significantly higher in the CP group than the control, and this level was reduced significantly by the treatment compared to the baseline values whereas baseline TIMP-1 levels were detected lower than post-treatment levels and control group. Hence, authors concluded that, clinical improvements together with the reduction of MMP-3 and increase of TIMP-1 levels in GCF were achieved with SRP. According to the results of the study, the authors emphasized that mechanical periodontal treatment alone caused a reduction in MMP-3 level whereas an increase was observed in TIMP-I level.

2.7. Treatment of Chronic Periodontitis

Chronic periodontitis is initiated by microorganisms living in biofilm communities. Prevention of initiation is related to preventing formation and/ or eradication of the microbial biofilm (61). Periodontal treatment traditionally comprises non-surgical debridement followed by a reevaluation, than surgical periodontal therapy if any need for further treatment (53).

Non-surgical therapy is referred to by a number of names, including **phase I therapy**, **initial therapy**, **cause related therapy** and **etiotropic phase of therapy** (106). The aim of non-surgical periodontal therapy is to control the periodontal

inflammation by reducing the periodontopathogen microorganisms and their products and by giving the host a chance to heal (65, 107). 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 institution of a comprehensive daily plaque control. Control or elimination of contributing local factors includes the following therapies as required (106):

- Complete removal of calculus
- Correction or replacement of poorly fitting restorations and prosthetic devices
- Restoration of carious lesions
- Orthodontic tooth movement
- Treatment of food impaction areas
- Treatment of occlusal trauma
- Extraction of hopeless teeth

Non-surgical therapy for the control of periodontitis consists of supra and subgingival debridement combined with oral hygiene instructions (53). Scaling and root planing (SRP) is considered as a gold standard for treating CP (108). The clinical benefits of SRP come from the removal of subgingival plaque and calculus and disruption of the subgingival microbial flora and as a result a delay in the repopulation of pathogenic microorganisms (109). However mechanical debridement has been shown to fail in eliminating periodontal pathogens from the subgingival niche, due to the tissue invading activity of some periodontopathogenic bacteria and due to the limited access (12, 111). Furthermore, re-colonization of the pretreatment microbiota by periodonto-pathogens occurs within weeks to months (7, 8, 10, 11, 12, 110, 111, 112). Due to the multifactorial etiological nature of periodontitis, choosing appropriate treatment options can be quite difficult; so many adjunctive treatment approaches have been proposed (108).

2.7.1. Systemic Antimicrobials

The adjunctive use of systemic antimicrobials to non-surgical periodontal therapy in the treatment of periodontitis has been evaluated in many studies. The rationale for their use being the suppression of periodontal pathogens persisting in biofilms in deep pockets, furcation areas, root concavities or within the periodontal tissues (108, 113). A range of systemic antimicrobials, including azithromycin (110, 114, 115), spiramisin (116), tetracycline (116, 117), clindamycin (118), doxycycline (118, 119), metronidazole (118, 120, 121, 122), amoxicillin and clavulanic acid (118, 123) or the combination of metronidazole and amoxicillin has been most widely reported (124, 125). A recent systematic review reported that the systemic use of amoxicillin and metronidazole in combination with SRP in patients with CP improves the clinical parameters in favor of this antibiotic combination compared with SRP alone. The results of the meta-analyses showed significantly more clinical attachment gain (0.2 mm) and PD reduction (0.4 mm) in favor of SRP+ amoxicillin/metronidazole combination (126). Despite the obvious benefits of the usage of systemic antimicrobials, there has been a consensus that, owing to the risk of adverse effects, including the development of bacterial resistance, interactions with other drugs and possible allergic reactions, systemic antimicrobials should be used in patients with aggressive periodontitis or in cases where the presence of high levels of periodontopathogens (*A. a and P. gingivalis*) are detected (113).

2.7.2. Local Antimicrobials

Local delivery of antimicrobial agents and delivery systems have been developed with the purpose of maintaining high levels of antimicrobials in GCF with minimal systemic uptake in order to prevent systemic complications. In a systematic review, adjunctive use of tetracycline, minocycline, metronidazole and chlorhexidine resulted in modest improvements in terms of PD reduction and clinical attachment gain compared with SRP alone (127). Although these locally applied sustained-release antimicrobial systems have been shown to offer benefit over SRP alone, the effects were minimal and

short-term (113-127). Previous investigations indicated that the use of essential oils and chlorhexidine, both in mouth rinses and in subgingival irrigation, may provide benefits in the therapy of periodontal disease (128). However, these approaches considerably promote staining and calculus formation, which could interfere with patient compliance in long-term treatment regimes, especially when mouthwash is prescribed (129).

2.7.3. Pocket Irrigation With Antiseptics

The use of antiseptics in conjuction with SRP may include pocket irrigation. Antiseptics, such as povidone-iodine, chlorhexidine gluconate have been used for periodontal pocket irrigation following SRP, aiming the suppression of the biofilm. As GCF is replaced every 90 seconds within the periodontal pocket, the effects of pocket irrigation are transient and systemic effects are minimized (113). The adjunctive use of povidone-iodine irrigation of pockets during non-surgical periodontal therapy offers a small increase in probing depth reduction (130), however, care should be taken as povidone-iodine can cause allergic reactions and should not be used in patients with thyroid dysfunction or during pregnancy and breastfeeding (131).

2.7.4. Lasers

Laser irradiation, in combination with conventional mechanical treatment has been proposed to improve the condition of the periodontal pockets than mechanical therapy alone. Basically, lasers have the potential advantages of bactericidal effect, detoxification effect, and removal of the epithelium lining and granulation tissue, which are desirable properties for the treatment of periodontal pockets. Some lasers may be capable of effectively removing not only dental plaque but also calculus from the root surface with extremely low mechanical stress and no formation of a smear layer on the treated root surface. Furthermore, potential bio-stimulation effects of scattering and penetrating lasers on the cells surrounding the target tissue during irradiation might be helpful for the reduction of inflammation and healing of periodontal tissues (132).

Although laser treatment generally showed tendencies for better results in animal studies most clinical studies failed to show significant differences between laser and conventional therapies (133).

2.7.5. Antimicrobial Photodynamic Therapy

Another promising approach was the use of antimicrobial photodynamic therapy. Long-wavelength visible light (red light) activates photosensitizing agents (photosensitizers) that produce reactive oxygen species, such as free radicals and singlet oxygen. These toxic oxygen derivatives then react with essential cellular components such as DNA, proteins and lipids, leading to cell death (108). Most of the studies, when photodynamic therapy was used as an adjunct to SRP, did not show any beneficial effects over SRP alone. Therefore, the authors concluded that long-term randomized controlled trials reporting data on microbiological changes and costs are needed to support the long-term efficacy of adjunctive antimicrobial photodynamic therapy (133, 134, 135, 136).

Although the pathogenesis of periodontal disease is not completely understood, it is well established that it is an infectious disease and that the host immune and inflammatory response to the microbial challenge mediates tissue destruction (137). Based on this view, the therapeutic strategies for the treatment of periodontal disease have been directed towards two different and complementary paths: Antimicrobial therapy and host modulation. Considering that the primary etiology of the disease is bacteria in the plaque and their products, mechanical and chemical approaches to reduce the presence of periodontopathogens in the plaque have been largely used in the treatment of periodontal patients (138). Recently, a better understanding of the participation of host immune-inflammatory mediators in the disease progression has increased the investigation of the use of modulating agents as an adjunctive therapy to the periodontal treatment.

2.7.6. Host Modulation

Host modulatory therapy 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 (139). Inhibition or blockade of proteolytic enzymes, pro-inflammatory mediators and of osteoclast activity has been the focus of these agents, which has led to encouraging results in pre-clinical and clinical studies. More specifically, three categories of host-modulating agents have been investigated in the periodontal therapy: antiproteinases (represented by tetracyclines); anti-inflammatory drugs; and bone-sparing drugs (represented by antiresorptive agents such as bisphosphonates) (137). Subantimicrobial dose doxycycline (Periostat®) is the only systemic host response modulator specifically indicated as an adjunctive treatment for periodontitis that blocks bacterial and host-derived enzymes associated with alveolar bone and connective tissue loss (19). Subantimicrobial doses of doxycycline have shown to reduce collagenase levels in GCF. Since the dose is subantimicrobial, development of the bacterial resistance is not seen (107). Although it appears to be a safe regimen, with the adjunctive use of 20 mg doxycycline hyclate (Periostat®) to SRP, clinical differences between the therapies were small (19, 102).

2.7.7. Probiotics

The consensus definition of probiotics put forward by the World Health Organization and by the Food and Agriculture Organization of the United States. They defined probiotics as ″live microorganisms which when administered in adequate amounts confer a health benefit on the host″ (417). The idea of probiotics dates back to the first decade of 1900 when the Ukrainian bacteriologist and Nobel Laureate Ilya Metchnikof (1908) 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. 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 (18). According to the Food and Agriculture Organization (FAO) and WHO guidelines (FAO/ WHO, 2002), probiotic microorganisms must be able to survive through the gut passage, must be able to resist gastric juices and exposure to bile. Also they must proliferate and colonize the gastrointestinal tract. Furthermore, they must be safe and effective (140). In order to maintain effective concentrations, probiotics should be ingested on a regular basis (141). To date, 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) (142).

The term 'replacement therapy' which is also called 'bacteriotherapy' or bacterial interference' is usually confused with probiotics. Even both approaches use live bacteria for the prevention or treatment of infectious disease, some slight differences exist (Table 1) (19).

Table 1. Differences between 'replacement' and 'probiotic' therapy (19).

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 at displacing or preventing colonization of a pathogen	
Has a minimal immunological impact	Exerts beneficial effects by influencing the immune system

2.7.7.1. Classification of Probiotics

Probiotic products can contain a single microorganism or a mixture of several species. They are available as capsules, tablets, lozenges, chewing gums, yogurt or diary drinks (143). The most widely used probiotics belong to lactic acid bacteria, especially *Lactobacillus* and *Bifidobacterium* species, *Propionibacterium* and *Streptococcus* (Table 2). The yeast *Saccharomyces boulardii* also reported to have health benefits (141, 143).

Table 2. Microorganisms Used as Probiotics (141).

***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*

2.7.7.2. Probiotic Products

Probiotics are provided in products in one of four basic ways (20):

- 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, yoghurt, yoghurt drink, cheese, kefir and bio-drink.

- As concentrated and dried cells packaged as dietary supplements such as non-dairy products such as powder, capsule, lozenge and gelatine tablets.

Probiotics that are present in the world are shown in Table 3.

Table 3. Over the counter probiotic products in the world. Modified from Caglar et al (20).

Strain	Carrier	Country produced
Bifidobecterium 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, UK, USA
L. casei	Drink	USA
	Yogurt	USA
	Kefir	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

2.7.7.3. Prebiotics

Prebiotics are generally defined as non-digestible carbohydrates used as a nutrient source by probiotic species in the gut 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 improve host health (19, 142). These prebiotics include inuline, fructo-oligosaccharides, galacto oligosaccharides and lactulose. In order to be effective, prebiotic must resist to digestion in the upper gastrointestinal tract and released in the lower tract and used by beneficial microorganisms, mostly *Bifidobacteria* and *Lactobacilli.* Prebiotic effects depend on solubility, distribution, branching and length of the chains of the molecules and they exert their effects mainly through the metabolism of the bacteria they promote (144). In gastro-intestinal applications, it is recommended for probiotics to be combined with prebiotics, thus forming a symbiotic composition with proven health benefits. Prebiotics may be beneficial for the probiotic, especially with regard to *bifidobacteria*, which is known as the symbiotic concept. Symbiotics 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 (19). However, the use of prebiotic concept in order to stimulate the putative probiotic strains to remain longer in the mouth still needs to be evaluated.

2.7.7.4. Mechanisms of Action of Probiotics

Probiotic organisms are thought to act through a variety of mechanisms including the exclusion and competition with potential pathogens for nutrients and epithelial cell adhesion, including degradation of toxins, production of antimicrobial substances, and local and systemic immunomodulation, enhancement of the mucosal barrier function (145, 146). Some probiotic mechanisms induce several beneficial host responses thereby regulating anti-inflammatory and pro-inflammatory cytokine production (141, 147). A combination of probiotic strains is often used to increase the beneficial effects (148, 149).

Lactic acid bacteria produce several metabolites like bacteriocin, adhesion inhibitors, biosurfactans, co-aggregation molecules, hydrogen peroxide and lactic acid (143, 147). These metabolites prevent the growth of food-borne pathogens in diary products. Certain strains of *Lactobacilli* and *Bifidobacteria* can influence immune function by including effects on enterocytes, antigen presenting cells (including both circulating monocytes and local dendritic cells, regulatory T cells, and effector T and B cells (143).

2.7.7.5. Safety of Probiotics

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 (18).

The approach proposed by the Food and Agriculture Organization of the United Nations (FAO) and WHO may be useful in evaluating putatve probiotics (140). Due to this scheme (Figure 4), the phenotype and genotype of probiotic strains should first be established. Thereafter, 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 (e.g., adherence to epithelial cell lines or ability to reduce pathogen adhesion to surfaces). 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 efficacy and 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 (140, 150).

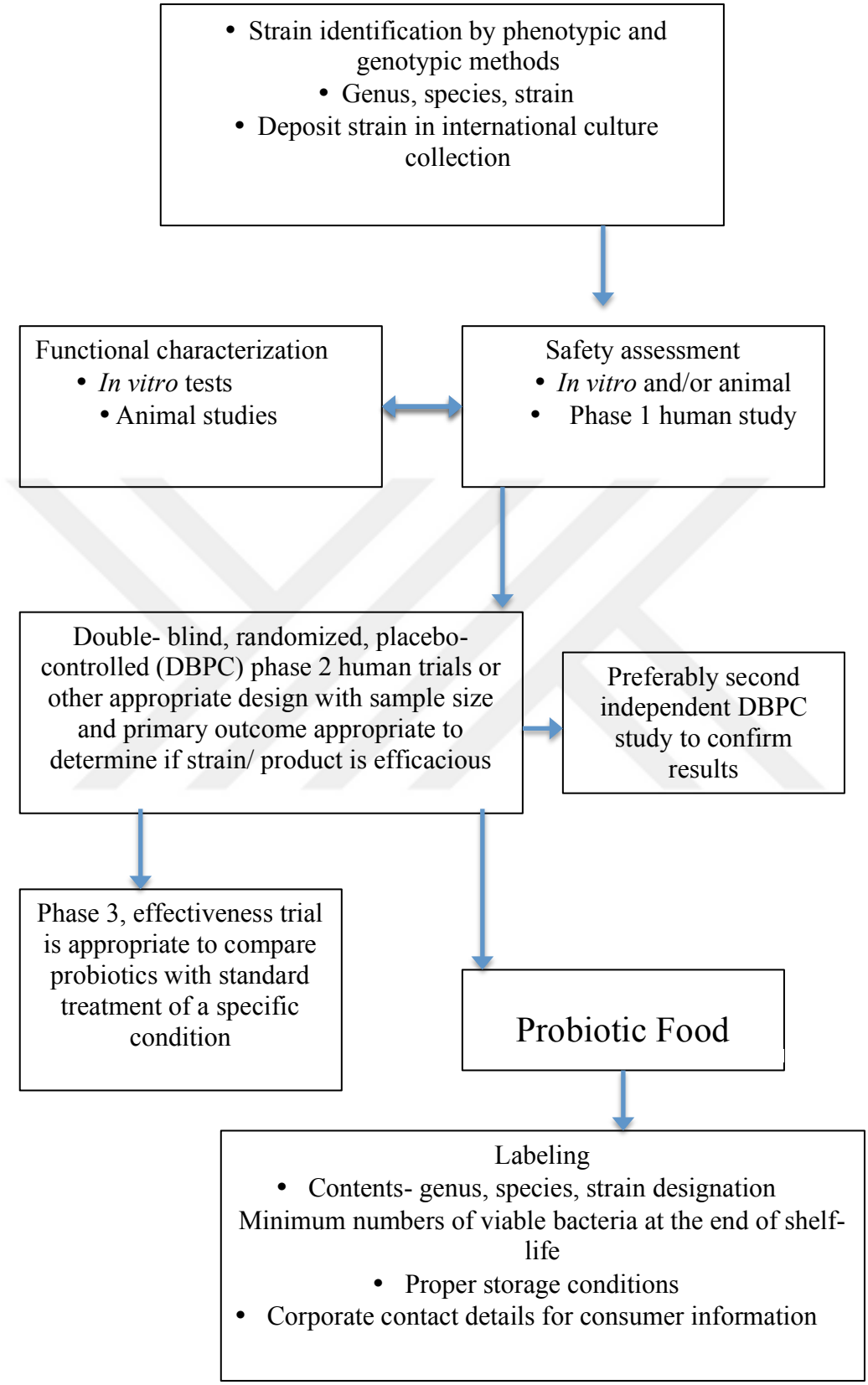

Figure 4. FAO and WHO guidelines for the evaluation of probiotics for food use. Available at: ftp://ftp.fao.org/es/esn/food/wgreport2.pdf (140).

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 (18). Any viable microorganism is capable of causing bacteremia, however, especially in patients with severe underlying diseases or in immune-compromised state. Nevertheless, the present literature supports the conclusion that the incidence of *Lactobacillus* bacteremia is unsubstantial and that all the cases where it has been registered are individuals with other systemic diseases such as diabetes, cardiovascular diseases, gastrointestinal disorders, malignancies, or organ transplant patients. However, it is evident that careful monitoring is needed in this regard in the future (18).

2.7.7.6. Probiotics and General Health

Evidence suggests that probiotics may have a role in general health. The well-substantiated indications of probiotics are:

- **Gastrointestinal disturbances:** Lactose maldigestion, antibiotic-associated diarrhea, rotavirus-associated diarrhea in children (149, 151), maintaining remission in ulcerative colitis, prevention of relapse of chronic pouchitis, and prevention of pouchitis (152).
- **Treatment of urogenital infections:** The presence of *lactobacilli* in the urogenital microbiota of healthy women dominantly, and the obliteration of lactobacilli in patients who develop urinary tract and other genital infections, has led to a thought on *lactobacilli* as potential probiotics for the prevention of urogenital disease (19).
- **Atopic disease:** The preventive potential of *L. rhamnosus GG* in atopic disease has been demonstrated. The possible mechanism is based upon the ability of *lactobacilli* to reverse increased intestinal permeability, promote gut barrier function through the restoration of normal levels of microbes (19, 149).
- **Treatment of oropharyngeal infections:** Acute otitis media, streptococcal pharyngotonsillitis, for the survival of voice prostheses (19).

- **Reduction of the risk for colorectal cancer:** Some epidemiological studies suggest that regular consumption of fermented diary products might reduce the risk for colorectal cancer. This hypothesis is based on the observation that selected *lactobacilli* reduce the activity of certain fecal enzymes that convert pro-carcinogens into carcinogens (153).
- **Immunomodulatory effects:** Probiotics have been demonstrated in experimental animal models of allergy, autoimmunity, however, information from clinical trials in humans is scarce (150).
- In the literature, probiotics are also reported to have immune response induction, reduction of the development of liver cancer and blood pressure lowering effect (18, 19).
- In recent years probiotics have been used as a treatment approach to promote oral health. A survey of the literature reveals that, probiotics have been shown to have anti-cariogenic effects, improve periodontal condition, and reduce halitosis (18, 19, 20).

2.7.7.6.1. Probiotics and Oral Health

Oral microbiota is at least as complex as the gastro-intestinal or vaginal microbiota and dental biofilms are considered to be difficult therapeutic targets (2), the encouraging effects of probiotics in different fields of healthcare have resulted recently in the introduction of probiotics for oral healthcare (18, 19).

Biofilms are the ecological communities evolved to allow survival of the community as a whole, that exhibit metabolic cooperation (67). Microbial shift, more commonly known as dysbiosis, refers that some diseases are due to a decrease in the number of beneficial symbionts and/or an increase in the number of pathogens (51).

An essential requirement for a microorganism to be an oral probiotic is its ability to resist the oral environmental conditions and defense mechanisms. To be able to exert probiotic properties in the oral cavity, it is essential for the microorganism 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 (142). The pattern of adhesion of different probiotic strains to oral epithelial cells has been investigated and most of the experiments on adhesion have been carried out with strains broadly used as probiotics in dairy products such as yogurt and cheese (18). Colonization of *L. rhamnosus* GG (LGG) in the oral cavity of healthy students was assessed by Yli-Knuuttila et al (154). 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 was tested by Kang et al. (155) for its co-aggregation ability with *Fusobacterium nucleatum* which plays an important role as a bridge-organism that facilitates the colonization of other bacteria by co-aggregation (156). Results of this study showed efficient co-aggregation of *W. cibaria* with *F. nucleatum*. Co-aggregation abilities of *lactobacilli* species might enable them to form a barrier that prevents colonization of pathogenic bacteria (157), due to the production of a microenvironment around these pathogens in which inhibiting substances were generated by *Lactobacillus* species.

Haukioja et al. (158) tested the colonization potential of different commercially available probiotics and *Lactobacillus* and *Bifidobacterium* strains obtained from the dairy industry. 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, it has been stated that, *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. In another study, same research group 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 (81).

2.7.7.6.2 Probiotics and Caries Management

Dental caries constitute the most common form of infection in humans. The concept of microbial ecological change for dental caries management has an importance while altered microbial ecology may lead to dental caries (20).

The impact of oral administration of probiotics on dental caries has been studied in several experiments by using different test strains. LGG (21, 22, 23) and *L. casei* (24) have proved their potential to suppress the growth of oral streptococci. Caglar et al. (25) reported significant reduction of *S. mutans* count after a 2-week consumption of yoghurt containing *L. reuteri*. A temporary reduction in *S. mutans* was observed during the period of yogurt intake and few days after cessation of consumption, indicating the necessity of continual administration of the probiotic in order to achieve an effect. Nase et al. (22) tested, LGG, on its caries inhibiting ability in vivo. Five hundred and ninety-four children, 1–6 years old were included in the study. The children drank either milk containing low concentrations (5–10 $\times 10^5$ CFU⁄ml) of live LGG, or control milk without LGG, 5 days a week for 7 months. No significant differences in *S. mutans* scores and caries prevalence was found between the groups, however, reduction of calculated caries-risk score was significant in probiotic group. Ahola et al. (23) examined whether the short-term consumption of cheese containing LGG and *L. rhamnosus* LC 705 would beneficially affect the oral cariogenic microbial flora of young adults when compared with the consumption of regular cheese. During the 3-week intervention period, the 74 adult subjects aged between 18 to 35 years ate either the probiotic cheese containing LGG (1.9×10^7 CFU⁄g) and *L. rhamnosus* LC 705 (1.2×10^7 CFU⁄g), or the control cheese without bacteria. Significantly more patients with decreased *S. mutans* counts were observed after 6 weeks in probiotic group. Montalto et al. (159) reported increased amount of *Lactobacillus* in saliva, with no reduction of *S. mutans* level, when administering to their patients *L. sporogens*, *L. bifidum, L. bulgaricus, L. termophilus, L. acidophilus*, *L. casei* and *L.rhamnosus* in a fluid form or in capsules in a 45-day double-blind, randomized, placebo-controlled intervention study. Nikawa et al. (26) examined the effects of *L. reuteri*-containing yogurt on the *S. mutans* levels. Probiotic yogurt significantly decreased *S. mutans* counts in contrast to

control group. Results also suggested that, *L. reuteri* in yogurt reduces the *S. mutans* levels in saliva for at least up to 2 weeks after discontinuing consumption. In accordance with studies mentioned above, Caglar et al. (25) reported, reduced *S. mutans* levels in patients receiving fluid or tablet probiotic forms. The same research group (27) compared *S. mutans* reduction in subjects using probiotics, xylitol, or probiotics plus xylitol-enriched chewing gums, and a placebo control group. Reduced *S. mutans* levels were observed in subjects using probiotics or xylitol-enriched chewing gum; however, no synergistic effect was seen when combining both agents. In another study by Çaglar et al. (28) probiotics effects were assessed after administration in a pacifier including a tablet that was dissolved in mouth after a 10 to 12-minute suction; reduced *S. mutans* levels versus control group was found.

2.7.7.6.3. Probiotics and Halitosis

Halitosis, the oral malador, is associated with an imbalance of the commensal microflora of the oral cavity. It results from the action of anaerobic bacteria that degrade salivary and food proteins to generate amino acids, which are transformed into volatile sulphur compounds (VSC), including hydrogen sulphide and methanethiol (160). Kang et al. (43) reported the inhibition of the production of VSC by various strains of *W. cibaria*. They concluded that this beneficial effect resulted from the production of hydrogen peroxide by *W. cibaria,* which inhibited the proliferation of *F. nucleatum*. Authors also reported that gargling with a solution containing *W. cibaria* was associated with a reduction in the production of hydrogen sulphide and methanethiol and consequently a reduction in halitosis. Burton et al. (44, 45, 46) also demonstrated the inhibitory effect of *Streptococcus salivarius* on VSC by competing for colonization with species that cause an increase in levels of VSC.

2.7.7.6.4. Probiotics and Fungal Infections

Next to bacterial infections, the periodontal tissues are susceptible to fungal infections (19). Several *Candida* spp., mostly *C. albicans*, cause the most common oral

and oropharyngeal fungal infections. Up to 40- 60 % of healthy population harbours oral *Candida* spp. Multiple and broad-spectrum antibiotics, immunosuppressive drugs, anticholinergic agents, endocrine dysfunction, bone marrow depression, immunodeficiency disorders, malignancies, nutritional deficiencies, radiation treatment, dentures, xerostomia and extreme old age are the predisposing factors for oral candidiasis (candidosis) (161). Since these infectious agents are ubiquitous in nature and slow to respond to drug therapy, fungal infections anywhere in the body are difficult to treat. Similar to bacteria, fungi also have developed a resistance to chemotherapeutic agents, which led researchers for alternative treatments. The use of probiotics is one of these emerging treatment approaches (19). Elahi et al. (47) reported a rapid decline in *C. albicans* levels in mice after the intake of probiotic strains *L. acidophilus* and *L.fermentum*. Continuous consumption of probiotics led to undetectable numbers of fungi in the oral cavity, maintaining the protective effect for a prolonged period after cessation of application. Hattaka et al. (48) performed a randomized double-blind, placebo-controlled study on 192 elderly people aged between 70 to 100 years, in order to investigate the effect of probiotics on the prevalence of oral candida. Reduction in the prevelance of *C. albicans* after consumption of probiotic cheese containing LGG and *Propionibacterium freudenreichii* spp. *Shermanii* JS has been reported after 16-week consumption period. Also an increase of salivation in the probiotic group whereas decreased salivation in the control group was observed. Therefore, the authors concluded that the reduction in prevalence of high candida levels in the probiotic group might be a reflection of the change in the salivation.

Extending research on oral infections including yeast infections, with respect to probiotics and analyzing their molecular mechanisms of activity might broaden their potential applications (18).

2.7.7.6.5. Probiotics and Periodontal Diseases

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 shifts in the microbial community, leading to the onset of periodontal inflammation (142). Current view on the aetiology 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 of beneficial bacteria (145, 162, 163). Since the shift towards a less pathogenic microbiota is temporary after SRP, re-establishment of more pathogenic microbiota occurs within weeks to months (7, 10, 12, 111). This re-colonization process depends on the level of personal oral hygiene, the efficacy of the subgingival debridement and the residual probing depths (7, 12).

Recently, the prominence has been given to the use of probiotics as adjunctives to SRP. The effects of probiotics can originate from three main modes of action: (1) modulation of host defenses including both innate and the acquired immune system, (2) production of antimicrobial substances against periodontopathogens and (3) competitive exclusion mechanisms (19, 142, 145). Probiotics can act on cells of immune system and modulate anti-inflammatory action. Probiotic bacteria and their metabolites can be recognized by epithelial and immune cells of the host (164). Several publications revealed 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. actinomycetemcomitans* on epithelial cells (145, 166). Della Riccia et al. (29) tested the immunomodulatory effects of *Lactobacillus brevis* on periodontal disease. The in vivo use of this probiotic led to a significant decrease in inflammatory markers in the saliva, such as metalloproteinase and nitric oxide synthase activity, prostaglandin E2 (PGE2) and interferon gamma (IFN- γ) levels. Probiotic bacteria can produce a wide range of substances that act as antimicrobial agents such as lactic acid, hydrogen peroxide, bacteriocins and bacteriocin-like inhibitory substances (164). Lactic acids are short-chain fatty acids that can pass across bacterial cell membranes and acidify the cytoplasm, which in turn can inhibit bacterial proliferation (145). Kõll-Klais et al. (167) showed higher prevalence of obligatory homofermentative *lactobacilli*, especially *Lactobacillus gasseri*, among healthy individuals when compared to patients with

periodontitis. Homofermentative *lactobacilli* produce higher concentrations of lactic acid in comparison with heterofermentative *lactobacilli* and induced therefore a more pronounced inhibition of *P. gingivalis*, *Prevotella intermedia and A. actinomycetemcomitans*. Furthermore, production of hydrogen peroxide by probiotic strains has shown to inhibit the growth of pathogenic bacterial species (168). Hillman and Shivers (169) created artificial oral *A. actinomycetemcomitans* infections in gnatobiotic rats. The level of *A. actinomycetemcomitans* colonization in those rats was detected 45-fold lower in animals infected with a hydrogen peroxide-producing *S. sanguinis* strain when compared with rats infected with a hydrogen peroxide-deficient mutant of this *S. sanguinis* strain. Bacteriocins are cationic peptides with a narrow spectrum of antimicrobial activity, whereas bacteriocin-like inhibitory substances have a broader spectrum (146). *S. salivarius* produces two potent bacteriocins, salivaricin types A and B. This strain has been used to prevent dental caries caused by *Streptococcus sobrinus* and *S. mutans*. Salivaricin B was found to be effective to treat halitosis caused by *Prevotella* spp. and *Micromonas micra* (44, 45). Another mechanism of actions of probiotics, 'The competitive exclusion' principle states that two species that compete for the same resources cannot 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, which occurs via hindering the adhesion of pathogenic bacteria as well as competing for the same nutrients (145). It has been shown that several bacterial strains, mostly *streptococci* can impede the colonization of periodontopathogens to hard and soft tissue surfaces in vitro by production of biosurfactants that prevent adhesion (30, 170). Furthermore, probiotics have been shown to inhibit adhesion by modifying the protein composition of the binding site (171). The possible mechanisms that probiotic species might positively affect periodontal health are demonstrated in Figure 5 (142).

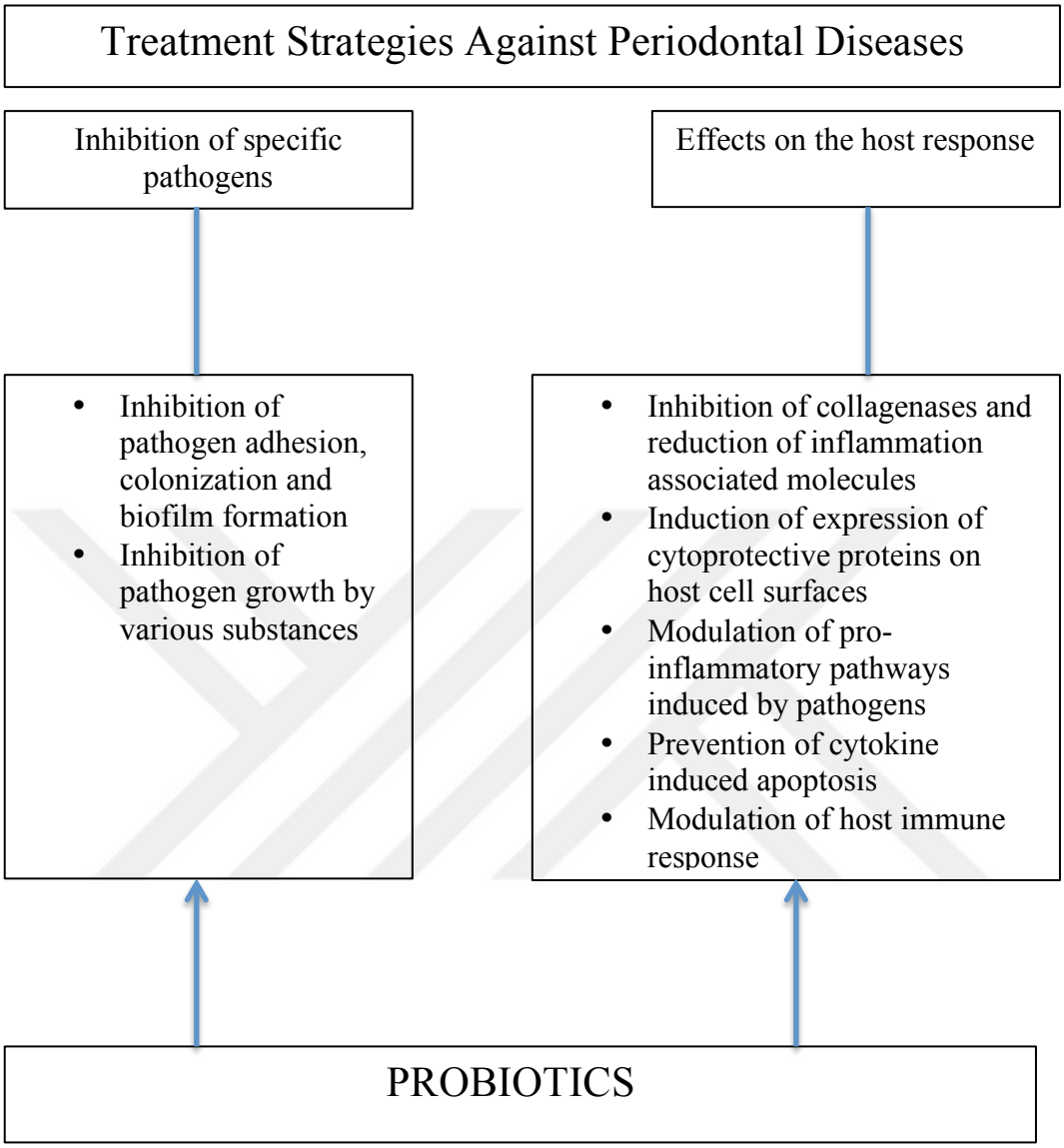

Figure 5. Theoretical possibilities for probiotics to affect periodontal health (142).

The use of probiotics for the beneficial effect in the prevention and treatment of periodontal diseases goes back to 1950s (172). In the 1990s, the use of a complex of five lactic acid bacteria was stated to improve clinical and microbiological parameters in patients with gingivitis and mild periodontitis (19, 31, 32). A periodontal dressing consisted of collagen and *L. casei* 37 was reported to have a beneficial effect on the subgingival microbiota of periodontal pockets (19, 33).

Since the beginning of 21st century, the use of beneficial bacteria for prevention and treatment of plaque-related periodontal inflammation gained acceleration. Ishikawa et al. (34) observed the inhibition of *P. gingivalis, P. intermedia* and *P. nigrescens* after 6-12 hours in coculture with *L. salivarius* TI 2711 in vitro. Also these researchers tested this observation in *in vivo* human studies. 76 volunteers were included in the study and no pretreatment was performed. While control group did not receive any probiotic, the other two groups received *L. salivarius* TI 2711 in tablets either 2×10^7 cfu/day or 1×10^8 cfu/day, five times a day for 8 weeks. Significant reduction in the number of black pigmented anaerobic rods were observed for probiotic groups, however, no significant change in total number of bacteria, number of *S. mutans* or number of *lactobacilli* in saliva were detected.

Krasse et al. (35) evaluated the effect of *L. reuteri* in the treatment of gingivitis in 59 patients. After plaque removal, while probiotic groups received the different strains of *L. reuteri* at a concentration of 1×10^8 cfu by chewing gum, placebo group received an identical chewing gum without probiotic two times a day for 2 weeks. Significantly higher gingivitis reduction was observed in the probiotic group. The plaque scores were also reduced for probiotic groups but no reduction was observed in placebo. These findings led the authors to conclude that, *L. reuteri* is efficacious in reducing gingivitis and plaque scores although the differences were rather small.

In order to identify beneficial bacteria that can retard and prevent periodontopathogen recolonization after SRP, Teughels et al. (15) performed a series of *in vitro* and *in vivo* adhesion experiments. *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. Therefore, researchers tested this hypothesis in an *in vivo* beagle dog model. Although application of beneficial bacteria did not exclude pathogen recolonization, it delayed the recolonization process. Also, Nackaerts et al. (173) reported improved bone density in surgically created periodontal defects in beagle dogs by using the same bacterial mixture. Both of these studies indicated the potential effect of subgingival application of beneficial species in periodontal pockets.

Shimauchi et al. (36) administered freeze-dried *L. salivarius* WB21 (WB21)-containing tablets or a placebo to 66 volunteers without severe periodontitis three times a day for 8 weeks. Periodontal clinical parameters and whole saliva samples were obtained at baseline, at week 4 and at week 8. Salivary lactoferrin levels were measured by ELISA assay. *Lactobacillus* counts in saliva and plaque samples were detected by semiquantitative RT-PCR using 16S rRNA primers. 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. Same authors also reported a significant decrease on the numerical sum of the five selected periodontopathogenic bacteria including *A. actinomycetemcomitans, P. intermedia, P. gingivalis, T. denticola, and T. forsythia.* Results indicated that probiotics could be useful in the improvement/maintenance of oral health in subjects with a high risk of periodontal disease. (37).

To analyze the anti-inflammatory effects of *L. brevis* extracts on periodontitis patients and to investigate the involved mechanisms in vitro on activated macrophages, Della Riccia et al. (29) conducted a study with eight healthy subjects and 21 CP patients. Saliva samples, collected before and after probiotic treatment, were analyzed for metalloproteinase and nitric oxide synthase activity, immunoglobulin-A, PGE2 and IFN- γ levels. The treatment resulted in significant decrease of all clinical parameters such as PI, GI and BoP. This was paralleled to a significant reduction of nitrite/nitrate, PGE2, MMP, and IFN γ levels in saliva samples. Authors concluded that, probiotic treatment suggests an innovative, simple and efficacious therapeutical approach of

periodontal disease.

To examine the immunomodulatory effects of probiotics, Staab et al. (38) administered probiotic milk containing *L. casei* Shirota to fifty volunteer students. For 8 weeks, the study group drank probiotic milk drink, which contained *L. casei* Shirota. No placebo was allocated and no influence on personal oral hygiene procedures was exerted. The subjects were clinically examined at baseline, after 8 weeks consumption of probiotic milk drink and 4 days after a period of experimental gingivitis. GCF levels of PMN elastase, myeloperoxidase (MPO) and MMP-3 were analysed with ELISA. At the end of the study, even clinical parameters were not different between the test and the control group; elastase activity and MMP-3 amounts were significantly lower after the intake of the probiotic milk drink in the test group. Also, there was a significant increase of MPO activity in the control group. So, this data suggested a beneficial effect of the probiotic milk drink on gingival inflammation.

In another study, Twetman et al. (39) investigated the effect of a chewing gum containing probiotic bacteria on moderate gingival inflammation and the levels of IL-1β, TNF-α, IL-6, IL-8 and IL-10 in GCF. The subjects were instructed to chew the gums for 10 min over the course of 2 weeks. GCF sampling was conducted at baseline and after 1, 2 and 4 weeks. The levels of inflammatory mediators were determined using luminex technology and multiplex immunoassay kits. The levels of TNF-α and IL-8 decreased significantly in probiotic groups compared with baseline after 1 and 2 weeks, 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 after 1 and 2 weeks. Findings of this study stated that, probiotic treatment could be a valuable approach for combating inflammation in the oral cavity by reduction of pro-inflammatory cytokines in GCF.

As it is well known, *Lactobacillus reuteri* is an obligate heterofermentative bacteria in the gastrointestinal tracts of humans, and it is reported to produce various compounds such as reuterin (174) and reutericyclin (175), which are water soluble,

broad-spectrum antimicrobials, effective over a wide pH range, and resistant to proteolytic and lipolytic enzymes (159, 176).

To investigate the effects of an orally administered probiotic tablet containing *L. reuteri* on the oral microbiota, Iniesta et al. (177) conducted a placebo-controlled, parallel study in 40 gingivitis subjects during 8 weeks. Treatment consisted on the administration of a daily tablet, either 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. No significant changes were observed between and within the groups in the clinical variables. In saliva, total anaerobic counts after 4 weeks and counts of *P. intermedia* after 8 weeks, had shown reductions in the test group. In subgingival samples, significant reductions in the changes baseline to 4 weeks were observed for *P. gingivalis* counts. With PCR, *L. reuteri* ATCC-PTA-5289 was more frequently detected than *L. reuteri* DSM-17938. Within the limitations of the study, authors concluded that *L. reuteri* containing probiotic tablets were able to colonize the saliva and the subgingival habitat of some gingivitis patients. Thus, 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 only 2 studies evaluating the efficacy of probiotic lozenges used as an adjunct to initial periodontal treatment and which are included in the present thesis. The first one of these studies is by Vivekanda et al. (40) and serves as a pioneer study in this context. The antimicrobial effect of *L. reuteri* Prodentis was shown by Vivekananda et al. (40) *in vivo*. Authors reported the plaque inhibition, anti-inflammatory, and antimicrobial effects of *L. reuteri* Prodentis containing lozenges after administration twice a day for three weeks in CP. Vicario et al. (41) also stated the similar findings after 30 days usage of tablets containing the same strain of *L. reuteri.* However, no attempt was performed to distrupt the biofilm in this study. The patient compliance factor and potential side effects of the probiotic agent were also evaluated. At the end of the study period, no adverse reactions were reported and authors concluded that, one tablet per day allowed for complete compliance from

patients. Teughels et al. (42) clinically and microbiologically evaluated the efficacy of probiotic containing lozenges in 30 CP patients. All patients received one-stage full-mouth disinfection. 15 of the patients were administered probiotic containing lozenges whereas the remaining 15 were administered with xylitol containing placebo lozenges in addition to SRP. The lozenges were used twice a day for a period of 3 months. BoP, PD and attachment gain were evaluated as clinical parameters whereas microbiological evaluation was performed with PCR method. Clinical parameters were significantly reduced in both group, however there was significantly more PD reduction and attachment gain in moderate and deep pockets. On the other hand, microbiologically, more *P. gingivalis* reduction was observed in the test group. As a result, the authors indicated that oral administration of *L. reuteri* lozenges could be a useful adjunct to SRP in CP.

To the best of our knowledge, there is no study in the literature evaluating the possible beneficial effect of probiotics on clinical and biochemical parameters in CP. From this standpoint, we aimed to investigate the efficacy of *L. reuteri* containing lozenges on the periodontal tissue breakdown by analyzing the levels of MMP-8 and TIMP-1 levels in the 12-month follow-up period with a randomized, double blind, placebo controlled clinical trial. The null hypothesis was that neither the clinical profile nor the concentrations of selected biochemical parameters in GCF would differ between the test and the placebo group.

3. MATERIAL AND METHODS

3.1. Patient Selection

The patient population consisted of 40 systemically healthy CP patients aged between 35-50 years, who were seeking for periodontal care and referred to the Yeditepe University Faculty of Dentistry Department of Periodontology. The study design of the present clinical trial was approved by the Yeditepe University Ethical Committee (Appendix 1).

Patient selection criteria were as follows:

1) CP patients with radiographically detected horizontal bone loss
2) Presence of at least 2 teeth, having one approximal site with PD of 5-7 mm and GI of ≥ 2 in each quadrant
3) No periodontal or antimicrobial treatment within 6 months
4) No systemic disease
5) No smoking
6) No pregnancy
7) No use of probiotic supplements
8) No adverse reactions to lactose or fermented milk products.

Patients fulfilling the inclusion criteria were invited to participate in the study. A written informed consent was obtained from all participants after a thorough explanation of the purpose, the nature and the implications of participating in this study (Appendix 2). No changes in the trial design were made after approval by the local Ethics Committee.

3.2. Probiotic Containing Product Under Investigation

The probiotic lozenges consisted of *L. reuteri* (1×10^8 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. Sample Size Calculation

According to the power and sample size program, when the considered parameter is PD, to detect a 0.82 mm difference between the groups, the analysis indicated that 7 patients is required per group (41). The α error was set at 0.05.

3.4. Study Groups

I. Group (SRP+ Prodentis®) (test group n= 20):

Lozenges containing probiotic and xylitol were used for the test group. Each subject was instructed to place one tablet in the mouth and allow it to dissolve without chewing, two times a day for 3 weeks after tooth brushing.

II. Group (SRP+ Placebo) (control group n= 20):

Only xylitol 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, two times a day for 3 weeks after tooth brushing.

The patients returned the bottles containing the probiotic or placebo lozenges at day 21 visit, to check for compliance. Each time in clinical examination, the patient was inquired in relation to general health changes, use of anti-inflammatory drugs, use of mouth rinses, use of probiotic products and any adverse events that the patient might

have noticed (e.g. gastrointestinal disturbances). No adverse event was reported throughout the study period.

3.5. Study Design

The study was designed as a randomized, placebo-controlled clinical trial. Patients were randomly assigned into two treatment groups according to a computer-based randomization table (www.randomizer.org / Copyright ©1997-2011 by Geoffrey C. Urbaniak and Scott Plous) (Appendix 3). 20 patients received SRP+ Probiotic Containing Lozenges (Prodentis®) in test group whereas the other 20 patients received SRP+Placebo in control group.

The study design was shown in Figure 6. Every patient included to the study was given oral hygiene instructions one week before to the experimental period. Patients were instructed to brush with a medium toothbrush in combination with flossing and/or interdental brushing twice a day. The patients were then randomly divided into SRP+ ProDentis® 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 and BoP. Both SRP + ProDentis® 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. Occlusal adjustment was performed if indicated. Probiotic and placebo containing lozenge administration was started after first SRP appointment. At day 21 intraoral photographs, GCF sampling and clinical examination were again performed. Subjects were seen for checking their oral hygiene at day 35 and day 60, and

[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

finally at days 90, 180 and 360, a complete biochemical and clinical examination were repeated.

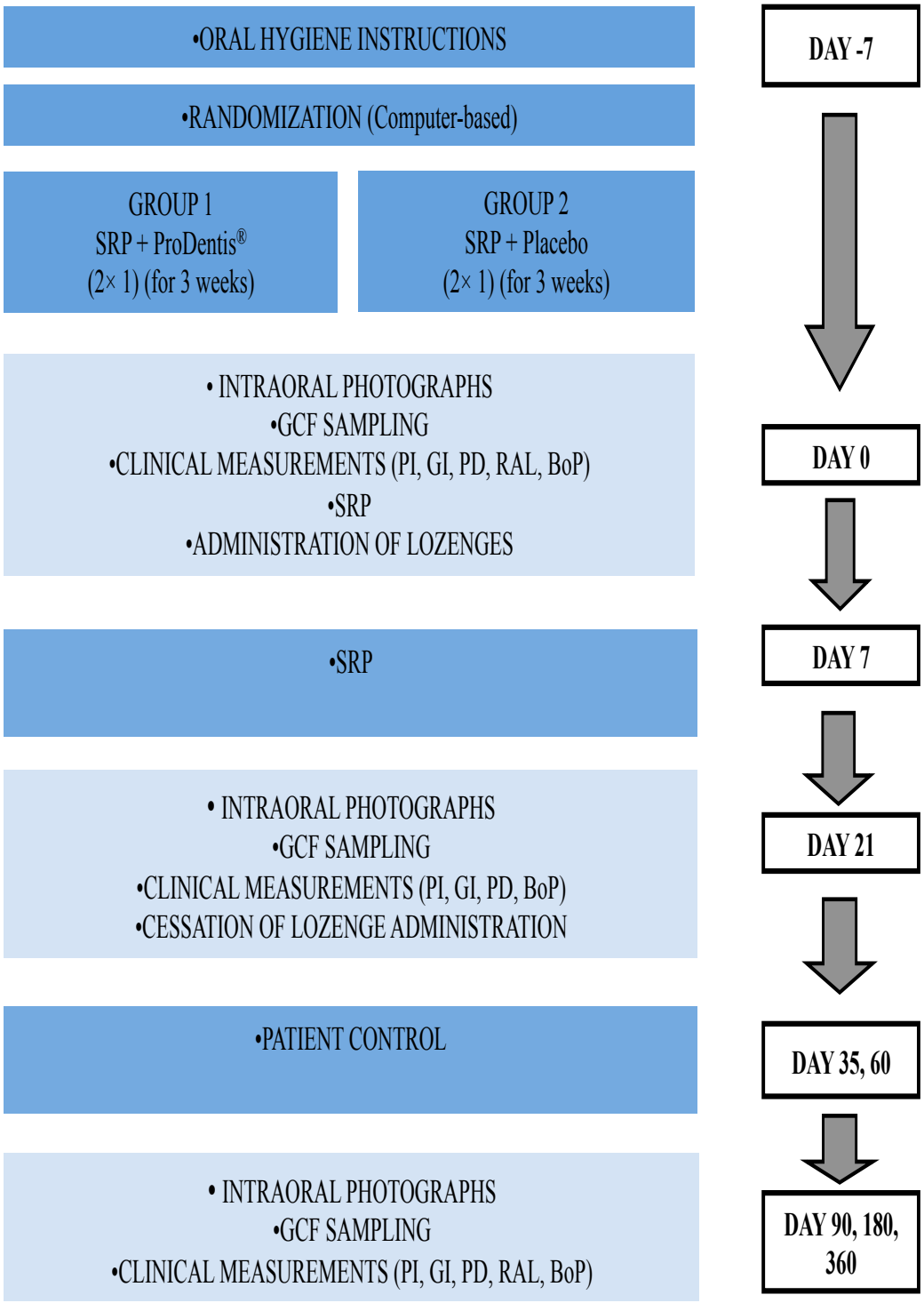

Figure 6. Study Design.

3.6. Clinical Indices and Measurements

All measurements were performed at baseline, and at days 21, 90, 180 and 360 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 7).

The following indices and measurements were used:

3.6.1. 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 (178).

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.

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

3.6.2. 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 mid-lingual margin) and scores between 0-3 were given for each point (179).

Scoring was made as follows:

0 – Normal gingiva

1 – Mild inflammation, slight change in color, slight edema, no bleeding on probing (BOP)

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

3 – Severe inflammation, ulceration and spontaneous bleeding.

3.6.3. 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.

3.6.4. 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.

3.6.5. Bleeding on Probing

Bleeding on probing was assessed simultaneously to the probing measurements, and the presence or absence of bleeding up to 30 s 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 (180).

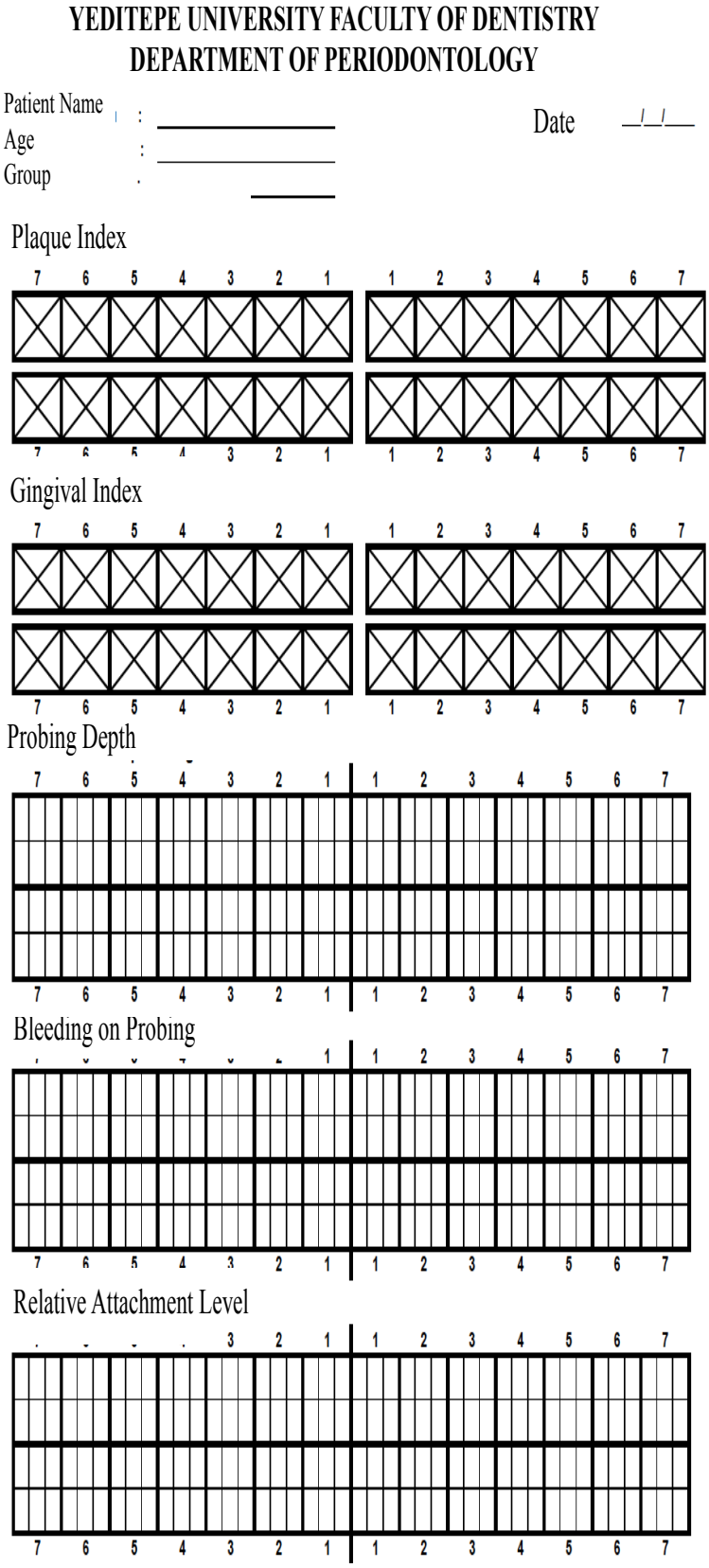

Figure 7. Data Sheet.

3.7. Clinical Procedures

3.7.1. Gingival Crevicular Fluid Sampling

2 single rooted-teeth with approximal PD of 5-7 mm and GI ≥ 2 in each quadrant were selected for GCF sampling. Samples were taken from the same teeth at baseline and days 21, 90, 180 and 360. GCF samples were collected using periopaper®. The sample sites were gently dried and all supragingival plaque were removed. The areas were carefully isolated to prevent samples from being contaminated by saliva. The paper strips were inserted into the crevice until mild resistance was felt or in any event not more than 1mm and left in place for 30 s (181). Care was taken to avoid mechanical injury of the gingival tissues. Strips contaminated by blood or exudate was discarded (182).

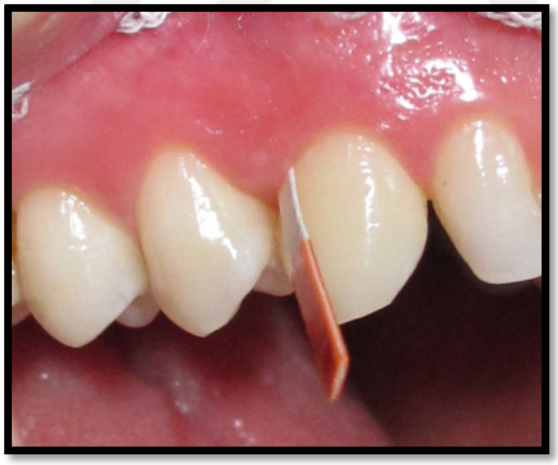

Figure 8. Collection of GCF Samples.

The volume of GCF was determined by means of a previously calibrated electronic device (Periotron[5]) and converted into an actual volume (ml) by reference to

[5] **Periotron 8000** Smithtown, New York USA

the standard curve. All strips with GCF were immediately and individually placed in Eppendorf vials and stored at -70° C until further analysis.

3.8. Laboratory Procedures

3.8.1. Elution of GCF Samples

GCF samples were eluted from the strips by acentrifugal method (181). 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 9). 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 10). 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 above-mentioned commercial ELISA kits.

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

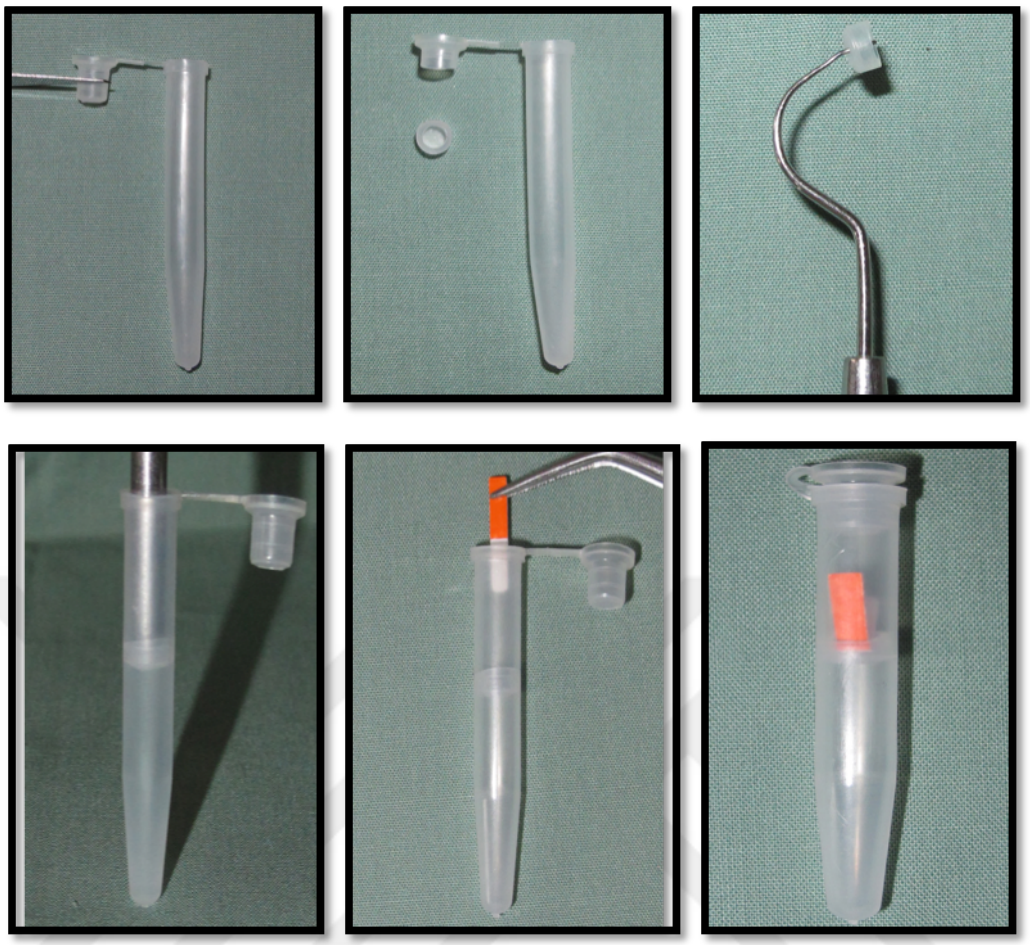

Figure 9. Preparation of the Beckman tubes for elution.

Figure 10. Centrifuge machine.

3.8.2. 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 11). GCF samples were assayed at dilutions 1:20 and 1:100 for MMP-8 and TIMP-1, respectively.

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

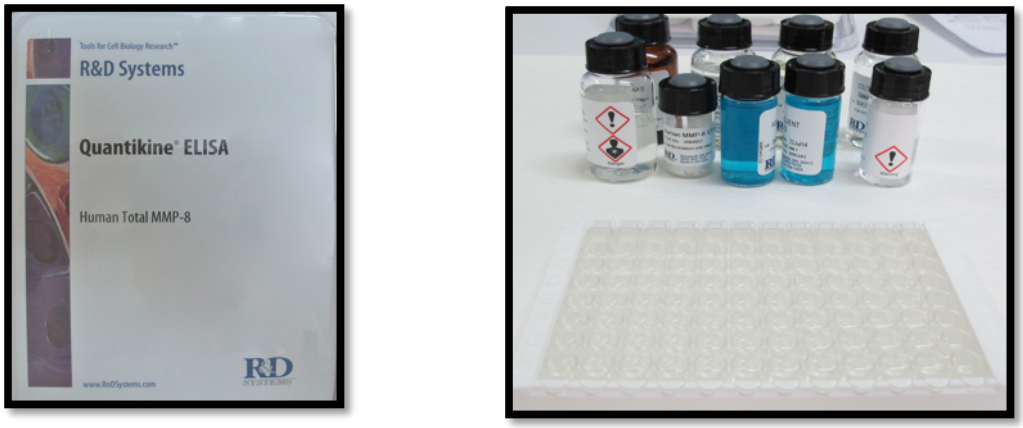

Figure 11. Specific MMP-8 sandwich ELISA kit.

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 two 2 hours at room temperature on a horizontal orbital microplate shaker set at 500±50 rpm. Later on, each well were 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 12).

Figure 12. 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 13). Later on, each well were aspirated and washed, repeating the process 3 times for a total of 4 washes with autowasher (Figure 14). 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 15). Optical density was determined within 30 minutes, using a microplate reader set to 450 nm (Figure 16).

Figure 13. Horizontal orbital incubator.

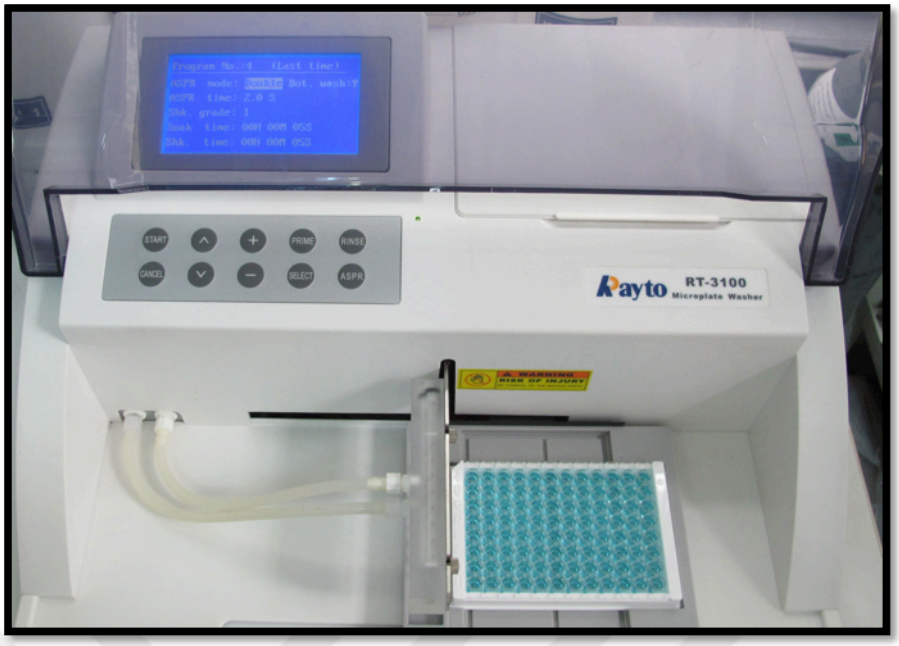

Figure 14. Microplate washer.

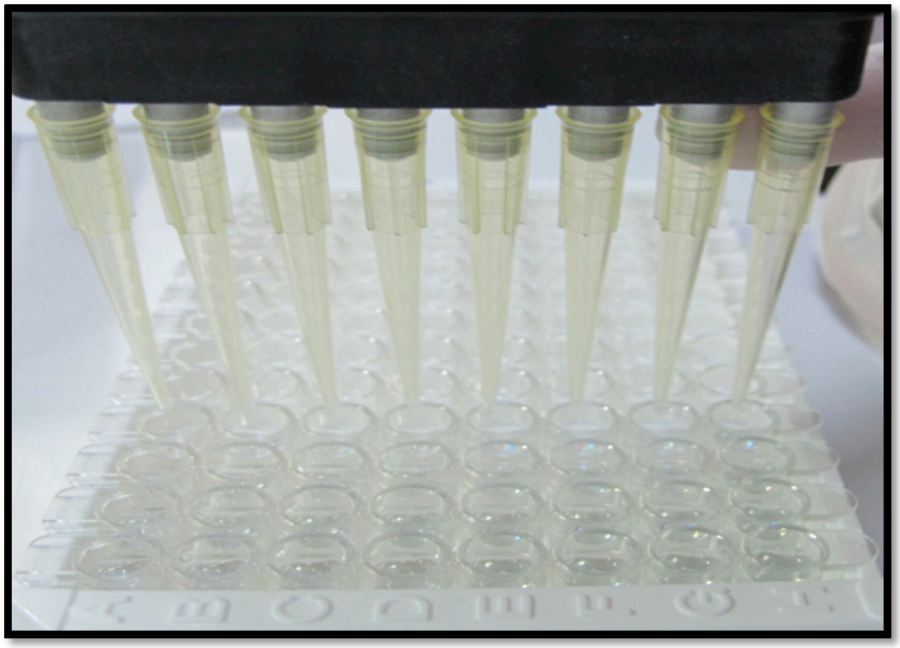

Figure 15. Addition of Stop Solution with multichannel pipettes.

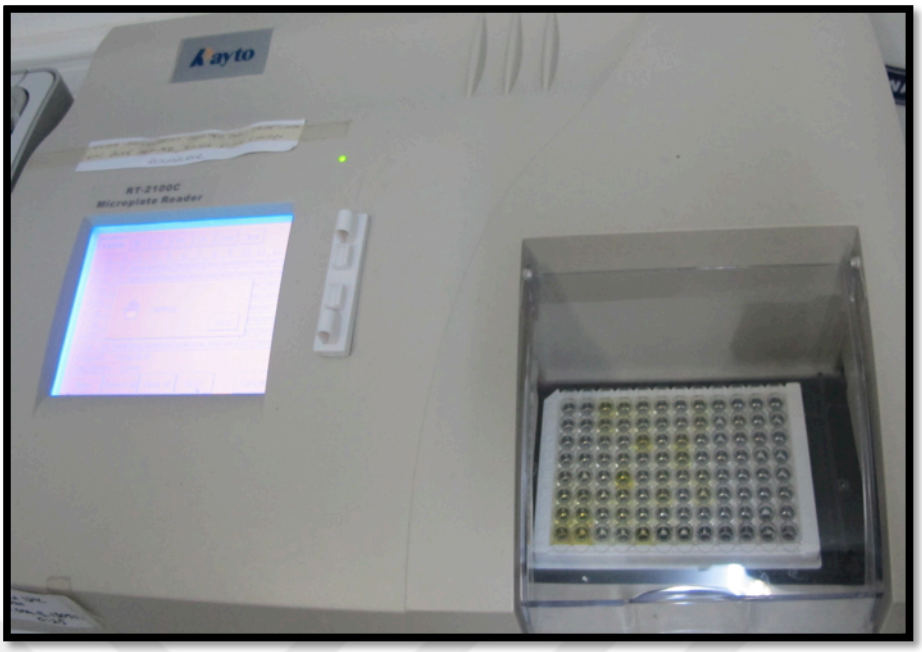

Figure 16. Absorbance measurement with Microplate Reader.

3.9. Statistical Analysis

For all statistical evaluations, the patient was maintained as the unit of measurement. Data analysis was done for full mouth for PI, GI, BoP, PD, GCF volume, MMP-8 and TIMP-1 concentration by using a statistical package (NCSS 2007 & PASS 2008 Statistical Software, USA). The compliance of parameters to the normal distribution was evaluated using Kolmogorov-Smirnov test. The balancing of groups by age and gender was tested by Student t-test and Chi-square test, respectively. Quantitative data was recorded as the mean value ± standard deviation for all investigated parameters. Intra-group multiple comparisons of the parameters in different evaluation periods were evaluated with Repeated measures analysis of variance. When significance was detected after this test, intra-group comparisons in pairs were evaluated by Bonferroni corrected paired sample t-test, whereas the Student t-test was used to evaluate the intergroup differences. For the Bonferroni corrected paired sample t-test statistical significance was set at $p<0.005$ and for the Student t-test statistical significance was set at $p<0.05$.

4. RESULTS

4.1. Demographic and Baseline Datas

40 patients (18 male, 22 female) aged between 35-50 years were included in the study. The mean years of age was 43±5.01 in SRP+ ProDentis® group and 41.40±8.86 in SRP+ Placebo group. No statistically significant differences were detected between the groups in terms of demographic and clinical baseline parameters (Table 4.1).

No adverse effects were reported and none of the subjects was excluded from the study. Intraoral photographs and radiographs of one representative case from each group are shown in Figures 17 (1.a-1.e) and (2.a-2.e).

Table 4.1. Demographic and baseline datas of the patients.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	p
AGE[++]	43±5.01	41.40±8.86	0.816
GENDER (M/F)[+]	8/12	10/10	0.659
PI[++]	2.29±0.28	2.31±0.41	0.659
GI[++]	2.12±0.15	2.12±0.21	0.566
BOP (%)[++]	88.90±7.66	88.65±4.11	0.634
PD (mm)[++]	5.23±0.68	5.36±0.72	0.082
GCF Volume[++] **(µl)**	0.54±0.22	0.59±0.36	0.553
MMP-8[++] **(ng/ml)**	35.91±5.97	35.52±5.80	0.836
TIMP-1[++] **(ng/ml)**	0.43±0.03	0.41±0.03	0.065

[+] Chi-square test, [++]Student t-test, $p<0.05$

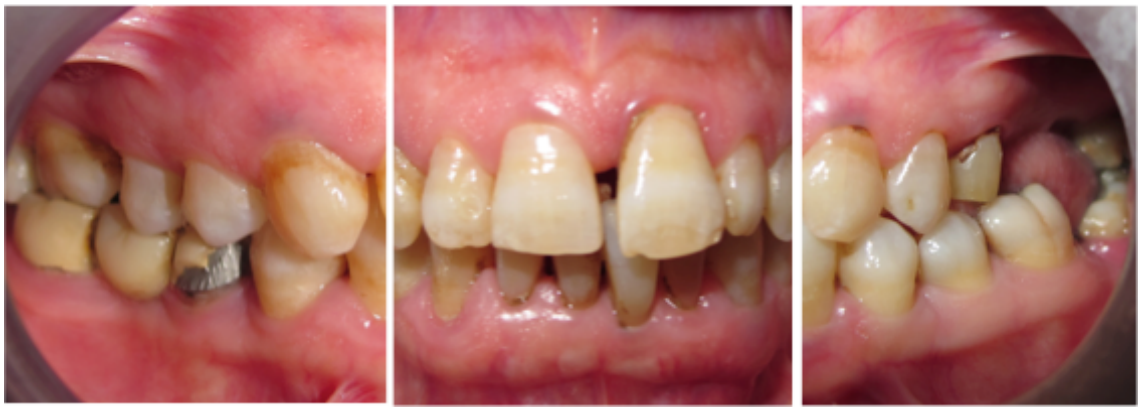

Figure 17.1.a. Clinical view of the patient in SRP+ ProDentis® group at baseline.

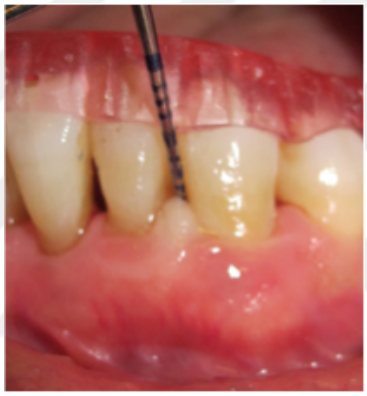

Figure 17.1.b. PD measurement at baseline.

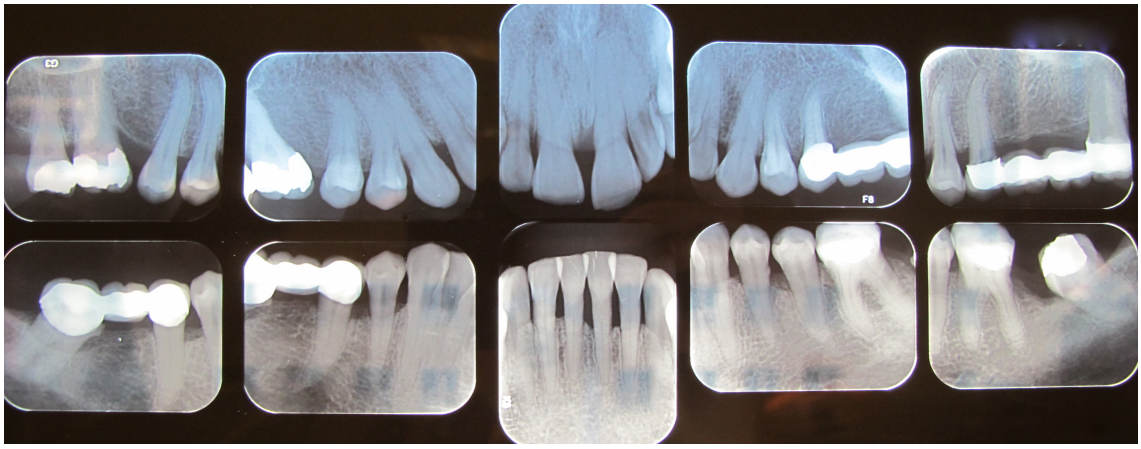

Figure 17.1.c. Radiographic view.

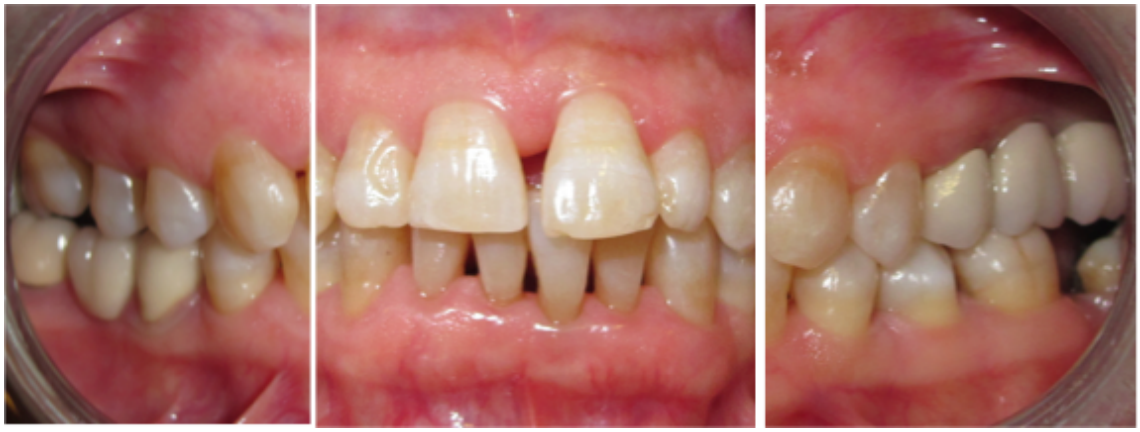

Figure 17.1.d. Clinical view of the patient in SRP+ ProDentis® group at day 360.

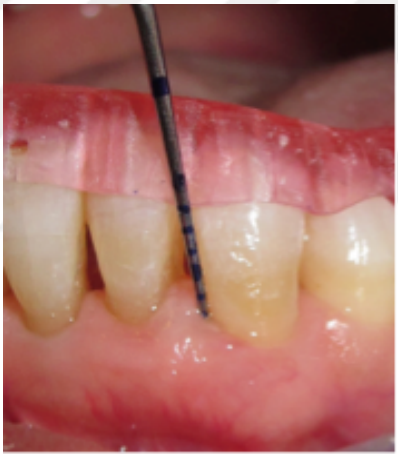

Figure 17.1.e. PD measurement at day 360.

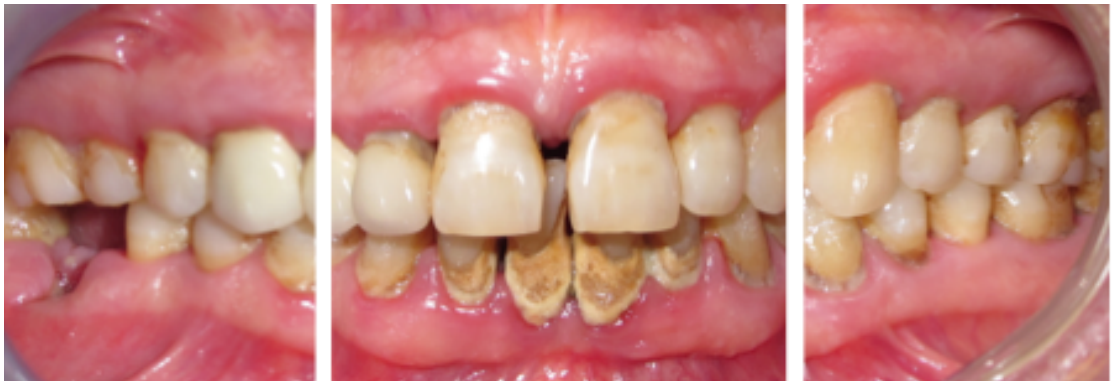

Figure 17.2.a. Clinical view of the patient in SRP+ Placebo group at baseline.

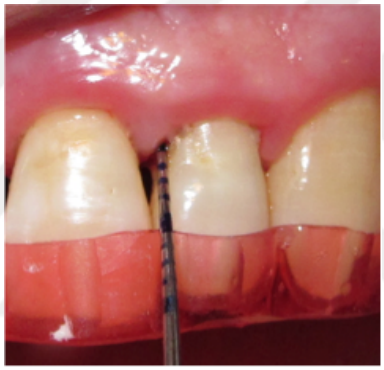

Figure 17.2.b. PD measurement at baseline.

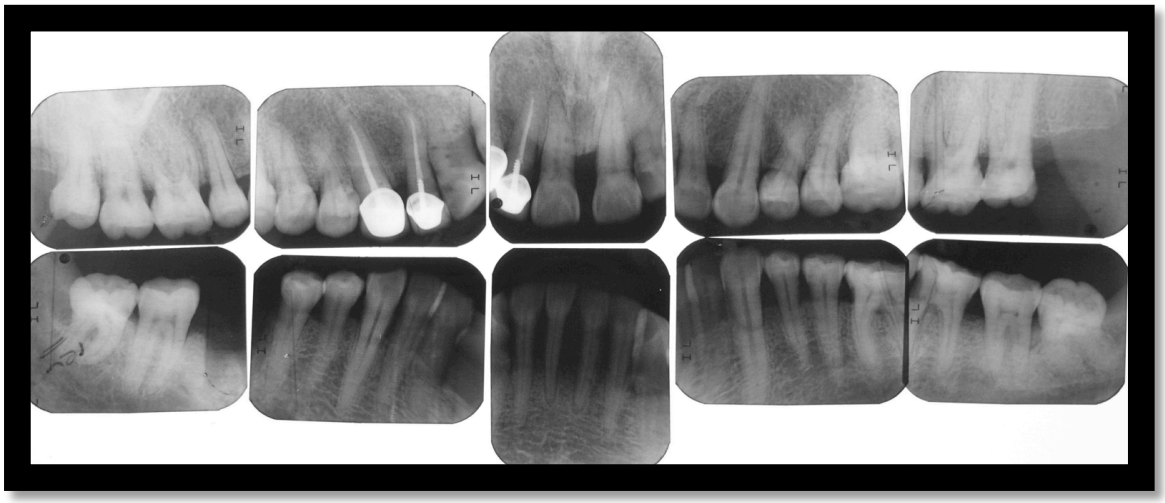

Figure 17.2.c. Radiographic view.

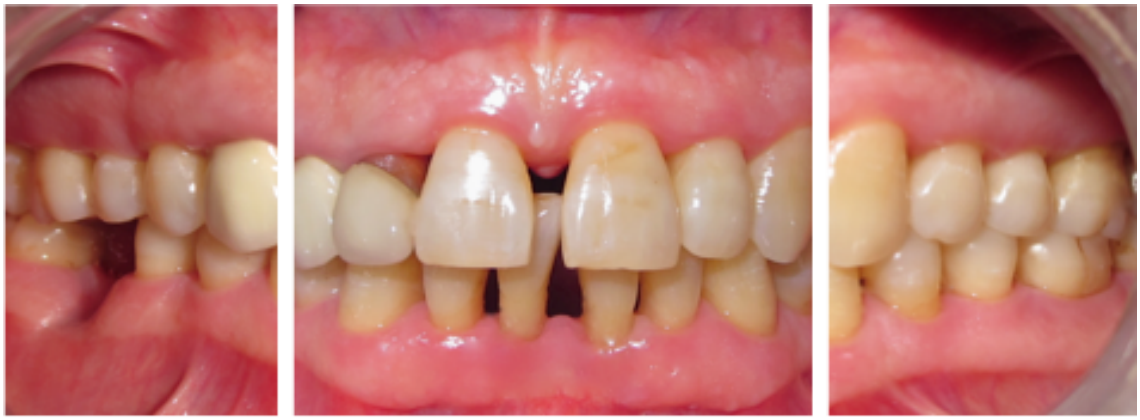

Figure 17.2.d. Clinical view of the patient in SRP+ Placebo group at day 360.

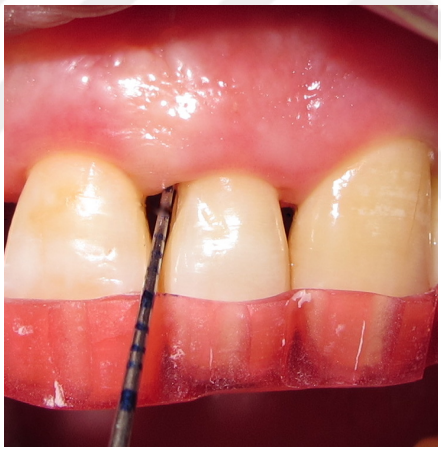

Figure 17.2.e. PD measurement at day 360.

4.2. Clinical Results

The mean values and standard deviations of clinical parameters in terms of PI, GI, BoP (%) and PD at baseline and days 21, 90,180 and 360 are presented in Table 4.1.

4.2.1. Plaque Index

In SRP + ProDentis® group, PI values were detected 2.29±0.28 at day 0 and it reduced to 0.48±0.17, 0.60±0.21, 0.63±0.24 and 0.73±0.24 at days 21, 90, 180 and 360 respectively. In SRP + Placebo group, PI values were detected 2.30±0.41 at day 0 and it reduced to 0.93±0.41, 1.14±0.29, 1.23±0.35 and 1.39±0.28 at days 21, 90, 180 and 360, respectively (Table 4.2.a-4.2.b).

In both groups, intra-group multiple comparisons of PI scores showed statistically significance at different time point measurements (p=0.001; p=0.001) (Table 4.2.a-4.2.b). Subsequent comparisons of mean values of PI scores in pairs in both groups at days 21, 90, 180 and 360 compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001; p=0.001) (Table 4.2.c-4.2.d).

Mean differences of PI scores were detected 1.82±0.35 and 1.37±0.61 between day 0-21; 1.70±0.33 and 1.16±0.54 between day 0-90; 1.66±0.37 and 1.07±0.60 between day 0-180; 1.56±0.37 and 0,91±0,53 between day 0-360 in SRP + ProDentis® and SRP + Placebo groups, respectively. Inter-group comparisons of the mean differences for PI scores between days 0-21, 0-90, 0-180 and 0-360 revealed statistically significant results in favor of the SRP + ProDentis® group (p=0.001; p=0.001; p=0.001; p=0.001) (Table 4.3.a, 4.3.b, 4.3.c, 4.3.d).

4.2.2. Gingival Index

In SRP + ProDentis® group, GI values were detected 2.12±0.15 at day 0 and it reduced to 0.61±0.28, 0.76±0.35, 0.69±0.37 and 0.8±0.38 at days 21, 90, 180 and 360, respectively. In SRP + Placebo group, GI values were detected 2.12±0.21 at day 0 and it reduced to 1.34±0.48, 1.53±0.48, 1.54±0.35 and 1.66±0.36 at days 21, 90, 180 and 360, respectively (Table 4.2.a-4.2.b).

In both groups, intra-group multiple comparisons of GI scores showed statistically significance at different time point measurements (p=0.001; p=0.001) (Table 4.2.a-4.2.b). Subsequent comparisons of mean values of GI scores in pairs in both groups at days 21, 90, 180 and 360 compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001; p=0.001) (Table 4.2.c-4.2.d).

Mean differences of GI scores were detected 1.51±0.31 and 0.78±0.41 between day 0-21; 1.37±0.39 and 0.59±0.39 between day 0-90; 1.43±0.43 and 0.58±0.38 between day 0-180; 1.33±0.43 and 0.46±0.4 between day 0-360, in SRP + ProDentis® and SRP + Placebo groups, respectively. Inter-group comparisons of the mean differences for GI scores between days 0-21, 0-90, 0-180 and 0-360 revealed statistically significant results in favor of the SRP + ProDentis® group (p=0.001; p=0.001; p=0.001; p=0.001) (Table 4.3.a, 4.3.b, 4.3.c, 4.3.d).

4.2.3. Bleeding on Probing (%)

In SRP + ProDentis® group, BoP values were detected 88.90±7.66 at day 0 and it reduced to 21.50±5.88, 16.65±4.21, 12.30±4.82 and 11.05±3.99 at days 21, 90, 180 and 360, respectively. In SRP + Placebo group, BoP values were detected 88.65±4.11 at day 0 and it reduced to 25.65±4.75, 21.85±3.98, 19.95±4.88 and 19,05±4,84 at days 21, 90, 180 and 360, respectively (Table 4.2.a-4.2.b).

In both groups, intra-group multiple comparisons of BoP scores showed statistically significance at different time point measurements (p=0.001; p=0.001) (Table 4.2.a-4.2.b). Subsequent comparisons of mean values of BoP scores in pairs in both groups at days 21, 90, 180 and 360 compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001; p=0.001) (Table 4.2.c-4.2.d).

Mean differences of BoP scores were detected 67.40±6.92 and 63.00±5.10 between day 0-21; 72.25±6.50 and 66.80±4.92 between day 0-90; 76.60±7.98 and 68.70±5.74 between day 0-180; 77.85±7.59 and 69.60±5.77 between day 0-360 in SRP + ProDentis® and SRP + Placebo groups, respectively. Inter-group comparisons of the mean differences for BoP scores between days 0-21, 0-90, 0-180 and 0-360 revealed statistically significant results in favor of the SRP + ProDentis® group (p=0.028; p=0.004; p=0.001; p=0.001) (Table 4.3.a, 4.3.b, 4.3.c, 4.3.d).

4.2.4. Probing Depth

In SRP + ProDentis® group, PD values were detected 5.23±0.68 mm at day 0 and it reduced to 4.03±0.74 mm, 3.80±0.75 mm, 3.38±0.86 mm and 3.49±0.87 mm at days 21, 90, 180 and 360, respectively. In SRP + Placebo group, PD values were detected 5.36±0.72 mm at day 0 and it reduced to 4.60±0.71 mm, 4.51±0.71 mm, 4.66±0.69 mm and 4.80±0.70 mm at days 21, 90, 180 and 360, respectively (Table 4.2.a-4.2.b).

In both groups, intra-group multiple comparisons of PD scores showed statistically significance at different time point measurements (p=0.001; p=0.001) (Table 4.2.a-4.2.b). Subsequent comparisons of mean values of PD scores in pairs in both groups at days 21, 90, 180 and 360 compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001; p=0.001) (Table 4.2.c-4.2.d).

Mean differences of PD scores were detected 1.20±0.37 mm and 0.76±0.36 mm between day 0-21; 1.44±0.33 mm and 0.85±0.32 mm between day 0-90; 1.77±0.69 mm

and 0.70±0.24 mm between day 0-180; 1.74±0.62 mm and 0.57±0.24 mm between day 0-360 in SRP + ProDentis® and SRP + Placebo groups, respectively. Inter-group comparisons of the mean differences for PD scores between days 0-21, 0-90, 0-180 and 0-360 revealed statistically significant results in favor of the SRP + ProDentis® group (p=0.001; p=0.001; p=0.001; p=0.001) (Table 4.3.a, 4.3.b, 4.3.c, 4.3.d).

4.2.5. Attachment Gain

Negative changes in RAL values were determined as attachment gain (mm). In SRP + ProDentis® group, attachment gains were detected 1.18±0.36, 1.67±0.24 and 1.39±0.26 at days 90, 180 and 360, respectively. In SRP + Placebo group, attachment gains were detected 0.79±0.32, 0.66±0.22 and 0.53±0.24 at days 90, 180 and 360, respectively.

In both groups, intra-group multiple comparisons of attachment gain values showed statistically significant differences between days 90, 180 and 360 (p=0.001; p=0.001) (Table 4.2.a-4.2.b).

Inter-group comparison of attachment gain values showed statistically significant differences in favor of SRP + ProDentis® group between days 0-90, 0-180 and 0-360 (p=0.001; p=0.001; p=0.001) (Table 4.3.b, 4.3.c, 4.3.d).

Table 4.2.a. Intra-group comparisons of clinical parameters of SRP + ProDentis® group at baseline and days 21, 90, 180 and 360.

	Group 1 SRP + ProDentis® (mean± SD)					
Clinical parameters	**DAY 0**	**DAY 21**	**DAY 90**	**DAY 180**	**DAY 360**	**P***
PI	2.29±0.28	0.48±0.17	0.60±0.21	0.63±0.24	0.73±0.24	**0.001**
GI	2.12±0.15	0.61±0.28	0.76±0.35	0.69±0.37	0.80±0.38	**0.001**
BoP (%)	88.90±7.66	21.50±5.88	16.65±4.21	12.30±4.82	11.05±3.99	**0.001**
PD	5.23±0.68	4.03±0.74	3.80±0.75	3.38±0.86	3.49±0.87	**0.001**
Attachment Gain			1.18±0.36	1.67±0.24	1.39±0.26	**0.001**

* Repeated measures analysis of variance, $p<0.05$
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 4.2.b. Intra-group comparisons of clinical parameters of SRP + Placebo group at baseline and days 21, 90, 180 and 360.

	Group 2 SRP + Placebo (mean± SD)					
Clinical parameters	**DAY 0**	**DAY 21**	**DAY 90**	**DAY 180**	**DAY 360**	**P***
PI	2.30±0.41	0.93±0.41	1.14±0.29	1.23±0.35	1.39±0.28	**0.001**
GI	2.12±0.21	1.34±0.48	1.53±0.48	1.54±0.35	1.66±0.36	**0.001**
BoP (%)	88.65±4.11	25.65±4.75	21.85±3.98	19.95±4.88	19.05±4.84	**0.001**
PD	5.36±0.72	4.60±0.71	4.51±0.71	4.66±0.69	4.80±0.70	**0.001**
Attachment Gain			0.79±0.32	0.66±0.22	0.53±0.24	**0.001**

* Repeated measures analysis of variance, $p<0.05$
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 4.2.c. Intra-group comparisons of clinical parameters of SRP + ProDentis® group in pairs.

Clinical parameters	DAY 0-21 p**	DAY 0-90 p**	DAY 0-180 p**	DAY 0-360 p**
PI	**0.001**	**0.001**	**0.001**	**0.001**
GI	**0.001**	**0.001**	**0.001**	**0.001**
BoP (%)	**0.001**	**0.001**	**0.001**	**0.001**
PD	**0.001**	**0.001**	**0.001**	**0.001**
Attachment Gain		**0.001**	**0.001**	**0.001**

** Bonferroni corrected paired sample t test, p<0.005
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 4.2.d. Intra-group comparisons of clinical parameters of SRP + Placebo group in pairs.

Clinical parameters	DAY 0-21 p**	DAY 0-90 p**	DAY 0-180 p**	DAY 0-360 p**
PI	**0.001**	**0.001**	**0.001**	**0.001**
GI	**0.001**	**0.001**	**0.001**	**0.001**
BoP (%)	**0.001**	**0.001**	**0.001**	**0.001**
PD	**0.001**	**0.001**	**0.001**	**0.001**
Attachment Gain		**0,001**	**0.001**	**0.001**

** Bonferroni corrected paired sample t test, p<0.005
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 4.3.a. Inter-group comparisons of mean differences of the clinical parameters between days 0-21.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
PI	1.82±0.35	1.37±0.61	**0.001**
GI	1.51±0.31	0.78±0.41	**0.001**
BoP (%)	67.40±6.92	63.00±5.10	**0.028**
PD	1.20±0.37	0.76±0.36	**0.001**

++Student t-test, p<0.05
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 4.3.b. Inter-group comparisons of mean differences of the clinical parameters between days 0-90.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
PI	1.70±0.33	1.16±0.54	**0.001**
GI	1.37±0.39	0.59±0.39	**0.001**
BoP (%)	72.25±6.50	66.80±4.92	**0.004**
PD	1.44±0.33	0.85±0.32	**0.001**
Attachment Gain	1.18±0.36	0.79±0.32	**0.001**

++Student t-test, p<0.05
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 4.3.c. Inter-group comparisons of mean differences of the clinical parameters between day 0-180.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
PI	1.66±0.37	1.07±0.60	**0.001**
GI	1.43±0.43	0.58±0.38	**0.001**
BoP (%)	76.60±7.98	68.70±5.74	**0.001**
PD	1.77±0.69	0.70±0.24	**0.001**
Attachment Gain	1.67±0.24	0.66±0.22	**0.001**

++Student t-test, p<0.05
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 4.3.d. Inter-group comparisons of mean differences of the clinical parameters between day 0-360.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
PI	1.56±0.37	0.91±0.53	**0.001**
GI	1.33±0.43	0.46±0.4	**0.001**
BoP (%)	77.85±7.59	69.60±5.77	**0.001**
PD	1.74±0.62	0.57±0.24	**0.001**
Attachment Gain	1.39±0.26	0.53±0.24	**0.001**

++Student t-test, p<0.05
PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

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 days 21, 90, 180 and 360 are presented in Table 4.1.

4.3.1. GCF Volume

In SRP + ProDentis® group, GCF volume (µl) values were detected 0.54±0.22 at day 0 and it reduced to 0.23±0.10 µl, 0.09±0.04 µl, 0.04±0.01µl and 0.50±0.19 µl at days 21, 90, 180 and 360, respectively. In SRP + Placebo group, GCF volume was detected 0.59±0.36 µl at day 0 and it reduced to 0.37±0.27 µl, 0.24±0.21 µl, 0.11±0.09 µl and 0.55±0.33 µl at days 21, 90, 180 and 360, respectively (Table 4.4.a-4.4.b).

In both groups, intra-group multiple comparisons of GCF volume (µl) values showed statistically significance at different time point measurements (p=0.001; p=0.001; p=0.006) (Table 4.4.a-4.4.b). Subsequent comparisons of mean values of GCF volume (µl) in pairs in both groups at days 21, 90, 180 compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001). However, no significant differences were observed between days 0-360 in both groups (p= 0.125; p= 0.184) (Table 4.4.c-4.4.d).

Mean differences of GCF volume values were detected 0.30±0.20 µl and 0.22±0.14 µl between day 0-21; 0.44±0.22 µl and 0.35±0.21 µl between day 0-90; 0.50±0.22 µl and 0.48±0.29 µl between day 0-180; 0.04±0.23 µl and 0.04±0.26 µl between day 0-360 in SRP + ProDentis® and SRP + Placebo groups, respectively. Inter-group comparisons of the mean difference values for GCF volume between days 0-21, 0-90 and 0-180 revealed significant results in favor of the SRP + ProDentis® group (p=0.038; p=0.008; p=0.002) (Table 4.5.a, 4.5.b, 4.5.c), however, inter-group comparison of the mean difference values revealed no significant differences in terms of GCF volume (µl) between days 0-360 (p= 0.860) (Table 4.5.d).

4.3.2. MMP-8 Concentrations

In SRP + ProDentis® group, MMP-8 concentrations (ng/ml) were detected 35.91±5.97 at day 0 and it reduced to 13.61±2.88 ng/ml, 8.56±1.46 ng/ml, 5.82±1.42 ng/ml and 31.51±5.86 ng/ml, at days 21, 90, 180 and 360, respectively. In SRP + Placebo group, MMP-8 concentrations were detected 35.52±5.80 ng/ml at day 0 and it reduced to 17.70±4.96 ng/ml, 13.86±3.50 ng/ml, 12.09±3.43 ng/ml and 32.19±6.15 ng/ml at days 21, 90, 180 and 360 respectively (Table 4.4.a-4.4.b).

In both groups, intra-group multiple comparisons of MMP-8 concentrations (ng/ml) showed statistically significance at different time point measurements (p=0.001; p=0.001) (Table 4.4.a-4.4.b). Subsequent comparisons of mean values of MMP-8 concentrations (ng/ml) in pairs in both groups at days 21, 90 and 180 compared to baseline values revealed statistically significant differences (p=0.001; p=0.001; p=0.001), however, no significant differences were detected between day 0-360 in both groups (p= 0.165; p= 0.257) (Table 4.4.c-4.4.d).

Mean differences of MMP-8 concentration (ng/ml) values were detected 22.30±5.66 ng/ml and 17.73±5.29 ng/ml between day 0-21; 27.34±5.38 ng/ml and 21.66±4.96 ng/ml between day 0-90; 30.08±5.19 ng/ml and 23.43±4.66 ng/ml between day 0-180; 4.4±3.54 ng/ml and 3.3±2.35 ng/ml between day 0-360 in SRP + ProDentis® and SRP + Placebo groups, respectively. Inter-group comparisons of the mean differences of MMP-8 concentration (ng/ml) values between days 0-21, 0-90 and 0-180 revealed statistically significant results in favor of the SRP + ProDentis® group (p=0.012; p=0.001; p=0.001) (Table 4.5.a, 4.5.b, 4.5.c). However no significant differences were detected between the groups in terms of MMP-8 concentrations (ng/ml) between day 0-360 (p= 0.775) (Table 4.5.d).

4.3.3. TIMP-1 Concentrations

In SRP + ProDentis® group, TIMP-1 concentrations (ng/ml) were detected 0.43±0.03 ng/ml at day 0 and it increased to 0.51±0.05 ng/ml, 0.55±0.04 ng/ml and 0.57±0.05 ng/ml at days 21, 90 and 180, respectively. However, TIMP-1 concentrations (ng/ml) decreased to 0.41±0.03 ng/ml at day 360. In SRP + Placebo group, TIMP-1 concentrations were detected 0.41±0.03 at day 0 and it increased to 0.44±0.02 ng/ml, 0.46±0.03 ng/ml and 0.50±0.05 ng/ml at days 21, 90 and 180, respectively. However, TIMP-1 concentrations (ng/ml) decreased to 0.40±0.02 ng/ml at day 360 (Table 4.4.a-4.4.b).

In both groups, intra-group multiple comparisons of TIMP-1 concentrations (ng/ml) showed statistically significance at different time point measurements (p=0.001; p=0.001) (Table 4.4.a-4.4.b). Subsequent comparisons of mean values of TIMP-1 concentrations (ng/ml) in pairs in both groups at days 21, 90 and 180 compared to baseline revealed statistically significant differences (p=0.001; p=0.001; p=0.001), however, no significant differences were detected between day 0-360 in both groups (p= 0.547; p= 0.652) (Table 4.4.c-4.4.d).

Mean differences of TIMP-1 concentration (ng/ml) values were detected -0.09±0.04 ng/ml and -0.04±0.02 ng/ml between day 0-21; -0.12±0.04 ng/ml and -0.05±0.02 ng/ml between day 0-90 and -0.14±0.04 and -0.09±0.05 ng/ml between day 0-180; 0.02±0.01 ng/ml and 0.01±0.02 ng/ml in SRP + ProDentis® and SRP + Placebo groups, respectively. Inter-group comparisons of the mean differences for TIMP-1 concentration values between days 0-21, 0-90 and 0-180 revealed statistically significant results in favor of the SRP + ProDentis® group (p=0.012; p=0.001; p=0.001) (Table 4.5.a, 4.5.b, 4.5.c). However no significant differences were detected between the groups in terms of TIMP-1 concentrations (ng/ml) between day 0-360 (p= 0.850) (Table 4.5.d).

Table 4.4.a. Intra-group comparisons of the biochemical parameters of SRP + ProDentis® group at baseline and days 21, 90, 180 and 360.

	Group 1 SRP + ProDentis® (mean± SD)					
Biochemical parameters	**DAY 0**	**DAY 21**	**DAY 90**	**DAY 180**	**DAY 360**	**P***
GCF Volume (µl)	0.54±0.22	0.23±0.10	0.09±0.04	0.04±0.01	0.50±0.19	**0.001**
MMP-8 (ng/ml)	35.91±5.97	13.61±2.88	8.56±1.46	5.82±1.42	31.51±5.86	**0.001**
TIMP-1 (ng/ml)	0.43±0.03	0.51±0.05	0.55±0.04	0.57±0.05	0.41±0.03	**0.001**

* Repeated measures analysis of variance, $p<0.05$
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

Table 4.4.b. Intra-group comparisons of the biochemical parameters of SRP + Placebo group at baseline and days 21, 90, 180 and 360.

	Group 2 SRP + Placebo (mean± SD)					
Biochemical parameters	**DAY 0**	**DAY 21**	**DAY 90**	**DAY 180**	**DAY 360**	**P***
GCF Volume (µl)	0.59±0.36	0.37±0.27	0.24±0.21	0.11±0.09	0.55 ±0.33	**0.001**
MMP-8 (ng/ml)	35.52±5.80	17.70±4.96	13.86±3.50	12.09±3.43	32.19±6.15	**0.001**
TIMP-1 (ng/ml)	0.41±0.03	0.44±0.02	0.46±0.03	0.50±0.05	0.40±0.02	**0.001**

* Repeated measures analysis of variance, $p<0.05$
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

Table 4.4.c. Intra-group comparisons of the biochemical parameters of SRP + ProDentis® group in pairs.

Biochemical parameters	DAY 0-21 p**	DAY 0-90 p**	DAY 0-180 p**	DAY 0-360 p**
GCF Volume (µl)	**0.001**	**0.001**	**0.001**	0.125
MMP-8 (ng/ml)	**0.001**	**0.001**	**0.001**	0.165
TIMP-1 (ng/ml)	**0.001**	**0.001**	**0.001**	0.547

** Bonferroni corrected paired sample t test, p< 0.005
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

Table 4.4.d. Intra-group comparisons of the biochemical parameters of SRP + Placebo group in pairs.

Biochemical parameters	DAY 0-21	DAY 0-90	DAY 0-180	DAY 0-360 p**
GCF Volume (µl)	**0.001**	**0.001**	**0.001**	0.184
MMP-8 (ng/ml)	**0.001**	**0.001**	**0.001**	0.257
TIMP-1 (ng/ml)	**0.001**	**0.001**	**0.001**	0.642

** Bonferroni corrected paired sample t test, p< 0.005
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

Table 4.5.a. Inter-group comparisons of mean differences of the biochemical parameters between day 0-21.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
GCF Volume (µl)	0.30±0.20	0.22±0.14	**0.038**
MMP-8 (ng/ml)	22.30±5.66	17.73±5.29	**0.012**
TIMP-1 (ng/ml)	-0.09±0.04	-0.04±0.02	**0.001**

++Student t-test, p<0.05
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

Table 4.5.b. Inter-group comparisons of mean differences of the biochemical parameters between day 0-90.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
GCF Volume (µl)	0.44±0.22	0.35±0.21	**0.008**
MMP-8 (ng/ml)	27.34±5.38	21.66±4.96	**0.001**
TIMP-1 (ng/ml)	-0.12±0.04	-0.05±0.02	**0.001**

++Student t-test, p<0.05
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

Table 4.5.c. Inter-group comparisons of mean differences of the biochemical parameters between day 0-180.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
GCF Volume (µl)	0.50±0.22	0.48±0.29	**0.002**
MMP-8 (ng/ml)	30.08±5.19	23.43±4.66	**0.001**
TIMP-1 (ng/ml)	-0.14±0.04	-0.09±0.05	**0.001**

++Student t-test, p<0.05
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

Table 4.5.d. Inter-group comparisons of mean differences of the biochemical parameters between day 0-360.

	Group 1 SRP + ProDentis® (mean± SD)	Group 2 SRP + Placebo (mean± SD)	P++
GCF Volume (µl)	0.04±0.23	0.04±0.26	0.860
MMP-8 (ng/ml)	4.4±3.54	3.3±2.35	0.257
TIMP-1 (ng/ml)	0.02±0.01	0.01±0.02	0.850

++Student t-test, p<0.05
GCF: Gingival crevicular fluid, µl: microliter, ng: nanogram, MMP-8: Matrix metalloproteinase-8, TIMP-1: Tissue inhibitor of metalloproteinases-1.

5. DISCUSSION

Periodontal destruction results from the microbial load within a periodontal pocket, which overrules the local and systemic host defense mechanisms. Such an imbalance can result from: a specific increase in the total amount of bacteria, an outgrowth/overgrowth of pathogenic species above a certain threshold level, and/or a reduction in the efficiency of the immune response (16, 183). Therefore, treatment strategies have been developed aiming to suppress or eliminate periodontal pathogens and control their overgrowth (13).

Chronic periodontitis is the most prevalent form of periodontitis and generally considered to be a slowly progressing disease (62). SRP is considered as an essential and effective component for the treatment of periodontal diseases together with OHI and it has been considered as a gold standard (53, 107, 184, 185, 186). Over the past decades, novel techniques and modifications in SRP procedure have been proposed in order to control the periodontal infection (113, 184). Badersten et al. (187) reported that a single episode of SRP achieved results similar to multiple root planing procedures. Even after non-surgical periodontal therapy the subgingival microbial load is reduced a thousand times (8), within 1 week the periodontal pockets are already re-colonized by the initial number of bacteria, but with a less pathogenic composition (11, 188). Therefore, Quirynen et al. (189) introduced the one-stage, full-mouth disinfection (OSFMD), aiming to eradicate, or at least suppress periodontopathogens in a very short time span from all oropharyngeal habitats (mucous membranes, tongue, tonsils, saliva). Also, OSFMD approach aimed to prevent and retard a cross-contamination of the treated periodontal pockets by bacteria from untreated habitats. On the other hand, full-mouth SRP protocol includes full-mouth SRP without antiseptics. Results of the studies yielded controversial results about those treatment approaches (16, 190, 191), so it is concluded that, success of the non-surgical periodontal therapy remains the thoroughness of root debridement and the patient's standard of oral hygiene, rather than the treatment modality (113). Hence, operator and patient preference may play a large

part in determining the choice of instrumentation for non-surgical therapy, including the choice between conventional quadrant debridement, full-mouth disinfection and full-mouth SRP (113). Since the efficacy of a single session of SRP depends on the experience of the clinican, inflammatory status of the tissues and anatomy of the roots (188), in general, treated areas are need to be re-evaluated after a single instrumentation. Therefore, in the present study, SRP was performed in two sessions with one-week interval.

However, non-surgical therapy alone may not effectively control infection, particularly in deep pockets that are not easily accessed by periodontal instruments (13, 185, 192). Also, tongue, tonsils and other niches in the oral cavity harbor pathogenic bacteria that recolonize the periodontal pocket and may be sources for reinfection (190). Therefore, adjunctive use of local or systemic antimicrobials to SRP often needed to sustain periodontal health (107).

The adjunctive antimicrobial therapeutic basis depends upon the systemic and local application of antibiotics. However, adjunctive use of local or systemic antibiotics improves the outcome of periodontal therapy only temporarily (15, 193). Administration of systemic and local antibiotics into periodontal pockets to promote disinfection may induce the risk of developing resistant microbial species increases with the frequent use of antibiotics (132). Thus, the use of antimicrobial agents is associated with unsatisfactory drug concentrations in the periodontal pocket and lack of a stable therapeutic concentration to exert antimicrobial action (2, 129) whereas lasers and photodynamic therapy still need improvements in terms of clinical efficacy (194). Therefore, recently probiotics have been proposed as an adjunct to periodontal therapy.

The applications of so-called ‘bacteriotherapy’ or ‘probiotics’ on systemic health have been studied in numerous in vitro and in vivo studies (151, 152, 195, 196, 197) and mostly these probiotic species belong to the genera *Lactobacillus* and *Bifidobacterium* (18). The optimistic results of probiotic studies in different fields of healthcare have resulted in the introduction of probiotics for oral healthcare (18, 19,

142, 148). The mechanisms of probiotic action in the mouth are thought to be similar to that observed in gastrointestinal indications. However, it is not clear whether probiotic strains modulate immune responses in the oral cavity, as has been suggested to take place in the gut mucosa. The epithelial structure and chemical composition of excretions in the gastrointestinal tract and the resident microbiota differ from the mouth mucosa and saliva therefore, results from studies conducted in patients with gastrointestinal diseases can not be directly adopted in the field of periodontology (18).

Lactobacilli can produce different antimicrobial components including organic acids, hydrogen peroxide, low-molecular-weight antimicrobial substances, bacteriocins, and adhesion inhibitors (146). *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 (39). It has previously been demonstrated that a *L. reuteri* strain can exhibit a powerful suppressive effect on TNF-α production by human monocytes and macrophages (195). Furthermore, it has been proposed that *L. reuteri* may produce an unidentified immunomodulin capable of inhibiting TNF-α production by LPS-activated monocytoid cells (196). It has also been postulated that the antibacterial agent reuterin, produced from glycerol by *L. reuteri,* could play a role in the probiotic effects of *L. reuteri* (197). Furthermore, recent studies have shown that a wide spectrum of intestinal bacteria are very sensitive to reuterin (198, 199), but whether or not this is the case concerning bacteria associated with periodontal diseases remains an open question (39).

Since only a few dose response comparison studies have been performed, limited data is available about appropriate probiotic dosing regimens (200). In this study, *L. reuteri* containing lozenges were prescribed twice a day after toothbrushing without chewing. Twetman et al. (39) reported a dose–response relationship or a threshold level seemed to appear after using *L. reuteri* containing chewing gums twice a day instead of once. So, the rationale for our choice of dosage based from an analysis of previous studies and the manufacturer's recommendation (35, 39, 40, 42). 100% of patients completed the full cycle of probiotics demonstrated that this dosage of two lozenges per

day allows for complete compliance from patients, which is critical from a clinical point of view.

Generally, commensal or indigenous organisms are important and play a crucial role in regulating the host defense against exogenous pathogens. Their presence makes it difficult for exogenous pathogens to become established and lack of significant changes in the local environment, exogenous pathogens entering the mouth have difficulty surviving in competition with the established flora (52). Conversely, when considering the therapeutic application of probiotics to periodontal disease, total removal of plaque seems to be an important step for upsetting the pathogenic equilibrium and enhancing the replacement of indigenous microbiota for the same reason (36).

Removal of subgingival plaque and calculus disrupts the subgingival microflora and delays the repopulation of periodontopathogens (10, 185). However, subgingival microflora has supragingival origins (7, 185), as the quantity, composition and rate of subgingival plaque recolonization is dependent upon supragingival plaque accumulation (7, 10, 184). Effective supragingival plaque control is crucial for long-term control of inflammatory periodontal diseases (201), therefore every patient was given OHI in terms of a self performed plaque control programme 1 week prior to the study and maintained at a good level throughout the experimental period. After treatment with SRP, re-establishment of the epithelial attachment appears to occur within a week (202), for this reason time interval between two sessions was chosen as 1 week based on this fact. Proye et al. (186) reported that the greatest changes in PD reduction could be observed 3 weeks after SRP and no further changes could be obtained during the 3 months. Data in the literature shows that most of the healing complete at 3 months (53, 203). Thus the following examination periods were determined as days 21 and 90. However, healing may continue for a period up to 9-12 months following SRP (53), therefore, the following examination periods were chosen as days 180 and 360.

It is known from the literature that several subject-related and tooth site-related factors may compromise the healing response to periodontal therapy (129). Molar sites have been reported to represent a truly compromised healing response releated to the furcation anatomy. Poor accessibility for instruments to the furcation fornix, as well as the presence of concavities and other root surface irregularities in the furcation areas may limit the efficacy of root debridement (204). In addition, distal location of the molars may limit adequate plaque control by the patient on a daily basis, which may lead to the recolonization of a microflora similar to that before treatment (7). In several papers, it has been shown that inflammatory mediators have been shown to be present intensively in PD≥ 5 mm. Also, papers with 2 years of follow up period after SRP confirm that, single rooted teeth with PD≥ 5 mm exhibit greater reduction in PD and gain in clinical attachment level than molar sites (184, 192, 205). Therefore, single rooted-teeth with initial PD≥ 5 mm were chosen for sampling in order to compare the different treatment approaches in a standardized manner.

Clinical measures of periodontitis such as PD, CAL or BoP alone have limitations to provide the clinician with real-time evaluation of disease status. As the GCF traverses from the microcirculation across inflamed periodontal tissues, it carries biological molecular markers gathered from the surrounding site. Thus, GCF is an attractive oral fluid due to its ease of collection and ability for the clinician to sample multiple sites within the oral cavity simultaneously. The use of GCF suggests potential diagnostic value to identify periodontal disease activity and response to therapy (206).

Numerous molecules in the oral fluids namely; GCF and saliva, as well as molecules in the blood circulation; serum or plasma have been investigated in an attempt to provide a sensitive and specific marker for periodontal tissue destruction. GCF and saliva can easily be obtained non-invasively and with minimal discomfort to the patient and consist of both locally synthesized and systemically derived molecules and harbor serum proteins, inflammatory mediators, host cell degradation products and microbial metabolites. (207). Whole or mixed saliva is a complex fluid mixture derived from the salivary glands and contains contributions from the GCF, oral bacteria, cells and other sources that make identification of the exact site of disease activity difficult.

Additionally, the flow rate of saliva varies within and between subjects, which is sex and biorhythm dependent (208). On the other hand, collection of GCF with paper strips is easy, non-invasive and rapid method for assessment of inflammatory molecules (71, 208). In the present study, GCF samples were obtained from single rooted teeth having at least one approximal site with a PD≥ 5 mm and GI≥ 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.

Systemic disorders and pregnancy have profound effects on the physiological and inflammatory response; therefore they have the potential to modify the plaque microbiota, disease progression and response to treatment (209). For this reason, study population was consisted of systemically healthy, 40 non-smoker patients. Smoking is accepted to be a true risk factor for periodontitis (209). Smokers display higher levels of plaque (210), depressed inflammatory reaction, significantly less favourable clinical outcome and seem to have an altered host antibody response to antigenic challenge than non-smokers (211, 212). Söder et al. (213) analyzed the levels of granulocyte elastase, MMP-8 and prostaglandin E_2 (PGE_2) in GCF of smokers and non-smokers with persistent periodontitis. According to their results, 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 (209).

In the literature, 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 (214). Short sampling time periods are associated with decreased volume of GCF sample for subsequent laboratory analysis, whereas prolonged collection times change the nature of the GCF sample collected (71). 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 (71, 215).

For estimating the GCF volume collected, staining techniques (71, 214), weighing of strips before and after sample collection (214, 216) and an electronic measuring device, The Periotron® (71, 214) has been proposed. Since evaporative losses because of delays in determining the volume in staining technique and very sensitive balance required in weighing the accumulated fluid, Periotron 8000®, was used in this study, which allowed accurate determination of the GCF volume and subsequent laboratory investigation of the sample composition (71). Periotron® quantifies the volume of GCF or saliva collected on filter papers by measuring the electrical capacitance of a wet paper strip (217). Evaporation seems to be an important factor that varies in Periotron® readouts, therefore samples were immediately transferred to the device and than stored at – 70°C until analysis.

Periodontopathogens and host factors, such as infiltrating cell populations, cytokines and MMPs are responsible for the most of the periodontal tissue breakdown in CP, leading to clinical signs of disease (110). Among these, MMP-8 in GCF has been reported to have some value as a diagnostic marker for periodontitis (55). GCF MMP-8 levels in shallow crevices have been found to be associated with attachment loss therefore, it was suggested as a prognostic marker (78). The total collagenase activity is mainly derived from neutrophils, and the enzyme collagenase-2, belongs to the class of MMPs and is classified as MMP-8, which is specifically abundant in GCF (219). Mancini et al. (96) 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. (219) 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.

TIMPs consist of four members, TIMP-1, -2, -3 and -4. Although they have basic similarities, they exhibit structural and biochemical differences (101, 220). Among TIMPs, TIMP-1 was shown to be the major inhibitor of MMPs in gingival tissues of patients with periodontal disease (85). When comparing the samples from periodontitis and healthy subjects, controversial results on TIMP-1 levels in GCF have been reported. In GCF, both increased (105, 221, 222) and decreased levels of TIMP-1 in diseased sites have been described (90, 223, 224).

The quantities of MMPs in GCF are very low, typically in the ng range per µl of fluid sampled. Consequently, highly sensitive and specific reagents and test systems are required for meaningful analysis. Antibody-based detection systems extensively used for the specific measurement of low abundance molecules in various biological fluids include immunoblot, ELISA, time-resolved immunofluorescence and other related assays that utilize specific antibodies to common MMPs. In comparison to other assay systems, ELISA tests have several advantages including improved sensitivity (at least compared to zymography), ease of use, moderately rapid throughput, the ability to quantify enzyme levels and the flexibility to test for multiple MMPs in single samples. Moreover, ELISAs may be more adaptable to use in office settings than other assays because of their simpler and more robust instrumentation (103).

In order to identify the oral hygiene level and accumulation of dental plaque biofilm, PI (178) was used. This index concerns the thickness of plaque along the gingival margin, which is the primary etiologic factor of periodontal diseases. One week prior to the study period, all subjects were instructed to brush their teeth by modified Bass method (225) and to use interdental brushes. Throughout the study period, patients were seen regularly to check their oral hygiene levels. Mean baseline PI scores in both groups were found 2.29 and 2.31, respectively. Intra-group comparisons in both groups revealed statistically significant differences for PI reduction compared to baseline values ($p< 0.05$). This finding showed that all patients in the present study provided optimal oral hygiene level. PI reductions were detected 1.56 and 0.91 in test and control groups, respectively at the end of the day 360. However, inter-group comparisons of the mean differences of PI score revealed signficancy in favor of the test group ($p< 0.05$).

This finding could be attributed to the anti-plaque properties of *L. reuteri* containing lozenges as stated in the previous studies (35, 40, 41, 42). *L. reuteri* is known to produce an antimicrobial substance; reuterin, which inhibits a wide range of pathogenic bacteria. Also, strains of *L. reuteri* have demonstrated an ability to block binding of pathogenic bacteria to host tissue. These possible mechanisms of action of *L. reuteri* might be the basis of a direct or indirect effect of anti-plaque properties (35, 142). However, in the literature contradictory results have been reported by Staab et al. (38), Slawik et al. (226) and Hallström et al. (227). These controversial findings may be due to the different study designs and patient population, probiotic strains used and carrier systems.

To evaluate the inflammatory status of the gingiva, GI and BoP scores were used. Both groups showed significant improvements at the end of the days 21, 90, 180 and 360 ($p< 0.05$). SRP together with a high standard of oral hygiene level led to a reduction of the bleeding tendency and inflammation of the periodontium (9, 107, 186, 188, 203). Intergroup comparison of the mean differences of GI values revealed statistically significance in favor of SRP+ ProDentis® group ($p< 0.05$), indicating the lasting adjunctive anti-inflammatory effect of probiotic containing lozenges between the days 0-21, 0-90, 0-180 and 0-360. In the literature, similar results were reported as reductions in BoP and GI scores following probiotic application (29, 35, 39, 40). On the other hand, there are also contradictory studies, which showed no clinical benefits of probiotics (38, 226, 227). However, SRP intervention was not performed in most of these studies that has failed to show the clinical benefits of probiotics. As already known, biofilms are difficult to penetrate by therapeutic agents unless they are mechanically disrupted. So, the superior finding of the present study may be explained by the disruption of the biofilm before probiotic administration, which might be more reliable by means of the eradication of the periodontal inflammation.

In a clinical point of view, the absence of bleeding together with an increased resistance of the periodontal tissues to probing are signs of resolution of the inflammation related to a sufficient removal of biofilm, that are commonly used as end points of the treatment success (228). Reduction in swelling of the marginal gingival

tissues leads to the gingival recession. Inflamed tissue with its inflammatory cell infiltrate and increased number of capillaries gradually replaced by a more collagen rich tissues (9, 11). These changes result to the closure of the pocket, thus reduction of PD values. Before the study period, individual acrylic stents with reference grooves were prepared for each patient in order to standardize probe position. Reduction of the PD values were found 1.20 mm and 0.76 mm at day 21, 1.44 mm and 0.85 mm at day 90, and 1.77 and 0.70 mm at day 180, 1.74 and 0.57 mm at day 360 in test and control groups, respectively. PD reduction would have been possibly due to the reduction of inflammation, establishment of a long junctional epithelium and reorganisation of collagen, all of which would influence the probe penetration (228). In the literature, PD reduction ranges from 0.50 mm to 1.30 mm in moderately deep pockets (4-6 mm) after 12 months following SRP (9). Therefore, our findings are in accordance with the literature. In relation to the primary outcome variable, significantly higher PD reduction in the test group was observed at all evaluation time periods ($p< 0.05$). Similarly, Vivekananda et al. (40) reported 1.31 mm PD reduction, when probiotic containing lozenges were used twice a day for 3 weeks as an adjunct to SRP in a split- mouth study design. In that study, PD reduction was detected 0.49 mm in SRP + placebo group. On the other hand, Teughels et al. (42) evaluated the effects of the same probiotic containing lozenges as an adjunct to SRP in 30 CP patients in a similar study design of this present study. PD reduction was reported 1.84 mm and 1.72 mm in test and control groups in moderately deep pockets when lozenges were administered twice a day for 12 weeks. Authors attributed the potential factor that could explain their superior results to the use of chlorhexidine during SRP to further suppress the microbiological ecology.

The reduction in PD is the sum of a clinical attachment gain and recession of the marginal gingival tissues (9, 186). Therefore, PD measurements alone do not disclose the true connective tissue levels attached to a teeth (53). Both the presence of long junctional epithelium and the increased content in collagen fibers in the gingival connective tissue result in the gain in clinical attachment level (9). Clinical attachment levels can be measured, relative to a landmark, such as the cementoenamel junction, a restoration, occlusal surface, or stent (229). Since probing attachment levels made from cementoenamel junction are subject to reproducibility error (53), relative measurements

by the guidance of individual acrylic stents were preferred 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+ ProDentis® group attachment gain was found 1,18 mm at day 90 and 1,67 mm at day 180 and 1.39 mm at day 360. In SRP+ Placebo group, attachment gain was detected 0,79 mm at day 90, 0,66 mm at day 180 and 0.53 mm at day 360. In the literature, attachment gain is reported between 0.07 mm and 1.40 mm after SRP in CP (9).

At the end of the days 90, 180 and 360, significant improvements have been observed in terms of PD reduction and attachment gain in favor of the SRP + ProDentis® group ($p< 0.05$). These findings could possibly due to the significant reductions in the PI and GI scores, which is releated to the possible probiotic action as the promotion of the healing process (40).

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 sulci 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 (70). In the present study, In SRP+ ProDentis® group, GCF volume decreased from 0,54 µl to 0,23 at day 21, to 0,09 µl at day 90, to 0,04 µl at day 180 and to 0.50 µl at day 360. In SRP+ Placebo group, GCF volume decreased from 0,59 µl to 0,37 µl at day 21, to 0,24 µl at day 90, to 0,11 µl at day 180 and 0.55 µl at day 360. In both groups, treatment resulted in significant reductions of GCF volume values when compared to baseline up to day 180 ($p< 0.05$) whereas, GCF volume obtained at day 360 tended to reach to the baseline values. SRP + ProDentis® group showed significantly more reductions in GCF volume than SRP + Placebo group up to day 180 ($p< 0.05$). However, inter-group comparison of mean difference values of GCF volume did not show any statistical significance at day 360. Similar results were reported by Slawik et al. (226) after 2 weeks consumption of the probiotic milk drink. In another study, Twetman et al. (39) 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 intervention period, GCF volume reduction was found to be significant only in the probiotic groups.

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 (230), modulation of the host immune response (165) and production of antimicrobial substances (231). 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 (232).

Probiotic bacteria and their metabolites can be recognized by epithelial and immune cells of the host (164). 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. actinomycetemcomitans* on epithelial cells (165, 166). Köll et al. (167) 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.

Pathogens in microbial dental plaque biofilm are capable of stimulating host cells to increase their MMP release, which is considered as one of the indirect mechanisms of tissue destruction occurring in periodontitis (3, 233). 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 (5). 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 (84). 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 (67).

Several components in oral fluids have been identified as possible biomarkers for CP, however most of them reflect inflammation rather than periodontal attachment loss (55, 233). MMP-8 in GCF and saliva associates with the initiation and progression of periodontitis and reflects its severity (5, 98, 233, 234) 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 (87, 96, 233, 235, 236). TIMPs, on the other hand, regulate the activities of MMPs (5). In the present study, In SRP+ ProDentis® group, MMP-8 concentration decreased from 35,91 ng/ml to 13,61 ng/ml at day 21, to 8,56 ng/ml at day 90, to 5,82 ng/ml at day 180 and 31.51 ng/ml at day 360, whereas in SRP + Placebo group MMP-8 concentration was 35,52 ng/ml at baseline and decreased to 17,78 ng/ml at day 21, to 13,86 ng/ml at day 90, to 12,09 ng/ml at day 180 and to 32.19 at day 360. Conversely, in SRP + ProDentis® group; TIMP-1 concentration increased from 0,43 ng/ml to 0,51 ng/ml at day 21, to 0,55 ng/ml at day 90 and 0,57 ng/ml at day 180. In SRP+ Placebo group, TIMP-1 concentration increased from 0,41 ng/ml to 0,44 ng/ml at day 21, to 0,46 ng/ml at day 90 and to 0,50 at day 180. However, TIMP-1 concentrations at day 360 were detected 0.41 and 0.40 in test and control groups, respectively at day 360.

A survey of literature reveals only one study performed by Della Riccia et al (29) analyzing the effects of probiotics on salivary MMP levels. On the other hand, in the literature no studies have been performed similar to the present study design and follow-up period in order to evaluate the efficacy of probiotics in MMP-8 and TIMP-1 levels. Therefore, findings of the present study were compared with the studies evaluating the levels of MMPs and TIMPs after SRP intervention. In the literature, several studies have been reported about the effect of SRP in MMP-8 and TIMP-1 levels. Chen et al. (91) reported positive correlations of MMP-8 and elastase with clinical parameters. In that study, total amount of MMP-8 in GCF reduced from 19.6 ng/sample to 2.2 ng/sample and concentration of MMP-8 in GCF reduced from 47.9 ng/µl to 16.5 ng/µl 2 weeks after SRP. Kinane et al. (218) investigated the effect of SRP

in GCF MMP-8 levels in CP by time-resolved IFMA. They reported significant improvements in the clinical indices together with reductions in MMP-8 levels. GCF MMP-8 concentrations have been found to be reduced even more dramatically following a 3-month period of maintenance. Marcaccini et al. (237) compared the MMP-8, MMP-9, TIMP-1 and TIMP-2 levels of CP patients before and after SRP with controls. At baseline, higher levels of MMP-8 were found in patients compared with controls and these levels decreased significantly after therapy, however, no differences were observed in TIMP-1 levels between the groups. Significant positive correlations were found between periodontal disease severity and GCF MMP-8 activities together with negative correlations with TIMP-1 levels (223). In the present study, in both 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 speculate that the significant reductions in MMP-8 concentrations in favor of the SRP + ProDentis® group are due to the suppressive effect of probiotics on MMPs with host immunomodulation, similar to the results of Della Riccia et al. (29).

Increased TIMP-1 concentrations after SRP have been reported by previous studies (89, 90). However Alpagot et al. (105) reported 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 (89). Increased levels of TIMP-1 may reflect its involvement in the healing process (238, 239).

Lack of significant data in the literature regarding the usage of probiotics as an adjunct to SRP in CP on inflammatory markers in 12-month follow up makes the comparison of the results with the other studies impossible. However, 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. The non-significant results obtained at 12-month follow up in inflammatory markers may be explained by this finding (110).

In conclusion, this study showed that under the given conditions the adjunctive use of *L. reuteri* containing lozenges resulted in significant additional clinical and biochemical improvements in moderately deep pockets when compared to SRP alone up to day 180. The present study serves as a pioneer in the literature reporting the adjunctive immunomodulatory effects of probiotics to SRP in CP. However, further in vitro studies are warranted in order to identify the exact molecular mechanisms of action of these immunomodulatory effects.

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7. APPENDIX

Appendix 1. Ethical Approval

YEDİTEPE ÜNİVERSİTESİ
HASTANESİ

22/02/2012

SAYI: B.30.2.YTÜ.0.70.10.00-001/332
KONU: Yeditepe Üniversitesi Diş Hekimliği Fakültesi Prof.Dr.Selçuk Yılmaz ve Dt. Gizem İnce Urgur'un çalışması Hk ;

Sn.Prof.Dr.Selçuk YILMAZ,

Sorumlu Araştırmacılığını gerçekleştireceğiniz "Kronik Periodontisli Hastalarda Başlangıç Periodontal Tedaviye Ek Olarak Probiyotik İçeren Strip Kullanımının Klinik ve Biyokimyasal Olarak Uzun Dönem Etkinliğinin Değerlendirilmesi " başlıklı proje 14/02/2012 tarihli Klinik Araştırmalar Etik Kurulu toplantısında görüşülerek gerçekleştirilmesinde bir sakınca olmadığına karar verilmiş olan çalışmanız, 22/02/2012 tarihinde Bilimsel Komite toplantısında da görüşülmüş olup , projeniz hakkında Bilimsel Komite Üyelerimiz bilgilendirilmiştir.

Bilgilerinize sunar, çalışmalarınızda başarılar dileriz.

Saygılarımızla,

Prof. Dr. Kemal SARICA
Bilimsel Komite Başkanı
Yeditepe Üniversitesi Hastanesi

Prof. Dr. Canan AYKUT BİNGÖL
Tıbbı Koordinatör
Yeditepe Üniversitesi Hastanesi

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Appendix 2. Informed consent form

YEDİTEPE ÜNİVERSİTESİ HASTANESİ	**YEDİTEPE ÜNİVERSİTESİ TIP FAKÜLTESİ KLİNİK ARAŞTIRMALAR DEĞERLENDİRME KOMİTESİ BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU**

Araştırmanın Adı / Protokol Numarası

Kronik Periodontitisli Hastalarda Başlangıç Periodontal Tedaviye Ek Olarak Probiyotik İçeren Pastil Kullanımının (ProDentis®) 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 hastalarda başlangıç periodontal tedaviye ek olarak üretici firmanın önerisi doğrultusunda kullanılacak probiyotik pastillerin klinik ve biyokimyasal olarak uzun dönem etkinliğinin karşılaştırmalı olarak değerlendirilmesidir.

Araştırmanın Süresi: 01.04.2012 01.04.2013 tarihleri arasında yapılacaktır.

Araştırmaya Katılan Gönüllü Sayısı: 40

Araştırmada İzlenecek Yöntem

Araştırma Yeditepe Üniversitesi Dişhekimliği Fakültesi Periodontoloji Anabilimdalı'na dişeti hastalığı şikayeti ile başvuran 35–60 yaş arasında klinik ve radyografik bulgulara göre kronik periodontitis tanısı konulacak her bir yarım çenesinde en az 2 tek köklü sondalama derinliği ≥5, gingival indeks ≥ 2 olan dişe sahip 40 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.

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 pastillerle 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 hastalarınperiodontal 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 20

kişilik 2 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 hastalarda her bir kadranda daha önce tespit edilmiş sondalama derinliği ≥ 5 mm ve gingival indeks ≥ 2 olan en az iki dişe sahip bölgelerden steril paper pointlerle diş eti oluğu sıvısı örnekleri alınacak ve tüm ağız plak indeksi, gingival indeks, sondalama 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 pastil 3 hafta boyunca sabah ve akşam birer tane olmak üzere günde 2 kez kullandırılacaktır.

2. gruba diş yüzeyi temizliği ve kök yüzeyi düzleştirmesi ile beraber plasebo (etken madde içermeyen) pastil 3 hafta boyunca sabah ve akşam birer tane olmak üzere günde 2 kez kullandırılacaktır. 3. gruba sadece diş yüzeyi temizliği ve kök yüzeyi düzleştirmesi uygulanacaktır. 3. hafta, 3., 6. ve 12. aylarda 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.hafta, 3., 6. ve 12. aylarda 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 indeks

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.

Sondalama 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

Sondalama derinliği ölçüldükten sonra dişlerin çevresindeki 6 noktasından (meziyo-bukkal, mid-bukkal, distobukkal, mezyo-lingual, mid-lingual, disto-lingual) kanama var (+) ya da yok (-) şeklinde kaydedilecektir.

Rölatif Ataşman Seviyesi

Oklüzal stentler üzerinde sondalama 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. hafta, 3., 6. ve 12. aylarda hastaların her yarım çenesinde en az iki farklı bölgesinden alınmak üzere 5 farklı 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, defekt bölgelerinde dişeti oluğunun içine en fazla 1 mm girecek şekilde yavaşça konumlandırılacak ve 30 sn kadar beklenilecektir. Daha sonra nazikçe oluktan çıkarılan Periopaper®'larda biriken DOS hacmi Periotron® 8000 aracılığıyla belirlenip özel olarak hazırlanmış mikrosantrifüj tüplerine aktarılıp kodlanacaktır. Hesaplama sonrası örnekler -78°C 'de deney gününe kadar saklanılacaktır. Gözle görülür bir şekilde kan ve tükürük ile kontamine olan örnekler çalışmaya dahil edilmeyecektir.

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 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 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 inkübasyon 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 inkübasyon 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.

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. Gizem İnce 0530-8243499

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

Appendix 3. Randomization Table

Group 1 (SRP + ProDentis®)																			
P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
34	10	8	15	38	27	20	36	37	26	28	17	39	14	33	3	13	24	31	2
Group 2 (SRP + Placebo)																			
P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20
9	29	4	30	16	35	23	6	5	25	19	21	22	11	1	12	32	7	40	18

8. CURRICULUM VITAE

She was born in Antalya, in 1986. She started to her education at 1992 in İzmir Bülent Okan Elementry School. Between 1997-2004, she studied in Ankara Yıldırım Bayezit Anatolian High School.

She was graduated from Ege University, Faculty of Dentistry in 2009. In 2010, she started to the PhD programme in Yeditepe University, Faculty of Dentistry, Department of Periodontology.

She is a member of Turkish Dental Association, Turkish Society of Periodontology, DILAD Laser Association and International Association for Dental Resarch.