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CLINICAL AND MICROBIOLOGICAL EFFECTS OF PROBIOTIC LOZENGES IN THE TREATMENT OF CHRONIC PERIODONTITIS: 1-YEAR FOLLOW-UP

PhD Thesis

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I. SUMMARY

The objective of this study was to evaluate the effect of *Lactobacillus reuteri* (*L. reuteri*) containing lozenges as an adjunct to initial periodontal therapy in chronic periodontitis (CP) patients and to detect *L. reuteri* colonization in periodontal pockets.

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 scaling and root planing (SRP) + *L. reuteri* containing lozenges, whereas Group II received SRP + Placebo. Plaque index (PI) and (GI), bleeding on probing (BoP), PD, and relative attachment level (RAL) were measured. Microbiological sampling was performed at baseline and days, 21, 90, 180 and 360 and was analyzed by culture method including total viable count (TVC) and proportions of obligate anaerobes.

Repeated measure analysis of variance was used for intra-group comparison of the clinical parameters and proportions of obligate anaerobes whereas the Friedman test was used for the TVC values at different time point measurements. The Bonferroni corrected Paired sample t was used to evaluate intra-group comparisons of the clinical parameters and proportions of obligate anaerobes in pairs. The Bonferroni corrected Wilcoxon signed ranks test was used to evaluate the intra-group comparisons of the TVC values in pairs. The Student t or Mann-Whitney U tests were used to evaluate the inter-group comparisons of the mean differences according to their distribution.

Intergroup comparisons of PI, GI, BoP and PD revealed significance in favor of Group I at all time intervals ($p<0.05$). Intergroup comparisons of total viable cell count ($x10^5$ colony forming units (CFU/ml) and proportions of obligate anaerobes revealed significance in favor of Group I at all time intervals, except day 360 ($p<0.05$).

In conclusion, within the limits of this study it can be stated that *L. reuteri* containing lozenges might be an adjunctive useful agent for retarding recolonization and the improvement of periodontal health. Further studies are warranted to clarify the administration dose of the lozenges.

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III. CONTENTS

IIV. ABBREVIATIONS

CP: Chronic Periodontitis

PD: Probing Depth

GI: Gingival Index

PI: Plaque Index

BoP: Bleeding on Probing

SRP: Scaling and Root Planning

RAL: Relative Attachment Level

CFU: Colony Forming Unit

TVC: Total Viable Cell Count

GCF: Gingival Crevicular Fluid

TNF-α: Tumor Necrosis Factor alpha

IL-8: Interleukin-8

IL-1β: Interleukin-1 beta

CAL: Clinical Attachment Loss

WHO: World Health Organization

VSC: Volatile Sulphur Compounds

OHI: Oral Hygiene Instruction

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1. INTRODUCTION AND AIM

There is a natural balance between the periodontal microbiota and the immune system of the host. In case this balance is disrupted, periodontitis is likely to ensue. For periodontitis to develop, certain conditions must exist such as an increase in the mass or pathogenic characteristics of bacteria, suppression of beneficial bacterial species and a susceptible host (1).

Conventional periodontal treatment mainly comprises mechanical debridement, which essentially aims to disrupt the biofilm and remove bacterial accumulations on root surfaces (2). This procedure prevents further damage to the tissues and converts the existing pathogenic microbiota to a more favorable one. As a result, the degree of inflammation is reduced and periodontal attachment levels are re-established to more stable levels (3–5) Although mechanical debridement substantially reduces the number of pathogenic species, in a short period, recolonization by pre-treatment microbiota is observed (6, 7). Different therapeutic approaches such as antimicrobial agents, lasers and photodynamic therapy have been proposed as adjuncts for the extension of bacterial recolonization (8–10). Although these adjunctive approaches demonstrate promising clinical and microbiological results, only temporary improvements can be achieved. Recolonization by periodontal pathogens starts within 2-8 weeks (6, 11). Concordantly, probiotics are recommended as promising agents to increase the number of beneficial bacteria, to retard the recolonization and to modulate immunological parameters in the prevention and treatment of periodontal diseases (12). Probiotics comprise a wide range of microorganisms, such as *Lactobacillus spp.* and *Bifidobacterium spp.* which are the most commonly used strains.

Among *lactobacilli* species, *Lactobacillus reuteri (L. reuteri)* has specifically gained attention due to its ability to enable the formation of reuterin. It appears that reuterin is significant in the maintenance of healthy microbiota since it prevents the overgrowth of other pathogenic microorganisms (13). This type of microorganism also possesses other favorable properties such as reduction in inflammatory mediators like

tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8) and interleukin-1 beta (IL-1β) (14). Although Haukioja et al. (15), demonstrated saliva as a common habitat of *L. reuteri* ATCC 55730, it was rarely detected in subgingival samples. The detection of this microorganism in subgingival samples may guide the clinician in terms of probiotic usage. However, colonization and even viability are not specific requirements for probiotics to exert beneficial effects (12, 16).

A couple of studies evaluated the effect of probiotics in the treatment of different types of periodontal diseases in humans and the results were found to be controversial (14, 17–23). In most of these studies, probiotic application has shown to reduce bleeding on probing (BoP), plaque (PI) and gingival indices (GI), probing depth (PD) and obligate anaerobes in chronic gingivitis and periodontitis patients (14, 17, 18, 20, 22). Other studies failed to demonstrate additional effect of probiotics, clinically and/or microbiologically (19, 21, 23).

The Null hypothesis was that there would be no significant differences between the two treatment groups in terms of both, clinical and microbiological parameters. Therefore the aim of this study was to evaluate the adjunctive effects of *L. reuteri* (Prodentis®, BioGaia AB, Sweden) containing lozenges to scaling and root planing (SRP) in terms of clinical and microbiological outcomes over a 1-year follow-up period and to assess whether *L. reuteri* can colonize the periodontal pocket and if so, at which stage of periodontal treatment this colonization takes place.

2. LITERATURE REVIEW

2.1. Periodontal Disease

Periodontal diseases are specific infectious diseases, characterized by the pathological manifestation of the host response against the specific bacterial challenge from the dental biofilm such as progressive destruction of the alveolar bone, and apical migration of connective tissue and epithelial attachments (10, 22, 24, 25).

The periodontal flora is in balance and harmony with its host immune system. Periodontal diseases occur when the equilibrium between the bacterial load and host response is disrupted. Specific conditions such as increased mass and/or pathogenicity of the microbiota, suppression of commensal or beneficial bacteria and/or reduced host responses are key factors in the development of periodontal diseases.

Approximately more than 700 species are capable of colonizing the subgingival area, and any individual harbors about 150 or more species in its periodontal pocket (26–28). However, it is likely that only a small percentage of these are etiological agents (29). Bacterial virulence factors characterize an organism or group of organisms as an etiological factor. These are bacterial constituents or metabolites capable of disrupting homeostatic or protective host mechanisms or causing the progression or initiation of the disease. The pathogenesis of periodontal disease is partly dependent on the virulence as well as the concentration of microorganisms capable of producing disease.

The primary microbial factor contributing to periodontitis is a shift in the content of the oral microflora. Microbial shift, more commonly known as dysbiosis, refers to the concept that some disease are due to a decrease in the number of beneficial symbionts and/or an increase in the number of pathogens. Resent research has indicated

that dysbiosis in the oral cavity can lead to periodontitis. Within time, the symbiotic host-microbe relationship gradually changes into a pathogenic one leading to a state of disease. At the same time, a succession of different microbial complexes develops. The first associated complex with disease is the 'orange-complex', consisting of gram-negative anaerobic species like *Prevotella intermedia* and *Fusobacterium nucleatum.* As the disease progresses, the microbiota shifts so the so called 'red-complex', consisting of the periodontopathogens *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* (3, 30).

As periodontitis develops, the oral microbiota shifts from one consisting primarily of gram-positive aerobes to one consisting primarily of gram-negative anaerobes and from fermenting to proteolytic species (31). Multiple etiologies such as bacterial, genetic and immunological factors contribute to the development of periodontitis, make choosing appropriate treatment options quite difficult (32).

2.2. Chronic Periodontitis

CP is the most prevalent form of periodontitis and is generally considered to be a slowly progressing disease. Data from many sources confirm that patients with this form of periodontitis usually exhibit slow rates of progression (26,27). However, there are also data indicating that some patients may experience short periods of rapid progression (28,33). Therefore, in the American Academy of Periodontology 1999 workshop, it was concluded that rates of progression should not be used to exclude patients from receiving the diagnosis of CP. It is most frequently observed in adults, however it can occur in children and adolescents in response to chronic plaque and calculus accumulation. It is an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment and bone loss (34). Clinical findings of CP include gingival inflammation, pocket formation, loss of periodontal attachment, loss of alveolar bone, and occasional suppuration (35). Radiographically, horizontal and/or vertical bone loss is evident. In some cases, thickened, fibrotic marginal tissues may be present as a result of long-standing and low-grade

inflammation. In advanced cases, as the disease progresses, tooth mobility appears that may even result in tooth loss (35). CP can be further classified on the basis of extent and severity. As a general guide, extent can be characterized as Localized: ≤ 30% of sites involved and Generalized: > 30% of sites involved. Severity can be characterized on the basis of the amount of clinical attachment loss (CAL) as follows: Slight: 1 or 2 mm CAL, Moderate: 3 or 4 mm CAL, and Severe: ≥ 5 mm CAL (36).

CP is initiated and perpetuated by a small group of predominantly gram-negative, anaerobic or microaerophilic bacteria that colonize the subgingival area. However, these 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.

2.3. Initial Periodontal Treatment

Periodontal therapy aims to establish and maintain the health of the periodontium throughout the mouth. The main objective encompasses different areas of therapeutic objectives for each patient according to the patient's needs. It is based on the diagnosis, disease severity, and other factors and includes a reasoned decision on the possible and desirable therapeutic endpoints and the techniques to be used to reach this objective (37).

The primary goal is elimination of gingival inflammation and correction of the conditions that cause and/or perpetuate it. This includes not only elimination of root irritants, but also pocket eradication/reduction, establishment of gingival contours and mucogingival relationships conductive to the preservation of periodontal health, restoration of carious lesions, correction of existing restorations.

The treatment plan for periodontal disease includes four phases. Phase I (non-surgical phase) aims to eliminate the etiologic factors of gingival and periodontal

disease. It encompasses plaque control and patient education, SRP, correction of restorative and prosthetic irritational factors, antimicrobial therapy, occlusal therapy, minor orthodontic movement and provisional splinting and prosthetics. When successfully performed, this phase stops the progression of dental and periodontal disease. Immediately after Phase I therapy, the patient should be placed on the maintenance phase (Phase IV) to preserve the results obtained and prevent any further deterioration and recurrence of the disease. The procedures included in phase I therapy may be the only procedures required to solve the patient's periodontal problems, or they may constitute the preparatory phase for surgical therapy. While on the maintenance phase the patient enters into the surgical (Phase II) and/or restorative phase of treatment (Phase III). Phase II includes periodontal surgery to repair and improve the condition of the periodontal surrounding tissues and their esthetics, rebuilding of lost structures, placement of implants and construction of the necessary restorative work, whereas in Phase III final restorations, fixed and removable prosthodontic appliances and the response to restorative procedures are evaluated (37). After these procedures the patient is again placed on the maintenance phase.

Initial periodontal treatment involves supra- and subgingival mechanical debridement and instruction in self-administered oral health measures resulting in reductions in the total microbiota (38). The primary objective of initial periodontal treatment is to disrupt subgingival biofilm and remove bacterial deposits from root surfaces in order to stop further tissue destruction and eliminate or control etiological factors together with creating a microbial shift towards a flora more associated with health. These microbiological changes in turn result in lower levels of inflammation and relative stable periodontal attachment levels (2, 4, 5).

However, the efficacy varies in different situations and recolonization of the pre-treatment microbiota by periodontopathogens occurs within weeks to months which is considered as rather a short time period (2–4, 6, 7, 30, 38).

The adjunctive use of local and/or systemic antibiotics and antiseptics to conventional mechanical periodontal therapy improve the outcome of the treatment only temporarily (39). Increasing levels of antibiotic resistant bacteria favor the development of approaches that do not rely on antibiotics (40). Additionally, the widespread use of orally administered antibiotics is reflected with increased level of resistance in the subgingival microbiota of CP patients. This has encouraged researchers in various fields of healthcare to the development of alternative antimicrobial approaches.

Although many adjunctive treatment alternatives such as, antimicrobial agents, lasers and antimicrobial photodynamic therapy, have been proposed to solve the recolonization process, there is no clear-cut consensus on this subject. The combined use of these adjunctive approaches to non-surgical periodontal therapy has leaded temporary improvement of the results. The use of antimicrobial agents has been associated with the increasing levels of bacterial resistance as well as many side effects whereas lasers and photodynamic therapy still need improvements in terms of clinical efficacy (8, 9, 41, 42).

Recently probiotics have drawn attention as adjunctive to initial periodontal therapy in the field of periodontology.

2.4. Probiotics

2.4.1. History of probiotics

The use of fermented food to promote health goes back thousands of years (43). In the early 1900's the Ukrainian-born biologist and Nobel Prize winner Elie Metchnikoff introduced the probiotic concept by stating that 'lactic bacilli are good for health'(44). At that time, it was known that milk fermented with lactic-acid bacteria inhibits the growth of proteolytic bacteria because of the low pH produced during fermentation of lactose. He proposed that the lactic acid-producing strain *Lactobacillus bulgaricus* (which is contained in Bulgarian yoghurt) is able to displace pathological intestinal microbiota and thereby 'replace the harmful microbes by useful microbes' (44). Another researcher and pediatrician, his colleague; Henri Tissier found that *Bifidobacterium* (characterized by its Y-shaped morphology, and then called 'bifid' bacteria) dominated the gut of healthy children and was in low numbers in children with diarrhea. He later claimed that these *Bifidobacteria* restore balance in the gut microbiota and suggested that these bacteria could be administered to patients with diarrhea to restore the gut flora (45). The origin of the term "probiotic" derives from the combined word "pro" (Lat. "for") and "biotic" (Greek adjective from bios "life"), and was used by Werner Kollath (46) , a German bacteriologist, hygienist and food scientist, who used "Probiotika", to denote all good organic and inorganic complexes, in contrast to harmful antibiotics. In 1965, Lilly and Stillwell used the term 'probiotic' to describe substances secreted by one organism which stimulate the growth of other microorganisms (47). Parker extended the definition of probiotics to 'Organisms and substances which contribute to intestinal microbial balance', in 1974 (48). R. Fuller emphasized in 1989, that consumption of viable microbial cultures as dietary supplements will improve intestinal balance and defined probiotics as ' A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' (49). Since than, many more different definitions have been proposed (Table 1), but the Food and Agriculture Organization of the United Nations and the World Health Organization (WHO) put the currently used and most accepted definition of probiotics forward. In

2001, in a Joint Expert Consultation, they defined probiotics as ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf)

Table 1. Definitions of Probiotics

Year	Definition	Reference
1953	Probiotika are active substances that are essential for a healthy development of life	Kollath (46)
1965	Substances produced by microorganisms that promote the growth of other microorganisms	Lilly & Stillwell (47)
1974	Organisms and substances that contribute to intestinal microbial balance	Parker (48)
1989	A live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance	Fuller (49)
1992	A viable monoculture or mixed-culture of microorganisms that, when applied to animal or human, beneficially affects the host by improving the properties of the indigenous microflora	Havennaar & Huis In't Veld (50)
1996	Living microorganisms that, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition	Schaafsma (51)
1999	A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as by improving nutritional and microbial balance in the intestinal tract	Naidu et al. (52)
1999	Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host	Salminen et al. (53)
2001	A preparation of, or a product containing, viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and as such exert beneficial health effects in this host	Schrezenmeir & de Vrese (54)
2001	Live microorganisms which when administered in adequate amounts confer a health benefit on the host	FAO/WHO report

2.4.2 Prebiotics and Synbiotics

Different from probiotics, ‘prebiotics’ (i.e. insulin-type fructans, maltodextrin, fructo-oligosaccharides and galacto-oligosaccharides) have been defined as non-digestible oligosaccharides that affect the proliferation of resident commensal bacteria that may than exert probiotic effects (55). This definition has been refined to include selectively fermented ingredients that allow the resident microflora that confer benefits upon host well-being and health, by M. Roberfroid (56).

The concept of prebiotics has the same aim as probiotics namely, improvement of the host health via modulation of the intestinal flora, but with a different mechanism. The major mechanism of action of prebiotics is assumed to be indirect, i.e. facilitating the proliferation of beneficial components of resident microflora with probiotic effects. There is evidence that some prebiotics also show direct effects on the host, which include stimulation of expression of IL-10 and interferon-γ, enhancement of IgA secretion, modulation of inflammatory responses to pathogens and stabilization of the gut mucosal barrier (54, 56). Studies of prebiotics have mainly been focused on gastrointestinal microbiota and health benefits; there has been little work in the oral cavity (57).

However, there are some cases in which prebiotics may be beneficial for the probiotics, especially with regard to *bifidobacteria*, which is known as the symbiotic concept (16). Synbiotics are defined as ‘mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract of the host’ (58).

2.4.3. Replacement therapy

Another term used in the literature and within context is ‘replacement therapy’ (also called ‘bacterio-therapy’ or ‘bacterial interference’). Although both approaches

use live bacteria for the prevention or treatment of infectious disease, there are some slight differences (Table 2) (16).

Table 2. Differences between ‘replacement’ therapy and ’probiotic’ therapy (16).

Replacement therapy	Probiotic therapy
Effector strain is not ingested and is applied directly on the site of infection	Probiotics are generally used as dietary supplements
Colonization of the site by the effector strain is essential	Probiotics are able to exert a 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

Due to the widespread use of antibiotics, occurrence of side effects and the development of resistance, encouraged researchers in various fields of healthcare, to develop alternative antimicrobial treatments and made this type of health promoting bacterial therapy popular again. (57–59)

Review of the literature reveals only a few numbers of studies in terms of replacement therapy applied in periodontology. The first one by Teughels et al. (41) serves as a pioneer study in this context. In an *in vivo* beagle dog model for periodontitis these authors explored that the subgingival application of beneficial bacteria interferes or retards the recolonization of periodontal pockets after SRP.

2.4.4. Classification of Probiotics

The effectiveness of probiotics is strain specific and their contribution to host health is obtained by different mechanisms such as the suppression of virulence factors production, prevention or inhibition of the proliferation of pathogens or modulation of the immune response (60). There are a number of different organisms that can be classified as probiotics (61). Most commonly used probiotic strains belong to the *Lactobacillus* and *Bifidobacterium* genera, but use of certain other bacterial strains like *Escherichia, Enterococcus, Bacillus* as well as *Streptococcus* have been documented (Table 3) (62).

Table 3. Names of micro-organisms used as Probiotics (62).

Lactobacillus sps.	***Bifidobacterium sps.***	***Streptococcus sps.***	***Sacchoromyces sps.***	***Others***
L.acidophilus	*B. bifidum*	*S.thermophilus*	*S.boulardii*	*Bacillus cereus*
L.casei (rhamnosus)	*B.breve*	*S. salivarius subsp. thermophilus*		*Escherichia coli*
L.fermentum	*B.lactis*			*Enterococcus*
L.gasseri	*B.longum*			*Propioni-bacterium freudenreichii*
L.johnsonii	*B.infantis*			
L.lactis	*B.adolescentis*			
L.paracasei				
L.planrarum				
L.reuteri				
L.sallivarius				
L.bularicus				

2.4.5. General Features of Probiotics

For any health promoting properties to persist, probiotics need to be ingested regularly, since they do not colonize the host permanently (63). Therefore they need some functional properties as listed below (63–65):

1. Adherence and colonization for a certain time to human body.
2. Enhancement of non-specific and specific immune response of the host.
3. Production of antimicrobial substances and competition with pathogens for binding sites.
4. Inhibition of pathogen growth.
5. Resistance and survival to human defense mechanisms during the oro-gastrointestinal pathway (e.g. tolerance to acids (low pH) in the mouth and stomach, and tolerance to bile in the upper intestine).
6. Human safety.
7. Be of human origin.
8. Exert non-pathogenic properties.
9. Confer clinically established physiological benefits.
10. Maintain viability and activity throughout manufacture and processing.

These criteria are application-based and depend on the desired specific probiotic effects and the target site of action.

2.4.6. Application of Probiotics

At the present time, probiotics in the world are provided in products in one of four basic ways:

a) Inoculated into a milk-based food (dairy products such as milk, milk drink, cheese, kefir, biodrink),
b) Inoculated into prebiotic fibers,
c) As a culture concentrate added to a beverage or food (such fruit juice),
d) As concentrated and dried cells packaged as dietary supplements (non-dietary products such as powder, capsule, gelatin tablets) (66).

Generally, most probiotics are delivered in diary products (fermented milk products), tablets or in beverages. Different formulations of over the counter products used as probiotics are listed in Table 4. These might not be the best way for a long time contact with oral tissues, however probiotic adhesion is facilitated on saliva coated surfaces. Recommended formulation of these diary products are at a concentration of 10^6 probiotic bacteria per gram or milliliter (58, 66–68).

Table 4. Major probiotic products in the world (66).

Strain	Present in product	Country produced
B. bifidum	Infant formula	Turkey
B. breve	Drink	Japan
B. lactis	Infant formula Research Drink	Israel Switzerland South Africa Chile
B. lactis HN019	Research	New Zealand
B. longum	Infant formula	Turkey
B. longum SBT-2928	Milk	Japan
B. longum BB536	Milk	Japan
B. spp	Drink	UK
L. acidophilus	Yogurt Drink Yogurt drink	Chile, USA UK Austria
L. acidophilus 5	Yogurt drink	UK
L. acidophilus 7	Yogurt	Austria
L. acidophilus Lat 11/83	Drink	Russia
L. acidophilus NCFB 1748	Research	Denmark
L. acidophilus SBT-2062	Milk	Japan
L. bulgaricus	Milk	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 Yogurt Kefir	USA USA USA, Austria
L. helveticus	Milk Drink	Finland Iceland
L. johnsonii La1	Yogurt	Switzerland, Germany, Japan, Austria
L. lactis L1A	Yogurt	Sweden
L. plantarum	Kefir	USA
L. plantarum 299v	Fruit drink Ice cream Recovery drink Oat mixture	Sweden Sweden Sweden Sweden
L. plantarum JI:1	Research	Sweden
L. reuteri	Infant formula Cheese Milk Yogurt Yogurt drink Ice cream Fruit drink Tablet Straw	Israel Spain, Portugal, Finland Japan, Finland USA, Finland UK Finland Finland
L. rhamnosus ATCC53103(LGG)	Yogurt Yogurt drink	Australia, Papua New Guinea, Indonesia, Finland, Latvia, Estonia, Croatia, South Korea, Bosnia-Herzegovina, Slovenia, Ecuador, Israel, Italy, Netherlands, Japan, Norway, Switzerland Australia, Finland, Sweden, Croatia, Bosnia-Herzegovina, Slovenia, Ecuador, Uruguay, Netherlands, Taiwan, Norway Finland, Sweden UAE, Israel, Italy Germany, Portugal, Japan, Iceland, Greenland, Spain, Estonia,

	Fruit yogurt Milk Milk drink Fruit drink	Ireland, Israel, South Korea Finland
L. rhamnosus	Drink	Finland, Sweden, Chile, South Africa
L. rhamnosus LB21	Yoghurt	Sweden
L. rhamnosus 271	Drink	Sweden
L. salivarius U CC 118	Research	Ireland
L. rhamnosus VTTE-97800	Research	Finland
S. salivarius K12	Lozenge	New Zealand
S. thermophilus	Drink Yogurt drink Infant formula	France, Austria Austria Turkey
E. faecium	Yogurt	Denmark
E. faeciumFargo 688	Research	USA

2.4.7. *Lactobacillus reuteri*

Genera *Lactobacillus* and Bifidobacterium include a large number of species and strains exhibiting important properties especially in the area of food and probiotics.

Lactobacilli are categorized into one of three groups based on the type of metabolic pathway used to ferment carbohydrates.

1) The obligately homofermentative group (e.g., *L. acidophilus*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*) possess a fructose diphosphate (FDP) adolase pathway dictating a glycolytic conversion of sugars primarily into lactic acid.
2) The facultatively heterofermentative group (e.g., *L. casei, L. curvatus, L. plantarum, L. sake, L. rhamnosus*) can use either this FDP adolase pathway to ferment certain sugars, or they can induce the phosphoketolase pathway to ferment other sugars.
3) The obligate heterofermentative group (e.g., *L. brevis, L. buchneri, L. fermentum, L. reuteri*) has only the phosphoketolase-based option (69).

The genus *Lactobacillus* includes 106 validity described species and is the most extensive genus in the order *Lactobacillales* (70). *Lactobacilli* are rods, normally present in the gastrointestinal and vaginal tract in healthy humans, and thought to be involved in the maintenance of the microbiota (71). *L. reuteri* was described in 1980 by Kandler et al. (72), as a new subspecies of heterofermentative lactobacilli, based on DNA-homology. This species is a gram-positive, non-spore forming, non-motile, facultative anaerobic rod shaped bacillus. Optimum growth temperature for *L. reuteri* is between 37-41^0 and optimum growth pH is ~6.5. *L. reuteri* is normally cultivated in oxygen-limited atmospheres and does not require anaerobic conditions for growth. Strains of *L. reuteri* are fastidious and rely on the availability of fermentable sugars, amino acids, vitamins and nucleotides. The organism will grow very fast if these factors are provided (73).

L. reuteri is reported to produce compounds that exhibit antagonistic activity, i.e. reuterin (74) and reutericyclin (75). These are water-soluable, broad-spectrum antimicrobials, effective over a wide pH, and resistant to proteolytic and lypolytic enzymes (75, 76). In several studies, especially *L. reuteri ATCC 55730* (and its daughter strain *DSM 17938*), has been demonstrated to have probiotic properties (70, 77–80).

L. reuteri is also a member of the indigenous oral microbiota. Antibacterial activity of *L. reuteri* was demonstrated in vitro in non-oral pathogenic bacteria (e.g. *S. aureus, L. monocytogenes* etc.) and in *Candida albicans*, without affecting the indigenous health-related microbiota (81). *L. reuteri's* unique ability to synthesize and secrete the antimicrobial agent reuterin is relatively well understood (81, 82).

Reuterin was shown to be an intermediary metabolite involved in two-step pathway by which glycerol is first dehydrated to form reuterin some of which is then reduced to 1,3-propanediol (Figure 1) (83).

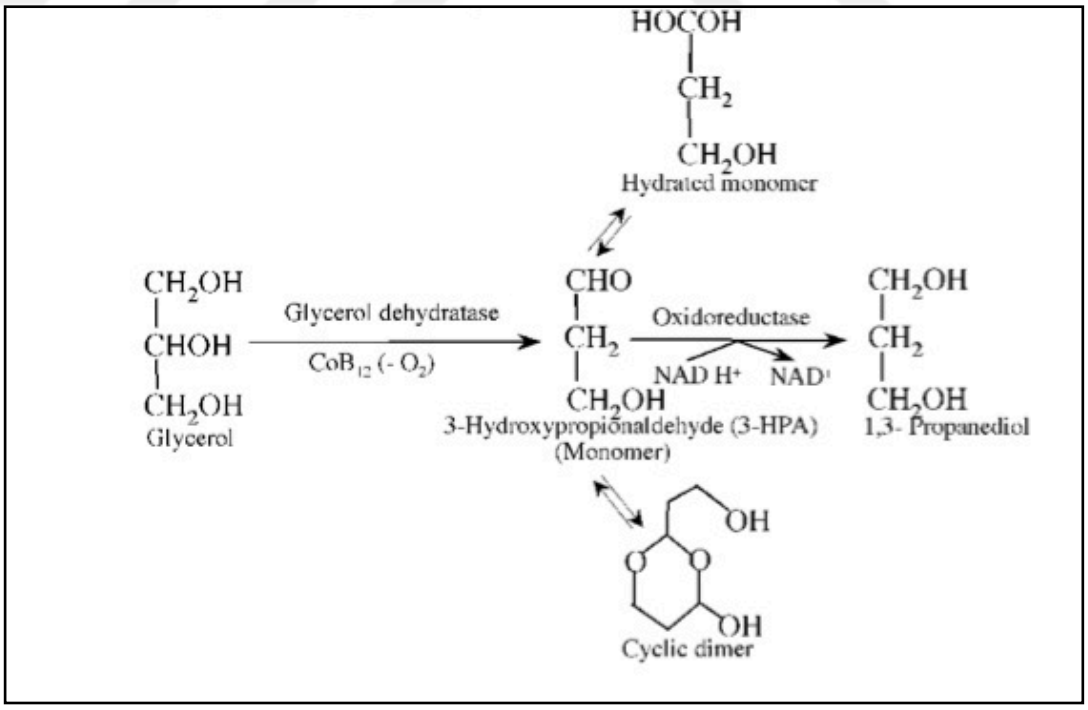

Figure 1. Reuterin synthesis pathway by *L.reuteri* (83)**.**

2.4.8. Probiotics and General Health

Various beneficial health effects from the consumption of probiotic bacteria have been proposed (83, 84). Although the specific mechanisms are not completely understood and known, evidence suggests that probiotics can influence various diseases positively. Traditionally probiotics have been used to treat diseases related to the gastrointestinal tract. Varying densities of different microbial species populate different niches of the digestive tract (85). Although not predominant in the gastrointestinal microbial ecology, widely used species belong to the genera *Lactobacillus* and *Bifidobacteria*. However, these organisms are already produced in the diary products and because they are very rarely implicated in infections of humans. Therefore, they are categorized as 'Generally Regarded As Safe' by the United States Food and Drug Administration (16).

The gastrointestinal microflora acts on its host mainly by performing a variety of metabolic activities, protecting against colonization by pathogens and stimulating the gut immune system (62, 85, 86). Bacterial products like, Lipopolysaccharides (LPS), Peptidoglycans and lipoteichoic acids have immunomodulatory properties and contribute to the mucosal and systemic immunomodulating effects that ileal and colonic bacteria have in the host. Interaction of probiotics with the immune system occurs at the level of cytokine production, mononuclear cell proliferation, macrophage phagocytosis, modulation of autoimmunity and immunity to bacterial and protozoan pathogens (62, 87). Also, lactic acid bacteria may improve the immune system by increasing the number of IgA-producing cells as well as increasing the proportion of T lymphocytes and Natural Killer cells (88). There is evidence that lactic acid bacteria modulate inflammatory conditions such as inflammatory bowel disease in adults and hypersensitivity responses such as milk allergies (88, 89). In general, accumulating evidence suggests that probiotics may have a role in gastrointestinal health.

Additionally, probiotics may be used in other clinical conditions such as, urogenital infections, atopic diseases and oro-pharyngeal infections like acute otitis media and steptococcal pharyngotonsillitis (16).

Most common clinical conditions with a positive outcome after probiotic administration are listed in the Table 5.

Table 5. Clinical conditions improved by probiotic intake.

Disorder	Probiotic	Patient group	Duration	Clinical effect	Reference
GI disorder					
Ulcerative colitis	*E. coli* Nissle 1917	116	12 months	Induction of remission; prevention of relapses	Rembacken et al.,(90)
	E. coli Nissle 1917	120	12 weeks	Maintaining the remission	Kruis et al., (91)
	B. longum	120	4 weeks	Improved systemic function	Fujimori et al., (92)
	VSL#3	29	12 months	Remission maintenance	Miele et al., (93)
	L. rhamnosus GG	187	12 months	Prolongation of relapse-free time	Zocco et al., (94)
	E. coli Nissle 1917	327	12 months	Induction of remission	Kruis et al., (95)
	Saccharomyces boulardii	25	4 weeks	Induction of remission	Guslandi et al., (96)
Crohn's disease	*Saccharomyces boulardii*	34	3 months	Improved intestinal permeability	Garcia Viela et al., (97)
	L. johnsonii	98	6 months	Postsurgical Crohn's disease recurrence	Marteau et al., (98)
	E. coli Nissle 1917	24	3 months	Relapse rate decreased	Guslandi et al., (99)
	Genetically modified *L. lactis* (LL Thy12)	10	7 days	Decreased in disease activity	Baraat et al., 2006

Pouchitis	VSL#3	36	12 months	Maintaining the remission	Mimura et al., (100)
	VSL#3	23	4 weeks	Prolongation of remission	Gionchetti et al., (101)
Lactose maldigestion	*L. acidophilus*	20	On intake	Decreased symptoms of lactose-maldigestion	Montes et al., (102)
Diarrhea episodes	*L .rhamnosus GG*	204	15 months	Reduction of diarrhea episodes in children	Oberhelman et al., (103)
	L. rhamnosus 19070-2; L. reuteri DSM 12246	69	5 days	Reduction of diarrhea phase	Rosenfeldt et al., (104)
	L. paracasei ST 11	230	5 days	Improved management of non-rotavirus diarrhea	Sarker et al., (105)
	L. rhamnosus GG	140	5 days	Shorten diarrhea duration	Guandalini et al., (106)
	Probiotic combination	75	5 days	Shorten diarrhea periods	Teran et al., (107)
Allergy states	*L. acidophilus NCFM; B. lactis*	47	4 months	Prevention of pollen-induced infiltration of eosinophils	Ouwehand et al., (108)
	Lactobacillus F19	89	7 months	Prevents early manifestation of allergy	West et al., (109)
	L. GG; L.gasseri TMC0365	40	10 weeks	Decreased allergic rhinitis	Kawase et al., (110)

2.4.9. Mechanism of Action of Probiotics

Action mechanism of probiotics in the mouth, are expected to be similar to those in other parts of the body. Several health benefits in the gastrointestinal tract have been suggested with the need of some additional properties. These include the ability to adhere and colonize the periodontal tissues including non-shedding surfaces and becoming part of the oral biofilm. Also, probiotics should not ferment sugars, which subsequently lowers the pH and can be detrimental, resulting in caries (111). However, to date there is no evidence supporting this suggestion.

Many of the probiotics used for gastrointestinal purposes exert their effect without colonizing or with only a temporary colonization of the host (12). It may be anticipated that, even without permanent colonization, repeated daily use of probiotic products over a long period will support an increase level of the probiotic in the oral cavity. This observation suggests that probiotic bacteria may exert beneficial effects without permanently colonizing the host, which can be attributed to their mechanism of action.

Theoretically 3 main modes of action have been suggested for probiotics (58, 112, 113).

1. Modulation of host defenses including innate and acquired immune system.
2. Production of antimicrobial substances against periodontal pathogens.
3. Competitive exclusion mechanisms.

Another feature of probiotics is that they can produce a diverse range of compounds that act as antimicrobial substances such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, bacteriocins, low molecular weight antimicrobial substances and adhesion inhibitors, that prevent growth of food-borne pathogens in the dairy products (12, 114).

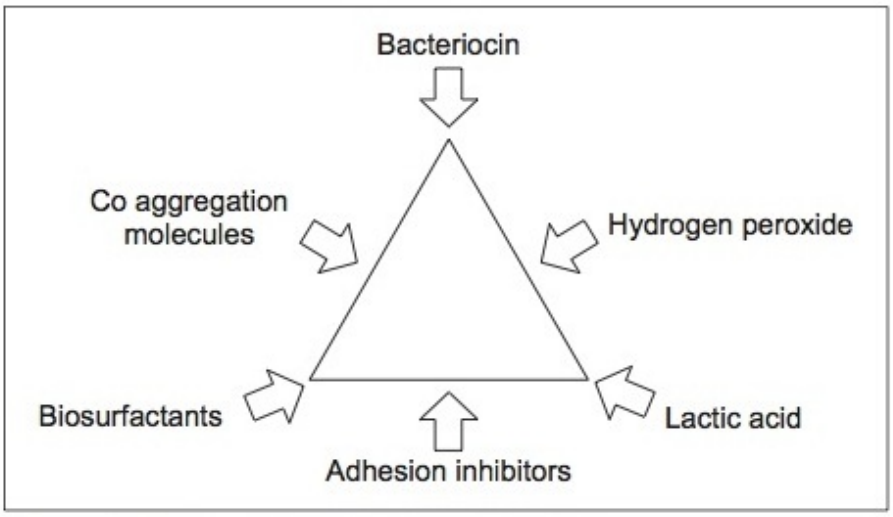

Figure 2. Metabolites of lactic acid bacteria (115).

Mechanisms of probiotics in the oral cavity may be either direct interaction with dental plaque or indirect by modulating host defenses.

Direct interactions include inhibition of specific pathogens by:

- Involvement in binding of oral micro-organisms to proteins (biofilm formation).
- Action on plaque formation and on its complex ecosystem by competing and intervening with bacteria-to-bacteria attachments.
- Involvement in metabolism of substrates (competing with substrates available).
- Production of chemicals that inhibit oral bacteria (antimicrobial substances).

Indirect interactions include effects on the host response such as:

- Inhibition of collagenases and reduction of inflammation-associated molecules.
- Induction of expression of cytoprotective proteins on host cell surfaces.
- Modulation of pro-inflammatory pathways induced by pathogens.
- Prevention of cytokine-induced apotosis.
- Modulation of host immune response.

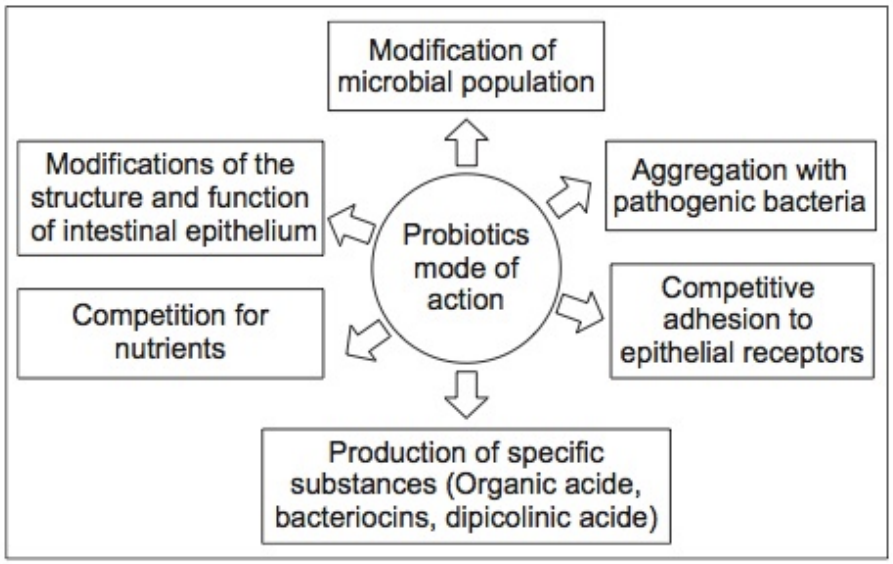

Figure 3. Mechanism of action of probiotics (115).

These potential mechanisms of probiotics in the mouth are not fully known and therefore need further investigation.

2.4.10. Probiotics and the Oral Microbiota

The microbiota of the oral cavity is unique and highly complex in its structure, containing a wide variety of bacterial species residing in oral biofilms as well as in a planktonic state. Predominant organisms in the oral cavity include *Streptococcus* species (particularly *S. sanguinis, S. mitis, and S. crista*), *Lactobacillus* species (particularly *L. gasseri, L. fermentum and L. salivarius*), *Fusobacterium*, *Bacteriodes, Porphynomonas, Prevotella, Haemophilus, Eubacterium, Bifidobacterium, Neisseria, Veillonella, Capnocytophaga, Peptostreptococcus, Staphylococcus, Proprionibacterium, Cornyebacterium, Actinomyces* and *Treponema* (116).

Resident commensal bacterial populations are protecting oral tissues from colonization by exogenous pathogens, promote normal development of host cell structure and function, ensure normal development of the immune system, and down

regulate immune responses (117). It is estimated that more than 1000 bacterial species colonize the mouth and oropharynx (116, 117), but only 50-60% of these microorganisms can be currently cultured. This may be due to the evolved biofilm community growth of some species, which therefore can not grow in monoculture (118). Within this biofilm life, resident bacteria gain significant advantages such as protection from the host defenses and antimicrobial agents; expression of resident phenotypes; and the development of food-webs and interactions such as quorum-sensing to communicate with each other (118–121).

To be an oral probiotic the essential requirement for a microorganism is its ability to adhere and colonize the surfaces in the oral cavity. Generally probiotics do not have the oral cavity as natural habitat and therefore the benefit on oral health may be questionable. However, studies suggest that *Lactobacilli* as members of the resident oral microflora could play an important role in the micro-ecological balance in the oral cavity (122).

In general, the oral microbiota plays an important role in human health and it is linked to common oral diseases such as dental caries and periodontal diseases.

2.4.11. Common Oral Diseases

The most common oral diseases are dental caries and periodontal diseases, which are both, related to dental plaque and mostly occur when the equilibrium between the bacterial load and host response is disrupted. This unbalanced oral microbiota can be associated with serious systemic diseases such as spontaneous preterm births, coronary heart disease, atherosclerosis and chronic kidney diseases (123).

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. Therefore, pattern of adhesion of different probiotic

strains to oral epithelial cells have been investigated and probiotics are broadly used in dairy products such as yoghurt and cheese (61).

Authors have reported that the co-aggregation abilities of *lactobacilli* species might enable them to form a barrier that prevents colonization of pathogenic bacteria, due to the production of a microenvironment around these pathogens in which inhibiting substances were generated by *Lactobacillus* species (111). In a study, Haukioja et al. (124), 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* showed better adherence than *Bifidobacteria*. Thus, *lactobacilli* may compete for the same binding sites on saliva-coated hydroxylapatite with *F. nucleatum*, which indicates that probiotics might affect the formation of oral biofilms, by modifying resident microflora.

Recently potential application of probiotics for oral health has attracted researchers to investigate oral probiotics, suggesting that probiotics could be useful in preventing and treating oral infections, such as dental caries (122,124), periodontal disease (17), Halitosis (125), and *Candia albicans* (126) infections.

2.4.12. Probiotics and Dental Caries

In case of dental caries, increased numbers of *streptococci* are associated at the site of the disease. A key factor in caries process is the production of carboxylic acids from dietary sugars such as sucrose, fructose and glucose. A wide group of acid-producing microorganisms such as low pH non-mutans *Streptococci*, *Veilonella, Propionibacterium, Bifidobacterium* are identified from carious lesions that may considered the main pathogenic species involved in the initiation and development of dental caries (125–127). *Lactobacilli* species are also associated with the development of carious lesions. These contain homo- and heterofermentative species, which are all

aciduric, and the low pH generated from acids challenge the homeostasis in the oral microbial community (128, 129). However, in light of the ecological plaque hypothesis (130, 131), caries is a result of a shift in the balance of resident microbiota driven by the environmental changes (132) and it is believed that all there parameters (microorganisms, susceptible host, and environment) must occur together for the development of carious lesions.

Several experimental studies utilizing different test strains evaluated the impact of oral administration of probiotics on dental caries. Strains of *lactobacilli* such as *L. rhamnosus GG* and *L. casei* have proved their potential to inhibit growth of oral streptococci. Çağlar et al. (133, 134), found definite *S. mutans* count reduction after a 2-week consumption of yoghurt containing *L. reuteri*. A temporary reduction in *S. mutans* was observed during period of yoghurt intake and few days after cessation of consumption, indicated the necessity of continual administration of the probiotic in order to achieve an effect. However, it has been suggested that the operative approach in caries treatment might be challenged by probiotic implementation with subsequent less-invasive intervention in clinical dentistry. Therefore more studies are needed before this goal could be definitely achieved.

2.4.13. Probiotics and Halitosis

Oral malodor (Halitosis) is mostly associated with an imbalance of the resident microflora of the oral cavity. Halitosis results from the proteolytic putrefaction of sulfur containing amino acids in dietary and salivary protein by mostly anaerobic, gram-negative bacterial species, which are transformed into volatile sulphur compounds (VSC), including hydrogen sulphide and methanethiol (135). In a study Burton et al. (136), reported inhibitory effect of *S. salivarius* on VSC by competing for colonization with species that cause increased levels of VSC. Also inhibition of the production of VSC by various strains of *W. cibaria* has been reported (137). The authors of the study reported that this beneficial effect resulted from the production of hydrogen peroxide by *W. cibaria,* which inhibits the proliferation of *F. nucleatum*. The 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.

2.4.14. Probiotics and Candida Infections

Oral Candidiasis is a yeast/fungal infection of *Candia spp.* on the mucous membranes of the mouth. Most oral fungal infections are caused by *Candida albicans*. This species is harbored in about 50% of the world's population as a normal component of the oral microbiota. However, the presence of *C. albicans* in the mouth is not considered as a disease, but when *candida* species become pathogenic and invade host tissues, oral candidiasis can occur. Candidiasis is an opportunistic infection caused by normally harmless microorganisms because of local or systemic factors altering the host immunity. Predisposing factors for oral candidiasis include usage of immunosuppressive drugs, multiple broad-spectrum antibiotics, anticholinergic agents. Also, endocrine dysfunction, bone marrow depression, immunodeficiency disorders, malignancies, nutritional deficiencies, radiation treatment and xerostomia are the cause for oral candidiasis.

The use of probiotics in the treatment of Candidiasis is an emerging field (16). A rapid decline in *C. albicans* levels in mice after the intake of probiotic strains *L. acidophilus* and *L. fermentum* was reported (138). A protective effect was observed after continuous consumption of the probiotic and maintained for a prolonged period after cessation of application. Another study by Hattaka et al. (126), reported a reduction in the prevelance of *C. albicans* after consumption of probiotic cheese containing *L. rhamnosus GG* and *Propionibacterium freudenreichii* spp. Shermanii JS.

2.4.15. Probiotics and Periodontal Disease

The current view on the etiology of plaque-related periodontal inflammation considers three factors that determine whether disease will develop in a subject: a

susceptible host; the presence of pathogenic species; and the reduction or absence of so-called beneficial bacteria (115) .

The first studies in the field of periodontal therapy are experimental gingivitis studies.

Staab et al. (139) evaluated the effect of a probiotic milk drink on gingival health and the development of experimental gingivitis. Fifty volunteer students took part in a parallel-designed non- blinded study. The test group drank a probiotic drink once a day; the control group did not receive any product to drink. After 8 weeks, individual mechanical plaque control was interrupted for 96 h. Clinical and immunological evaluations were recorded at baseline, after 8 weeks and again 96 h later. The authors determined that clinical parameters were not different between the groups. In the test group, some of the immunological parameters were significantly lower after the intake of the probiotic milk drink. The authors suggested a beneficial effect of the probiotic milk drink on gingival inflammation.

Twetman et al. (14) investigated the effect of a chewing gum containing probiotic bacteria on gingival inflammation and the levels of selected inflammatory mediators in gingival crevicular fluid. Forty-two healthy adults with moderate levels of gingival inflammation entered a double-blind placebo-controlled study design. The subjects were randomly assigned to one of three parallel arms: Group A/P was given one active and one placebo gum daily, Group A/A received two active chewing gums, and Group P/P two placebo gums. The chewing gums contained two strains of *Lactobacillus reuteri*. The subjects were instructed to chew the gums for 10 min over the course of 2 weeks. Bleeding on probing (BoP) and GCF sampling were conducted at baseline and after 1, 2 and 4 weeks. Immunological parameters were determined using luminex technology and multiplex immunoassay kits. BoP improved and GCF volume decreased in all groups during the chewing period, but the results were statistically significant only in Groups A/P and A/A. The levels of TNF-a and IL-8 decreased significantly in Group A/A compared with baseline after 1 and 2 weeks, respectively. A non- significant decreasing tendency was also observed concerning IL-1b during the

chewing period. The levels of IL-6 and IL-10 were unaffected in all groups after 1 and 2 weeks. As a result, the authors indicated that the reduction of pro-inflammatory cytokines in GCF might be proof of principle for the probiotic approach combating inflammation in the oral cavity.

Iniesta et al. (21) investigated the effects of an orally administered probiotic on the oral microbiota in 40 gingivitis subjects during 8 weeks. Treatment consisted on the administration of a daily tablet, either containing *L. reuteri* or placebo. Unstimulated saliva and subgingival samples were collected and analyzed by culture and PCR. Clinical and microbiological outcome variables were compared between and within groups. The authors determined no significant changes 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, showed reductions in the test group. In subgingival samples, significant reductions in the changes baseline to 4 weeks were observed for *P. gingivalis* counts. The authors concluded that 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.

Krasse et al. (17), assessed the effectiveness of the probiotic *L. reuteri* in the treatment of gingivitis and further evaluated the influence of the probiotic on plaque and the *lactobacilli* population in saliva. A randomized, placebo-controlled, double blind study was performed over 2 weeks. Fifty-nine patients with moderate to severe gingivitis were included and given one of two different *L. reuteri* formulations at a dose of 2 x 10^8 CFU per day, or a corresponding placebo. At baseline gingival index and plaque index were measured and saliva was collected for lactobacilli determination. After 14 days clinical measurements and saliva samples were assessed. Gingival index decreased significantly in all 3 groups. Plaque index decreased significantly in one of the active groups between day 0 and day 14 but there was no significant change in the placebo group. At day 14, patients were colonized with *L. reuteri* in the active groups. The authors indicated that *L.reuteri* was efficacious in reducing both gingivitis and plaque in patients with moderate to severe gingivitis.

Koll-Klais et al. (140) reported that probiotic strains included in periodontal dressings at optimal concentration of 10^8 CFU/ml were shown to diminish the number of most frequently isolated periodontal pathogens: *Bacteroides sp., Actinomyces sp*., and *S. intermedius*, and also *Candida albican*s. These authors registered that the resident *Lactobacilli* flora inhibits the growth of *Porphyromonas gingivalis* and *Prevotella intermedia* in 82% and 65%, respectively and a 10 to 12-month remission period after periodontal treatment by application of the periodontal dressing that consisted of collagen and *L. casei* was observed.

Ishikawa et al. (141) assessed the ability of *L. salivarius* TI 2711 (LS 1) to displace periodontopathogenic bacteria like *P. gingivalis* and *P. intermedia* in an in vivo study. LS 1 was one thousand fold more susceptible to lactic acid that *L. acidophilus*, a representative acid-resistant *Lactobacillus* strain found at the sites of caries, when these bacteria were exposed to lactic-acid. In an in vitro system, LS 1 completely killed *P. gingivalis* within 24 hours when these bacteria were co-cultured together. In a clinical study, 57 subjects took tablets containing 2x10 7 CFU or more LS 1 daily for 4 or 8 weeks. The black-pigmented anaerobic rods, which include most periodontopathogenic bacteria, in the saliva decreased to one-twentieth of the initial value after 4 weeks, whereas the numbers of whole bacteria, *S. mutans* and *lactobacilli* did not change. While saliva pH was widely distributed (ranging from 5.4 to 8.5) before LS 1 treatment, it converged into a neutral range of around 7.3 after treatment. Therefore, the possibility that LS 1 accelerates caries formation by lowering the pH in the oral cavity was excluded. The author's findings suggest that LS 1 may be a potentially useful probiotic agent against periodontopathogenic bacteria.

Studies by Mohammad S. Al-Zahrani (142) have shown an inverse association between the intake of dairy products and prevalence of periodontitis. Yoshihiro Shimazaki (143) concluded that the routine intake of lactic acid foods may have a beneficial effect on periodontal disease. *C. albicans* is among the most common infectious agents in the oral cavity. The incidence of yeast infections is higher at older age and under conditions of impaired immunity. Testing the pattern of colonization of L. acidophilus and *L. fermentum*, Elahi et al.(138) reported a rapid decline in *C.*

albicans in mice after the intake of probiotic strains. Continuous consumption of probiotics led to almost undetectable numbers of fungi in the oral cavity, maintaining the protective effect for a prolonged period after cessation of application.

Shimauchi et al. (144) evaluated the effect of probiotic intervention using lactobacilli on the periodontal condition of volunteers without severe periodontitis. Freeze-dried *L. salivarius* WB21 (WB21)- containing tablets or a placebo were given to volunteers in a double-blind randomized study. A total of 66 volunteers were randomly assigned to receive tablets containing WB21 (6.7×10^{8}CFU) with xylitol or xylitol alone (placebo) three times a day for 8 weeks. Periodontal clinical parameters and whole saliva samples were obtained at baseline, 4 weeks, and the end of 8 weeks. Salivary lactoferrin levels were measured by enzyme-linked immunosorbent assay. *Lactobacilli* in saliva and plaque samples were detected by semi- quantitative RT-PCR using 16S rRNA primers. The authors reported improvement in periodontal clinical parameters in both groups after an 8-week intervention. Current smokers in the test group showed a significantly greater improvement of plaque index and probing pocket depth from baseline when compared with those in the placebo group. Salivary lactoferrin level was also significantly decreased in the test group smokers. The authors concluded that probiotics could be useful in the improvement/ maintenance of oral health in subjects at a high risk of periodontal disease.

Mayanagi et al. (19) evaluated whether the oral administration of *lactobacilli* could change the bacterial population in supra/subgingival plaque in a randomized double-blind placebo controlled clinical trial. Sixty-six healthy volunteers without severe periodontitis were randomized into two groups to receive either *lactobacilli* (2.01×10^{9} CFU/day of *L. salivarius* WB21 and xylitol) or placebo (only xylitol) over a 8 week study period. The authors conluded that oral administration of probiotic lactobacilli decreased significantly the numerical sum of the five selected periodontopathogenic bacteria including *A. actinomycetemcomitans, P. intermedia, P. gingivalis, T. denticola,* and *T. forsythia.*

Vivekananda et al. (145) evaluated the effects of *L. reuteri* (Prodentis) alone and in combination with SRP in a double blind, randomized, placebo-controlled clinical trial of 30 volunteers with CP. The study period was 42 days. 'Split-mouth' design was used for the SRP, which was performed on day 0. The participants received. *L. reuteri* Prodentis lozenges ($1x10^8$ CFU DSM17938 and $1x10^8$ CFU ATCC PTA 5289) or the corresponding placebo lozenges twice a day from day 21 to day 42. Clinical parameters (PI, GI, BoP PD and CAL) and microbiological levels of the pathogens *Aggregatibacter actinomycetemcomitans* (A.a), *P. gingivalis* and *P. intermedia* were assessed at day 0 before SRP treatment, on day 21 before administration of the lozenges, and on day 42. At day 42, the PI, GI, and GBI were significantly reduced by all treatment modalities. For PD and CAL, the best result was obtained with the SRP + Prodentis treatment. PPD was reduced from 5.08±0.75 to 3.78±0.61 mm ($p<0.001$) and CAL from 3.93±0.93 to 2.85±0.74 mm ($p<0.001$). Prodentis, either alone or following SRP, reduced A.a, *P. gingivalis* and *P. intermedia* by 1 $\log_{10}$ unit ($p<0.01$). The authors reported plaque inhibition, anti-inflammatory, and antimicrobial effects of *L. reuteri* Prodentis lozenges after administration twice a day for three weeks in CP patients.

Vicario et al. (146) reported similar findings after 30 days usage of tablets containing the same strain of *L. reuteri.* The authors assessed the clinical effect of the administration of *L. reuteri* Prodentis as a probiotic agent in the treatment of initial to moderate CP. Patient compliance factor and potential side effects of the probiotic agent were also evaluated. Twenty systemically healthy, non-smoking subjects with initial-to-moderate CP were enrolled in a double-blind, placebo-controlled, randomized clinical trial for 1-month. Clinical parameters were collected at baseline and 30 days post-treatment. Clinical parameters were improved in the test group after a 30-day intervention. The test group demonstrated a statistically significant reduction ($p < 0.05$) in all the periodontal parameters included in the study (PI, BoP and PD), while the control group treated with placebo did not show any statistically significant change in periodontal parameters. At the end of the study period, no adverse reactions were reported. The authors concluded that, oral administration of *L. reuteri* Prodentis improved the short-term clinical outcomes in non-smoking patients with initial-to-moderate CP.

Teughels et al. (22) evaluated the effects of *L. reuteri* containing probiotic lozenges as an adjunct to SRP in thirty chronic periodontitis patients. Patients were recruited and monitored clinically and microbiologically at baseline, 3, 6, 9 and 12 weeks after therapy. All patients received one-stage full-mouth disinfection and were randomly assigned to a probiotic or placebo group. The lozenges were used twice a day for 12 weeks. At week 12, all clinical parameters were significantly reduced in both groups, however there was significantly more pocket depth reduction and attachment gain in moderate and deep pockets; more *P. gingivalis* reduction was observed in the test group. The results indicated that oral administration of *L. reuteri* lozenges could be a useful adjunct to SRP in CP.

However, there is not yet any true evidence on the effect of probiotic therapy on periodontal disease, and the effect of the ingested probiotics needs further investigation.

Therefore, the objective of this study was to evaluate the effect of *L. reuteri* containing lozenges adjunctive to SRP in terms of clinical and microbiological outcomes over a 12 months period in CP patients and to assess whether *L. reuteri* can colonize in the periodontal pockets.

3. MATERIALS AND METHODS

3.1. Patient Selection and Inclusion Criteria

Forty systemically healthy patients, diagnosed as CP according to their clinical and radiographic examination that were seeking for periodontal care or referred for periodontal treatment to Yeditepe University, Faculty of Dentistry, Department of Periodontology were screened for this study.

After this examination only patients who met the following inclusion criteria were included.

Inclusion criteria were:

1. CP patients with horizontal bone loss
2. Presence of at least 2 teeth having at least one approximal site with PD of 5-7 mm and gingival index (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. After a detailed explanation of the study purpose, the nature of probiotics, periodontal treatment and microbial sampling, and the implications and possible benefits of participation in the study, a written informed consent was obtained from all participants (Appendix 1).

The present randomized, parallel, controlled and double blind clinical trial was conducted according to the guidelines of Helsinki Declaration of Human Rights. The protocol of the study and consent form were approved by the Yeditepe University, School of Medicine Ethical Committee (Decision number: 164), (Appendix 2).

3.2. Probiotic and Placebo Lozenges

The probiotic lozenges[1] consisted of two strains of *L. reuteri (*DSM17938 and ATCC PTA5289) at a dose of $2x10^8$ CFU and xylitol. The placebo lozenges[2] consisted of xylitol with no active probiotic strains. Both of the probiotic and placebo lozenges were identical in shape, texture and taste and therefore could not be discriminated from each other. Patients were asked to led dissolve the lozenge after tooth brushing two times a day, one in the morning and one at night. Patients were also instructed not do eat or drink for one our after the use of the lozenge and not to use any probiotic containing products during the course of the study.

3.3. Sample Size Calculation

Sample size was calculated for the primary outcome variable, PD reduction, based on Vivekananda et al. (132). According to the results of power analysis, the sample size of 7 subjects for each group were defined for 80% statistical power $\beta=0.20$ and $\alpha=0.82$, standard deviation (SD); 0.5.

[1] BioGaia ProDentis ®, Stockholm, Sweden
[2] BioGaia xylitol lozenge, Stockholm, Sweden

3.4. Treatment Groups

I. Group (SRP+ Probiotic group n= 20):

For the SRP + Probiotic group lozenges containing probiotic and xylitol were used. 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 group n= 20):

For the SRP + Placebo group only xylitol containing lozenges were used. 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.

3.5. Randomization and Treatment Protocol

This study was designed as a randomized, double-blinded, placebo-controlled clinical trial. Before the start of the study patients personal information such as, patients name, surname, address, sex, age, current systemic status, and if use and dosage of any medications and history of any type of operation were all recorded

The randomization of the 40 patients eligible for the study and willing to participate in the study were randomly assigned into two treatment groups according to a computer-assisted randomization table[3] (Table 6).

[3] www.randomizer.org/Copyright© 1997-2011 by Geoffrey C. Urbaniak and Scott Plous

Table 6. Randomization Table.

Research Randomizer Results:
2 sets of 20 Unique Numbers Per Set, Range: 1 to 40 -- Unsorted

Group 1 (SRP + Probiotic)

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

P: Patient number

Every patient was given OHI (Oral Hygiene Instruction) one week prior to the experimental period. Patients were instructed to brush with Bass method and the use of interdental devices was thoroughly explained. The patients were then randomly divided into SRP+ Probiotic® and SRP+ Placebo treatment groups. Lozenges containing probiotic and xylitol were used for the test group and only xylitol lozenges were used for the placebo group.

At day 0, intraoral photographs were taken, after microbial sampling clinical measurements were conducted including plaque index (PI), gingival index (GI), probing depth (PD), bleeding on probing (BOP) and relative attachment level (RAL). SRP was performed under local anesthesia; probiotic and placebo lozenges were administered. At day 7, SRP was repeated. At day 21 intraoral photographs, clinical and microbiological examinations were repeated. Subjects were controlled for oral hygiene instructions at day 35 and day 60. At day 90, 180 and 360 microbiological and clinical examinations were repeated including.

After clinical and microbiological examination full-mouth SRP was performed in two sessions one starting at day 0 and one at day 7 together with oral hygiene reinforcement. SRP was achieved under local anesthesia by treating every quadrant using ultrasonic [4] and hand instruments[5]. After SRP, patients were instructed to perform regular oral hygiene habits, such as twice daily brushing by 'Bass technique' for a minimum of 2 minutes, using a manual toothbrush, toothpaste and interdental devices.

3.6. Adverse Events and Patient Compliance

At baseline (day 0) patients were given the lozenges (probiotic or placebo). At their visit at day 7 and day 21, the clinical examiner interrogated each patient to check for compliance or any adverse events that the patient might have noticed. Additionally, changes in general health, use of any anti-inflammatory drugs or mouth rinses were questioned. After cessation of the drug at day 21, all these questions were repeated at their day 35, day 60, day 90, day 180 and 360 day visits.

[4] Piezon® OEM Built- in Kit, EMS, Switzerland
[5] Gracey, SG 3/4, 5/6, 7/8, 11/12, 13 / 14 mini-five, SAS 3/4 ,Hu–Friedy, USA

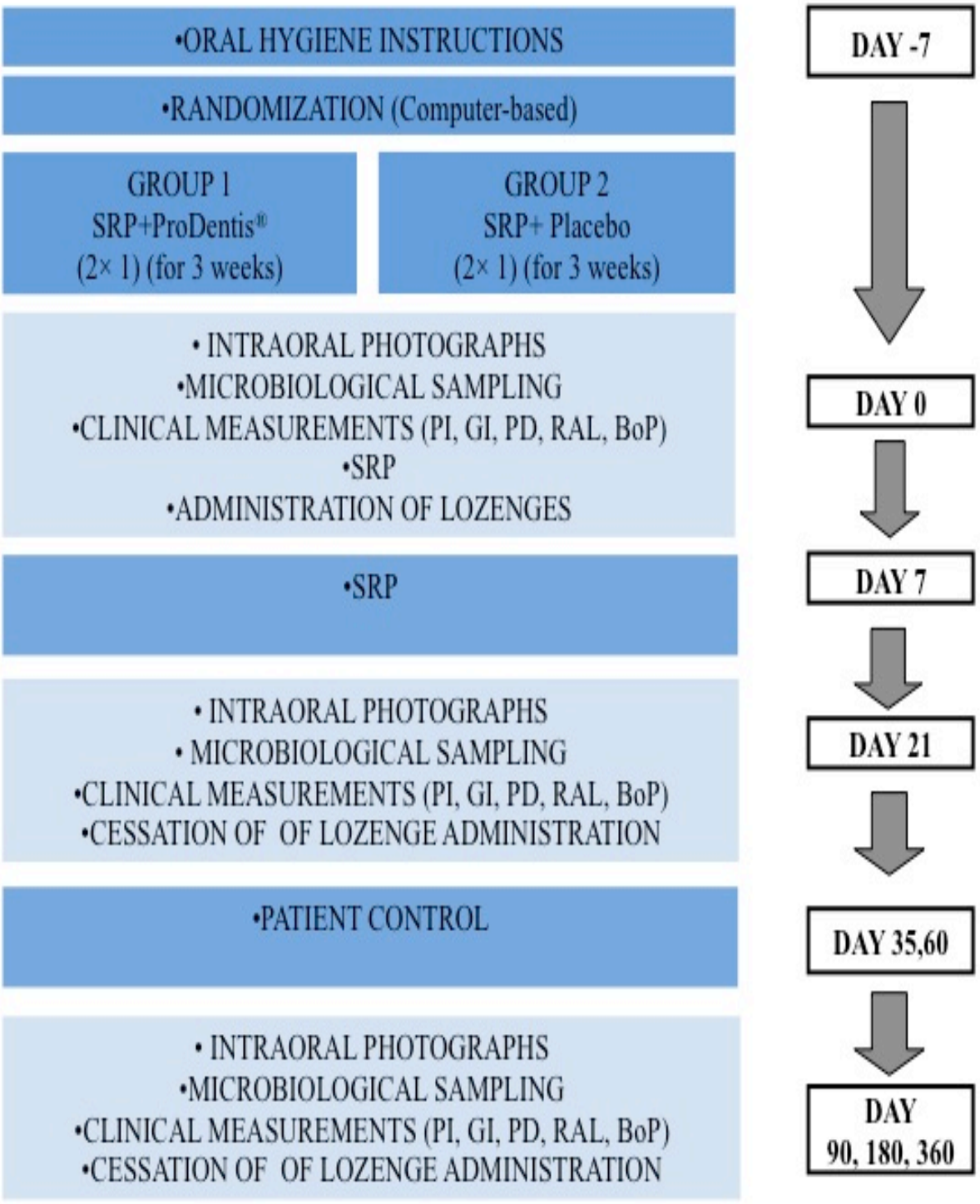

Figure 4. Flowchart of the study.

3.7. Clinical Indices and Measurements

All measurements were performed at baseline and at day 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[6]. 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.

3.8. Plaque Index

Teeth were isolated with cotton rolls and after gently drying of air syringe; microbial dental plaque biofilm was evaluated with the probe from 4 tooth surfaces. The plaque index is fundamentally based on the same principle as the gingival index, namely the desirability of distinguishing clearly between the severity and the location of soft debris aggregates. Each of the four gingival areas (buccal, mesial, distal, lingual/palatal) of the tooth is given a score from 0-3 (147).

Criteria for the plaque score index system

0 = No plaque in the gingival area.

1 = A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may only be recognized by running a probe across the tooth surface.

2 = Moderate accumulation of soft deposits within the gingival pocket, on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye.

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

[6] University of North Carolina PCPUNC 15, Hu-Friedy Ins Co, Chicago, IL, USA

3.9. Gingival Index

Each of the four areas of the tooth (buccal, mesial, distal, lingual/palatal), which make up the total circumference of the marginal gingiva, is given a score from 0-3, for the assessment of the gingival condition which clearly distinguishes between the quality of the gingiva (the severity of the lesion) and the location (quantity) (148).

Criteria for the gingival index system

0 = Normal gingiva.

1 = Mild inflammation - slight change in color, slight oedema. No bleeding on probing.

2 = Moderate inflammation – redness, edema and glazing, bleeding on probing.

3 = Severe inflammation – marked redness and edema, ulceration with tendency to spontaneous bleeding.

3.10. Probing Depth

Full mouth PD was measured at 6 sites per tooth (mesio- buccal, mid- buccal, disto- buccal, mesio- lingual/palatal, mid-lingual/palatal, disto-lingual/palatal). The probe was inserted parallel to the axis of the tooth into the periodontal pocket, using an individual occlusal stent as a reference point for probe placement. The distance between the gingival margin and the bottom of the periodontal pocket was measured and recorded.

3.11. 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 periodontal pocket.

3.12. Bleeding on Probing

BoP was assessed simultaneously to the pocket measurements from six aspects (mesio- buccal, mid- buccal, disto- buccal, mesio- lingual/palatal, mid-lingual/palatal, disto-lingual/palatal) and the presence or absence of bleeding up to 30 sec. after probing was recorded (149).

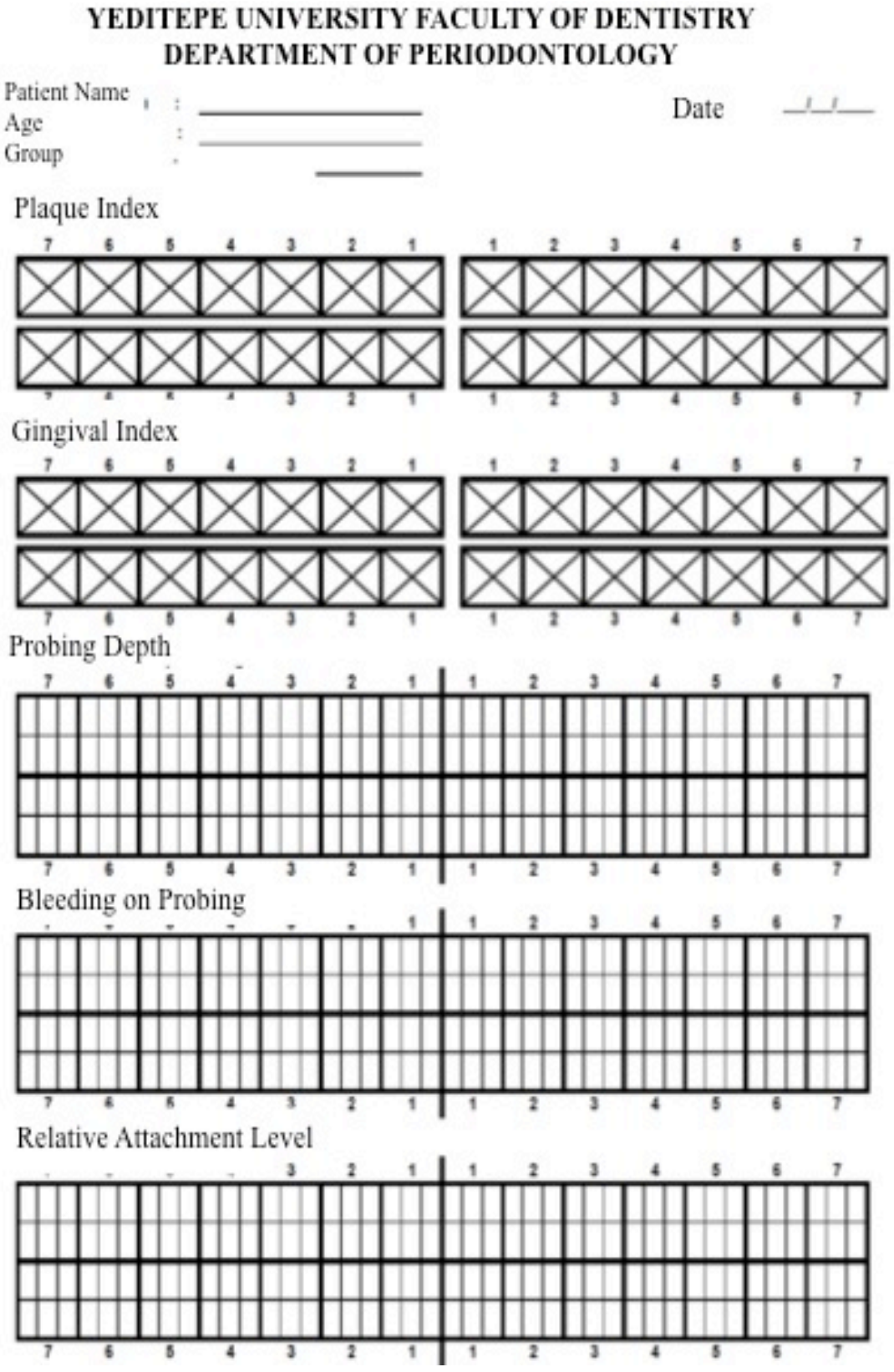

Figure 5. Data sheet.

3.13. Microbiological Procedures

3.13.1. Sample Collection and Microbiologic Culturing

For microbial sampling, 2 single rooted- teeth with approximal PD 5 - 7 mm and GI ≥2 in each quadrant were selected. Samples were taken from the same teeth at baseline (day 0) at day 21, day 90 and day 180 and day 360.

After superficial cleaning of the sites with cotton rolls and gently air-drying of the supragingival area with compressed air in order to avoid contamination. All the samples from each individual patient at each sampling time period were pooled before the microbial analysis. For this paper-points [7] were inserted until resistance was felt in each pocket of each tooth (Figure 6). After 30 seconds, the paper points (Figure 7) were transferred immediately to 4,5 ml Phosphate Buffered Saline[8] immediately dispersed using a vortex mixer at maximal setting for 30 seconds, and then serially tenfold diluted. From each dilutions (10^{-1},10^{-2},…. 10^{-5}) two portions of 0,1 ml was taken and plated separately onto tryptic soy agar[9] medium supplemented with %5 defibrinated sheep blood, 0.0005% hemin[10] and 0.00005% menadione[11] .
The first tripc soy agar plate was incubated at 37°C for 7 to 10 days in Gas Jars[12] , while the other plate was incubated at 37°C in %10 CO_2 for 4 days.

The total viable count (TVC) was determined as the total number of bacterial colonies on plates anaerobically incubated (Figure 8, 9). All the microbiologic data was transformed into colony forming units/milliliter (CFU/ml) of transport medium. In addition, obligate anaerobic bacteria was calculated as the TVC minus the total counts of colonies on plates incubated in 10% CO_2 condition and was expressed as a

[7] #30, DiaDent, Almere, The Netherlands
[8] Phosphate buffered saline tablets, Chalbiochem®, Merck KGaA, Darmstadt, Germany
[9] Oxoid Ltd, Basingstoke, Hampshire, England
[10] Sigma-Aldrich Chemie GmbH, Steinheim, Germany
[11] Sigma-Aldrich Chemie GmbH, Steinheim, Germany
[12] AnaeroGen kit, Oxoid Ltd, Basingstoke, Hampshire, England

percentage of TVC. Microbial samples were analyzed by culturing and TVC and proportions of obligate anaerobic bacteria were determined.

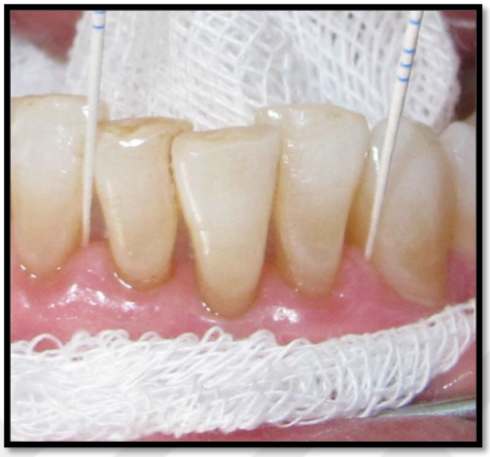

Figure 6. Subgingival plaque sampling.

Figure 7. Paper-points used for sampling.

Figure 8. Total Viable Cell Count (TVC) (x10^5 CFU/ ml).

Figure 9. Proportions of Obligate Anaerobic Bacteria in TVC.

3.13.2 Cultivation and Detection of *L.reuteri*

Another sample was obtained from the previously diluted solutions, that were prepared for total viable cell count and obligate anaerobes, in order to cultivate *L. reuteri* and plated on De Man, Rogosa, Sharpe agar (MRS, Acumedia, Ljusne, Sweden) modified by addition of 2% sodium acetate and 50 mg/L vancomycin. Plates were incubated anaerobically (AnaeroGen, Oxoid, Sollentuna, Sweden) at 37 °C for 48 h, after which colonies were confirmed as *L. reuteri* using a BioGaia AB proprietary method based on reuterin production in the presence of glycerol (150). In this test, the plates were overlaid with 5 ml soft agar (1% agar and 2% glycerol) and incubated at 37 °C for 30-45 min (Figure 10). Reuterin was detected by the addition of 5 mL 2,4-dinitrophenylhydrazine (DNPH) solution (0.1% DNPH, 1.7% HCl). After 5 min incubation, the solution was discarded and 5 ml 5 M potassium hydroxide was added for 30 s. A positive read-out was accepted as a reddish brown color around the colonies demonstrates the presence of reuterin (151) (Figure 11).

The active study product of Prodentis® lozenge was re-analyzed at the microbiology laboratory to confirm that the CFU count of both strains was above the stipulated shelf life limit of $1x10^8$ CFU.

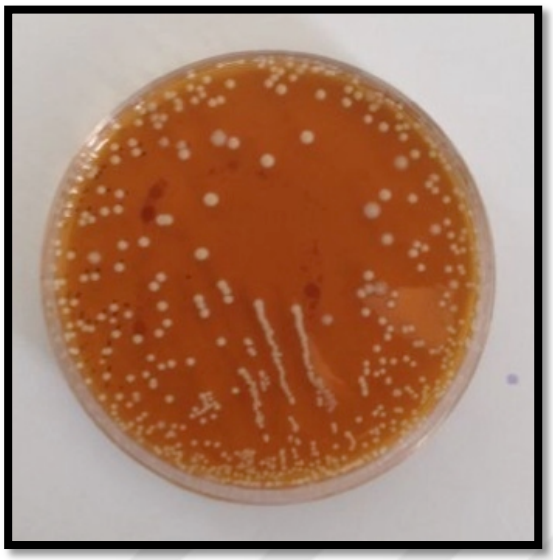

Figure 10. Growth of total *Lactobacilli* on specific media.

Figure 11. Detection of reuterin production of *L.reuteri.*

3.14. Statistical Analysis

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 are required per group (132).

The α error was set at 0.05. For all statistical evaluations, the patient was maintained as the unit of measurement. Data analysis was done for full mouth for PI, GI, BoP, RAL, PD, TVC, and proportions of obligate anaerobic bacteria 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's t-test and Chi-square test, respectively. Quantitative data was recorded as the mean value ± standard deviation for PI, GI, BoP, PD, attachment gain and proportions of obligate anaerobic bacteria, and median (min-max) for TVC.

Repeated measure analysis of variance was used for intra-group comparison of the clinical parameters and proportions of obligate anaerobes whereas the Friedman test was used for the TVC values at different time point measurements. The Bonferroni corrected Paired sample t was used to evaluate intra-group comparisons of the clinical parameters and proportions of obligate anaerobes in pairs. The Bonferroni corrected Wilcoxon signed ranks test was used to evaluate the intra-group comparisons of the TVC values in pairs. The Student t or Mann-Whitney U tests were used to evaluate the inter-group comparisons of the mean differences according to their distribution. For the Bonferroni corrected Paired sample t and Bonferroni corrected Wilcoxon signed ranks tests statistical significance was set at $p<0.005$. For the Student t and Mann-Whitney U tests statistical significance was set at $p<0.05$.

4. RESULTS

4.1. Demographic and Baseline Data

A total of 40 systemically healthy, chronic periodontitis patients, 18 males, 22 females, aged between 35 and 50 years were included in this study. Baseline clinical and microbiological parameters were similar in both groups ($p>0.05$) (Table 7). All subjects completed the 360 days study period and no adverse effects were observed. All the patients were compliant for the study requirements. Intraoral photographs (at day 0, day 21, day 90, day 180 and day 360) and periapical radiographs of one representative case from each group are shown in Figure 12.a. -f. and 13.a. -f.

Table 7. Baseline data of the patients in the treatment groups.

	Group 1 SRP + Probiotic (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	2.11±0.21	0.566
BoP (%)$^{++}$	0.88±0.07	0.87±0.04	0.634
PD (mm)$^{++}$	5.23±0.68	5.36±0.72	0.082
TVC (x10^5 CFU/ml) (median-range)$^{+++}$	35.50 (26-43)	41.50 (14-81)	0.493
Obligate anaerobes (%)$^{++}$	48.43±3.64	49.44±5.01	0.428

$^{+}$ Chi-square test, $^{++}$Student t-test, $^{+++}$Mann Whitney U test, $p<0.05$, PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing Depth, TVC: Total Viable Count

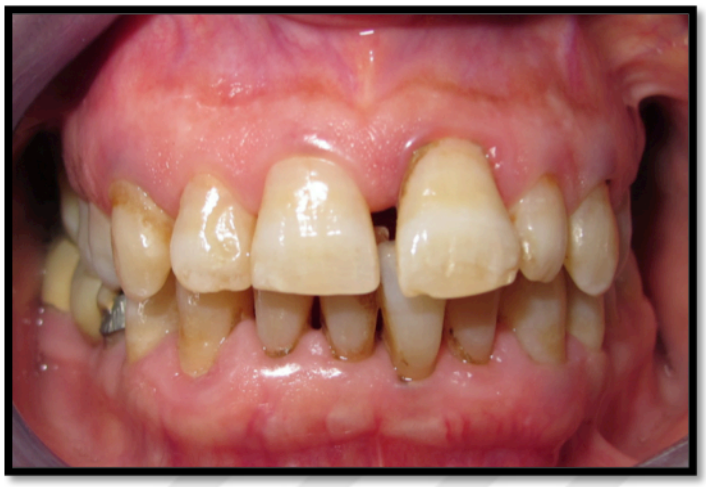

Figure 12.a. Intraoral photograph of a representative case from the SRP + Probiotic group at day 0.

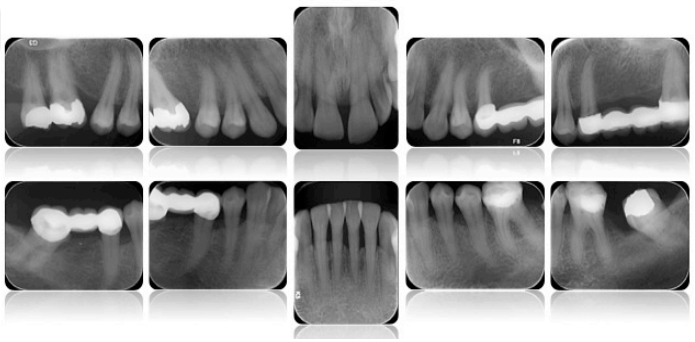

Figure 12.b. Intraoral periapical radiograph of a representative case from the SRP + Probiotic group.

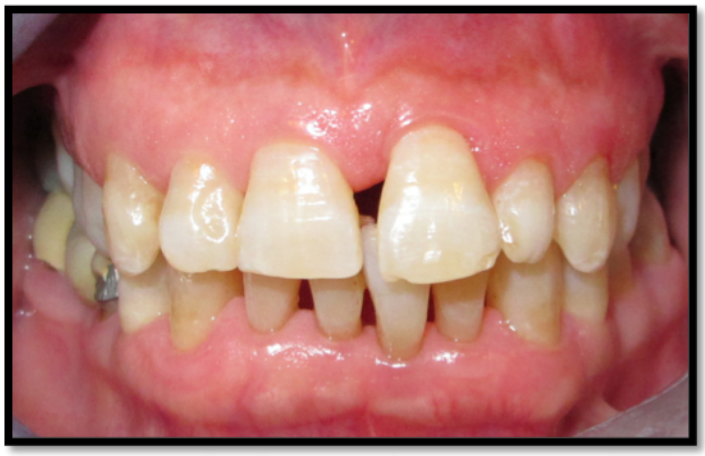

Figure 12.c. Intraoral photograph of a representative case from the SRP + Probiotic group at day 21.

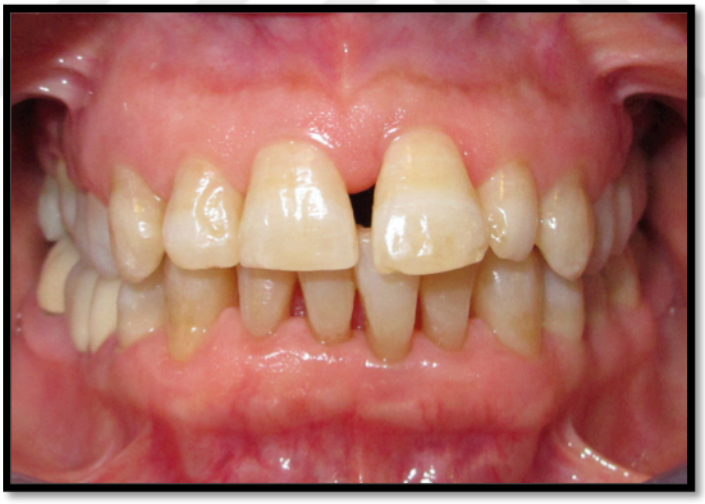

Figure 12.d. Intraoral photograph of a representative case from the SRP + Probiotic group at day 90.

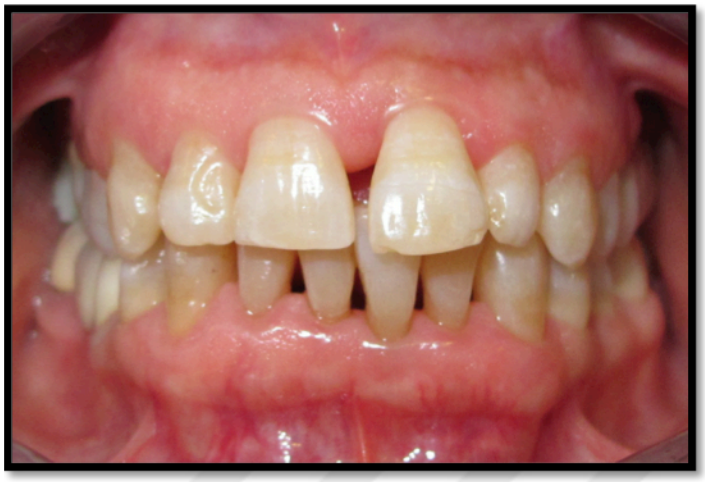

Figure 12.e. Intraoral photograph of a representative case from SRP + Probiotic group at day 180.

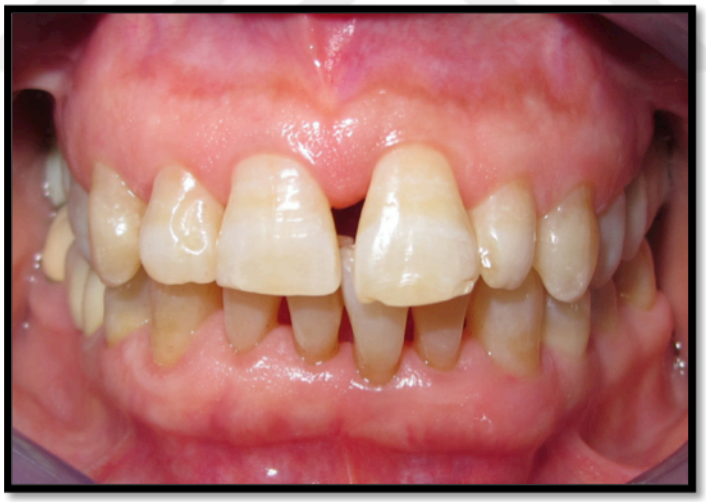

Figure 12.f. Intraoral photograph of a representative case from the SRP + Probiotic group at day 360.

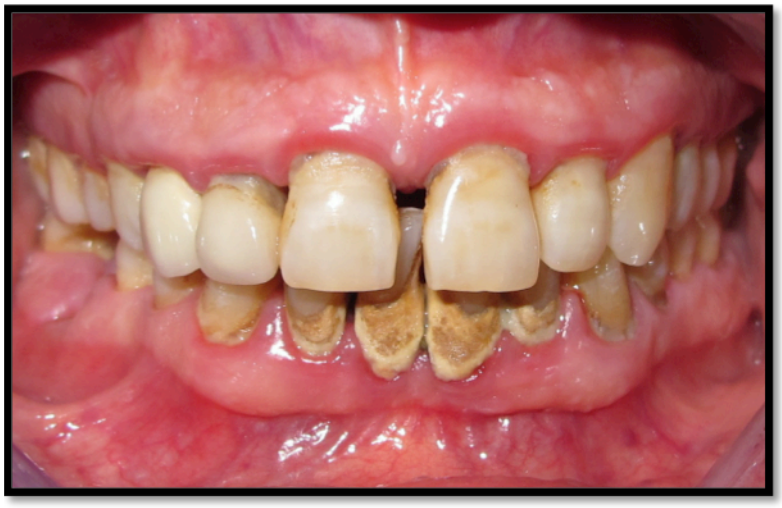

Figure 13.a. Intraoral photograph of a representative case from the SRP + Placebo group at day 0.

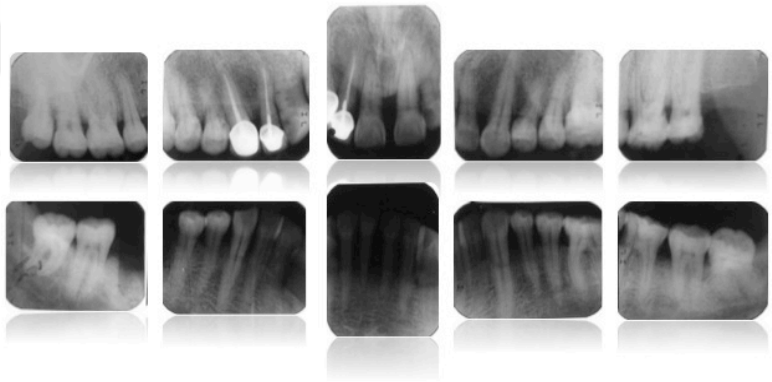

Figure 13.b. Intraoral periapical radiographs of a representative case from the SRP + Placebo group.

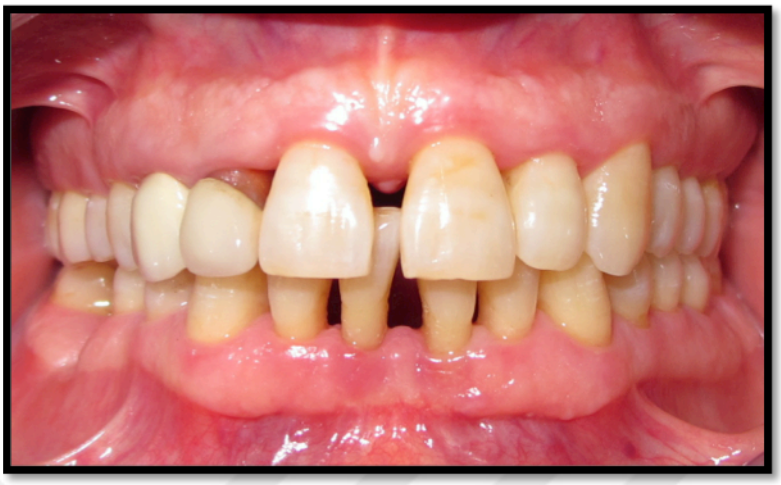

Figure 13.c. Intraoral photograph of a representative case from the SRP + Placebo group at day 21.

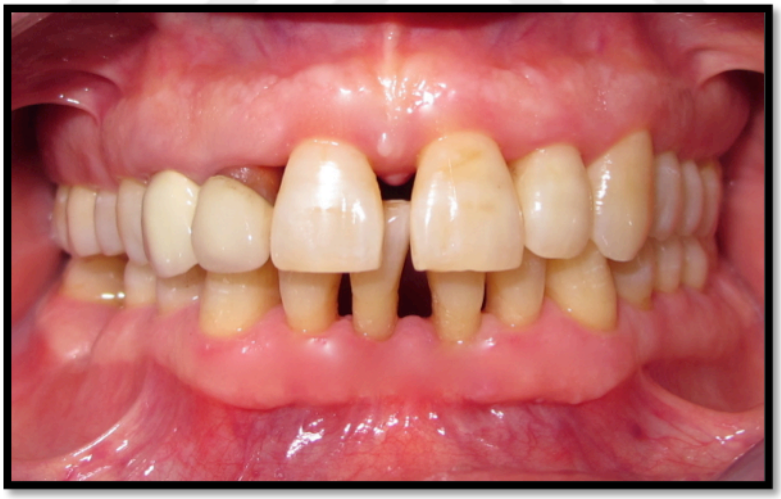

Figure 13.d. Intraoral photograph of a representative case from the SRP + Placebo group at day 90.

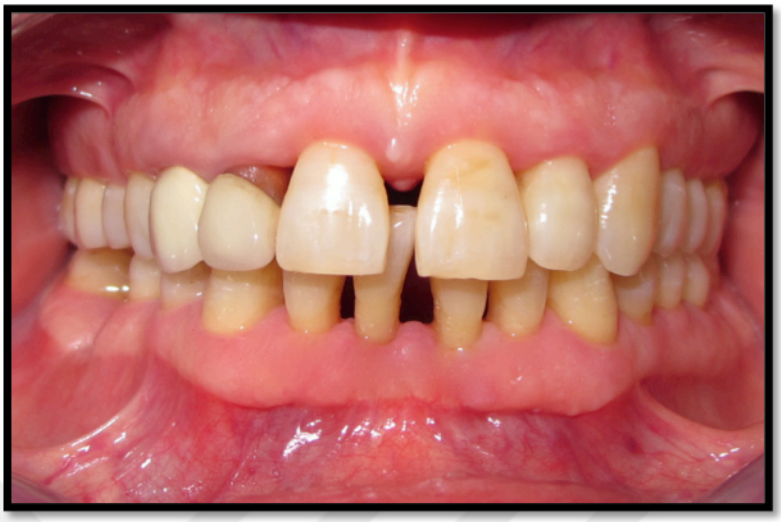

Figure 13.e. Intraoral photograph of a representative case from the SRP + Placebo group at day 180.

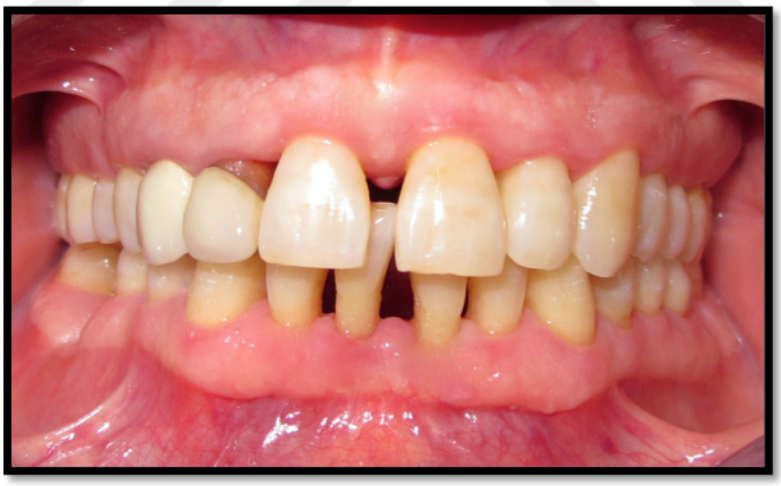

Figure 13.f. Intraoral photograph of a representative case from the SRP + Placebo group at day 360.

4.2. Clinical measurements

The mean PI, GI, BoP, and PD and RAL values for different time points for both groups are presented in Table 8.

4.2.1. Plaque index

In the SRP + Probiotic group, PI values were detected 2.29±0.28, 0.47±0.17, 0.60±0.21, 0.63±0.24 and 0.73±0.24 at days 0, 21, 90, 180 and 360 respectively. In the SRP + Placebo group PI values were 2.30±0.41, 0.93±0.41, 1.14±0.29, 1.23±0.35 and 1.39±0.28 at days 0, 21, 90, 180 and 360 respectively. Intra-group comparisons of PI values showed statistical significance at different time point measurements in both groups (0.001; 0.001, respectively) (Table 8). Further comparisons in pairs of PI values at days 21, 90, 180 and 360 compared to baseline values revealed statistical significant results in both groups (Table 9, 10). Mean differences of PI values in SRP + Probiotic group were detected 1.82±0.35, 1.70±0.33, 1.66±0.37 and 1.56±0.37, between days 0-21, 0-90, 0-180 and 0-360. Mean differences of PI values in SRP + Placebo group were detected 1.37±0.61, 1.16±0.54, 1.07±0.60 and 0.91±0.53 between days 0-21, 0-90, 0-180 and 0-360 (Table 11, 12, 13, 14). Inter-group comparisons of mean differences of PI values revealed statistical significant results in favor of the SRP + Probiotic group between days 0-21, 0-90, 0-180 and 0-360 ($p=0.008$; $p=0.001$; $p= 0.001$; $p=0.001$, respectively) (Table 11, 12, 13, 14).

4.2.2. Gingival index

In the SRP + Probiotic group, GI values were detected 2.12±0.15, 0.61±0.28, 0.76±0.35, 0.69±0.37 and 0.80±0.38 at days 0, 21, 90, 180 and 360 respectively. In the SRP + Placebo group GI values were 2.12±0.21, 1.34±0.48, 1.53±0.48, 1.54±0.35 and 1.66±0.36 at days 0, 21, 90, 180 and 360 respectively. Intra-group comparisons of GI values showed statistical significance at different time point measurements in both

groups (0.001; 0.001, respectively) (Table 8). Further comparisons in pairs of GI values at day 21, 90, 180 and 360 compared to baseline values revealed statistical significant results in both groups (Table 9, 10). Mean differences of GI values in SRP + Probiotic group were detected 1.51±0.31, 1.37±0.39, 1.43±0.43 and 1.33±0.43, between days 0-21, 0-90, 0-180 and 0-360. Mean differences of GI values in SRP + Placebo group were detected 0.78±0.41, 0.59±0.39, 0.58±0.38 and 0.46±0.4 between days 0-21, 0-90, 0-180 and 0-360 (Table 11, 12, 13, 14). Inter-group comparisons of mean differences of GI values revealed statistical significant results in favor of the SRP + Probiotic group between days 0-21, 0-90, 0-180 and 0-360 (p=0.001; p=0.001; p= 0.001; p=0.001, respectively) (Table 11, 12, 13, 14).

4.2.3. Bleeding on Probing

In the SRP + Probiotic group, BoP values were detected 88,90±7.66, 21.50±5.88, 16.65±4.21, 12.30±4.82 and 11.05±3.99 at days 0, 21, 90, 180 and 360 respectively. In the SRP + Placebo group BoP values were 88.65±4.11, 25.65±4.75, 21.85±3.98, 19.95±04.88 and 19.05±4.84 at days 0, 21, 90, 180 and 360 respectively. Intra-group comparisons of BoP values showed statistical significance at different time point measurements in both groups (0.001; 0.001, respectively) (Table 8). Further comparisons in pairs of BoP values at days 21, 90, 180 and 360 compared to baseline values revealed statistical significant results in both groups (Table 9, 10). Mean differences of BoP values in the SRP + Probiotic group were 67.40±6.92, 72.25±6.50, 76.60±7.98 and 77.85±7.59 between days 0-21, 0-90, 0-180 and 0-360 (Table 11, 12, 13, 14). Mean differences of BoP values in SRP + Placebo group were detected 63.00±5.10, 66.80±4.92, 68.70±5.74 and 69.60±5.77 between days 0-21, 0-90, 0-180 and 0-360 (Table 11, 12, 13, 14). Inter-group comparisons of mean differences of BoP values revealed statistical significant results in favor of the SRP + Probiotic group between days 0-21, 0-90, 0-180 and 0-360 (p=0.023; p=0.005; p= 0.001; p=0.001, respectively) (Table 11, 12, 13, 14).

4.2.4. Probing Depth

In the SRP + Probiotic group, PD values were detected 5.23±068 mm, 4.03±0.74 mm, 3.80±0.75 mm, 3.38±0.75 mm and 3.49±0.87 mm at days 0, 21, 90, 180 and 360 respectively. In the SRP + Placebo group PD values were 5.36±0.72 mm, 4.60±0.71 mm, 4.51±0.71 mm, 4.66±0.69 mm and 4.80±0.70 mm at days 0, 21, 90, 180 and 360 respectively. Intra-group comparisons of PD values showed statistical significance at different time point measurements in both groups (0.001; 0.001, respectively) (Table 8). Further comparisons in pairs of PD values at days 21, 90, 180 and 360 compared to baseline values revealed statistical significant results in both groups (Table 9, 10). Mean differences of PD values in SRP + Probiotic group were detected 1.20±0.37 mm, 1.44±0.33 mm, 1.77±0.69 mm and 1.74±0.67 mm, between days 0-21, 0-90, 0-180 and 0-360. Mean differences of PD values in SRP + Placebo group were detected 0.76±0.36 mm, 0.85±0.32 mm, 0.70±0.24 mm and 0.57±0.24 mm between days 0-21, 0-90, 0-180 and 0-360 (Table 11, 12, 13, 14). Inter-group comparisons of mean differences of PD values revealed statistical significant results in favor of the SRP + Probiotic group between days 0-21, 0-90, 0-180 and 0-360 (p=0.001; p=0.001; p= 0.001; p=0.001, respectively) (Table 11, 12, 13, 14).

4.2.5. Attachment Gain

Negative changes in RAL values were determined as attachment gain. In the SRP + Probiotic group, mean attachment gain values were detected 1.18±0.36 mm, 1.67±0.24 mm and1.39±0.26mm at days 90, 180 and 360 respectively. In the SRP + Placebo group mean attachment gain values were detected, 0.79±0.32 mm, 0.66±0.22 mm and 0.53±0.24 mm at days 90, 180 and 360 respectively (Table 8). Statistically significant differences were observed in both groups between days 90, 180 and 360 (p =0.001; p=0.001) (Table 8). Further comparisons in pairs of mean attachment gain values at days 90, 180 and 360 compared to baseline values revealed statistical significant results for both groups (Table 9, 10). Inter-group comparisons of mean attachment gain values revealed statistical significant results in favor of the SRP + Probiotic group between days 0-90, 0-180, 0-360 (p =0.001; p=0.001) (Table 12, 13, 14).

Table 8. Intra-group comparisons of clinical parameters.

	SRP+ Probiotic group (n=20) (Mean ± SD)						SRP+ Placebo group (n=20) (Mean ± SD)					
Clinical Parameters	**Day 0**	**Day 21**	**Day 90**	**Day 180**	**Day 360**	**p***	**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**	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±0.15	0.61±0.28	0.76±0.35	0.69±0.37	0.80±0.38	**0.001**	2.12±0.21	1.34±0.48	1.53±0.48	1.54±0.35	1.66±0.36	**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**	88.65±4.11	25.65±4.75	21.85±3.98	19.95±4.88	19.05±4.84	**0.001**
PD (mm)	5.23±0.68	4.03±0.74	3.80±0.75	3.38±0.86	3.49±0.87	**0.001**	5.36±0.72	4.60±0.71	4.51±0.71	4.66±0.69	4.80±0.70	**0.001**
Attachment gain (mm)	-	-	1.18±0.36	1.67±0.24	1.39±0.26	**0.001**	-	-	0.79±0.32	0.66±0.22	0.53±0.24	**0.001**

* Repeated measure analysis of variance, $p < 0.05$.

PI: Plaque index, GI: Gingival index, BoP: Bleeding on Probing, PD: Probing depth.

Table 9. Intra-group comparisons of the clinical parameters in pairs for the SRP + Probiotic group.

SRP + Probiotic Group (n=20)				
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 (mm)	**0.001**	**0.001**	**0.001**	**0.001**
Attachment gain (mm)	**-**	**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 10. Intra-group comparisons of the clinical parameters in pairs for the SRP + Placebo group**.**

SRP + Placebo Group (n=20)				
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 (mm)	**0.001**	**0.001**	**0.001**	**0.001**
Attachment gain (mm)	**-**	**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 11. Inter-group comparisons of the differences of the clinical parameters between days 0-21.

Day 0-21	SRP+ Probiotic group (n=20) (Mean±SD)	SRP+ Placebo group (n=20) (Mean±SD)	p*
PI	1.82±0.35	1.37±0.61	**0.008**
GI	1.51±0.31	0.78±0.41	**0.001**
BoP (%)	67.40±6.92	63.00±5.10	**0.028**
PD (mm)	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 12. Inter-group comparisons of the differences of the clinical parameters between days 0-90.

Day 0-90	SRP+ Probiotic group (n=20) (Mean±SD	SRP+ Placebo group (n=20) (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	**0.005**
PD (mm)	1.44±0.33	0.85±0.32	**0.001**
Attachment gain (mm)	1.18±0.36	0.79±0.32	**0.001**

*Student t-test, p<0,05

Table 13. Inter-group comparisons of the differences of the clinical parameters between days 0-180**.**

Day 0-180	SRP+ Probiotic group (n=20) (Mean±SD	SRP+ Placebo group (n=20) (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 (mm)	1.77±0.69	0.70±0.24	**0.001**
Attachment gain (mm)	1.67±0.24	0.66±0.22	**0.001**

*Student t-test, p<0,05

Table 14. Inter-group comparisons of the differences of the clinical parameters between days 0-360**.**

Day 0-360	SRP+ Probiotic group (n=20) (Mean±SD	SRP+ Placebo group (n=20) (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 (mm)	1.74±0.62	0.57±0.24	**0.001**
Attachment gain (mm)	1.39±0.26	0.53±0.24	**0.001**

*Student t-test, p<0,05

4.3. Microbiological data

The mean values for obligate anaerobes (%) and the values for TVC ($x10^5$ CFU/ml) (median-range) at baseline and days 21, 90, 180, 360 for both groups are presented in Table 15. Both treatments led to a significant decrease of TVC ($x10^5$ CFU/ml) and proportions of obligate anaerobes at days 21, 90, and 180 for both groups ($p<0.005$) (Table 15).

4.3.1. Total Viable Count and Proportions of Obligate Anaerobes

In the SRP + Probiotic group, values ($x10^5$ CFU/ml) (median-range) were detected 35.5 (26-43), 12.2 (1-16.7), 10 (0.5-14.2), 11.5 (1-15) and 35 (25-42) at day 0, 21, 90, 180 and 360 respectively. In the SRP + Placebo group TVC values were 41.5 (14-81), 23.2 (6.7-70), 16.8 (5.2-60), 17.5 (5-60) and 40.5 (12-78) at day 0, 21, 90, 180 and 360 respectively. Intra-group comparisons of TVC values showed statistical significance at different time point measurements in both groups (0.001; 0.001, respectively) (Table 15). Further comparisons in pairs of TVC values ($x10^5$ CFU/ml) (median-range) compared to baseline values revealed statistical significant results in both groups except day 360 (Table 16, 17). Mean differences of TVC values ($x10^5$ CFU/ml) (median-range) in SRP + Probiotic group were detected 27.4 (13-40), 29.8 (16-40.2), 27.5 (14-40) and 1 (0-5), between days 0-21, 0-90, 0-180 and 0-360. Mean differences of TVC values ($x10^5$ CFU/ml) (median-range) in SRP + Placebo group were detected 11.4 (2.3-39.3), 15.2 (3.2-40.8), 15.5 (3-41) and 1 (0-5) between days 0-21, 0-90, 0-180 and 0-360 (Table 18, 19, 20, 21). Inter-group comparisons of mean differences of TVC ($x10^5$ CFU/ml) (median-range) values revealed statistical significant results in favor of the SRP + Probiotic group between days 0-21, 0-90 and 0-180 ($p=0.001$; $p=0.003$; $p= 0.012$, respectively). This statistically significant difference could not be observed between days 0-360 ($p=0.976$) (Table 17, 18, 19, 20).

In the SRP + Probiotic group obligate anaerobes (%) values, were detected 48.43±3.64, 24.70±7.93, 21.51±8.72, 25.75±8.10 and 47.16±3.97 at day 0, 21, 90, 180 and 360 respectively. In the SRP + Placebo group obligate anaerobes (%) values were 49.45±05.01, 39.40±5.39, 35.84±6.01, 40.72±4.78 and 48.04±4.82 mm at day 0, 21, 90, 180 and 360 respectively. Intra-group comparisons of obligate anaerobes (%) values showed statistical significance at different time point measurements in both groups (0.001; 0.001, respectively) (Table 15). Further comparisons in pairs of obligate anaerobes (%) values ($x10^5$ CFU/ml) (median-range) compared to baseline values revealed statistical significant results in both groups except day 360 (Table 16, 17). Mean differences of obligate anaerobes (%) values ($x10^5$ CFU/ml) (median-range) in SRP + Probiotic group were detected 23.73±7.99, 26.92±8.44, 22.68±7.87 and 1.27±1.06, between days 0-21, 0-90, 0-180 and 0-360. Mean differences of obligate anaerobes (%) values ($x10^5$ CFU/ml) (median-range) in SRP + Placebo group were detected 10.05±4.27, 13.60±4.86, 8.72±4.46 and 1.40±0.87 between days 0-21, 0-90, 0-180 and 0-360 (Table 18, 19, 20, 21). Inter-group comparisons of mean differences of obligate anaerobes (%) values revealed statistical significant results in favor of the SRP + Probiotic group between days 0-21, 0-90 and 0-180 (p=0.001; p=0.001; p= 0.001, respectively). This statistically significant difference could not be observed between days 0-360 (p=0.688)(Table 17, 18, 19, 20).

Table 15. Intra-group comparisons of microbiological parameters**.**

	SRP+ Probiotic group (n=20)						SRP+ Placebo group (n=20)					
Microbiological Parameters	**Day 0**	**Day 21**	**Day 90**	**Day 180**	**Day 360**	**p**	**Day 0**	**Day 21**	**Day 90**	**Day 180**	**Day 360**	**p**
TVC ($x10^5$ CFU/ml) (median-range)[+]	35.5 (26-43)	12.2 (1-16.7)	10 (0.5-14.2)	11,5 (1-15)	35 (25-42)	**0.001**	41.5 (14-81)	23.2 (6.7-70)	16,8 (5.2-60)	17,5 (5-60)	40.5 (12-78)	**0.001**
Obligate anaerobes (%) [++]	48.43±3.64	24.70±7.93	21.51±8.72	25.75±8.10	47.16±3.97	**0.001**	49.45±5.01	39.40±5.39	35.84±6.01	40.72±4.78	48.04±4.82	**0.001**

[+]Friedman test, $p < 0.05$

[++] Repeated measure analysis of variance, $p < 0.05$.

TVC: Total Viable Count

Table 16. Intra-group comparisons of the microbiological parameters in pairs for the SRP + Probiotic group.

SRP+ Probiotic group (n=20)				
Microbiological Parameters	**Day 0-21 p**	**Day 0-90 p**	**Day 0-180 p**	**Day 0-360 p**
TVC (x10^5 CFU/ml) (median-range) [+]	**0.001**	**0.001**	**0.001**	0.873
Obligate anaerobes (%) [++]	**0.001**	**0.001**	**0.001**	0.814

[+] Bonferroni corrected Wilcoxon sign test, p<0.005.
[++] Bonferroni corrected paired sample t-test, p<0.005

Table 17. Intra-group comparisons of the microbiological parameters in pairs for the SRP + Placebo group.

SRP+ Placebo group (n=20)				
Microbiological Parameters	**Day 0-21 p**	**Day 0-90 p**	**Day 0-180 p**	**Day 0-360 p**
TVC (x10^5 CFU/ml) (median-range) [+]	**0.001**	**0.001**	**0.001**	0.912
Obligate anaerobes (%) [++]	**0.001**	**0.001**	**0.001**	0.701

[+] Bonferroni corrected Wilcoxon sign test, p<0.005.
[++] Bonferroni corrected paired sample t-test, p<0.005

Table 18. Inter-group comparisons of the differences of the microbiological parameters between days 0-21**.**

Day 0-21	SRP+ Probiotic group (n=20) (mean±SD	SRP+ Placebo group (n=20) (mean±SD)	p
TVC ($x10^5$ CFU/ml) (median-range) [+]	27.4 (13-40)	11.4 (2.3-39.3)	**0.001**
Obligate anaerobes (%) [++]	23.73±7.99	10.05±4.27	**0.001**

[+] Mann Whitney U
[++] Student t-test, p<0,05

Table 19. Inter-group comparisons of the differences of the microbiological parameters between days 0-90.

Day 0-90	SRP+ Probiotic group (n=20) (mean±SD	SRP+ Placebo group (n=20) (mean±SD)	p
TVC ($x10^5$ CFU/ml) (median-range) [+]	29.8 (16-40.2)	15.2 (3.2-40.8)	**0.003**
Obligate anaerobes (%) [++]	26.92±8.44	13.60±4.86	**0.001**

[+] Mann Whitney U
[++] Student t-test, p<0,05

Table 20. Inter-group comparisons of the differences of the microbiological parameters between days 0-180.

Day 0-180	SRP+ Probiotic group (n=20) (mean±SD	SRP+ Placebo group (n=20) (mean±SD)	p
TVC ($x10^5$ CFU/ml) (median-range) +	27.5 (14-40)	15.5 (3-41)	**0.012**
Obligate anaerobes (%) ++	22.68±7.87	8.72±4.46	**0.001**

+ Mann Whitney U
++ Student t-test, p<0,05

Table 21. Inter-group comparisons of the differences of the microbiological parameters between days 0-360**.**

Day 0-360	SRP+ Probiotic group (n=20) (mean±SD	SRP+ Placebo group (n=20) (mean±SD)	p
TVC ($x10^5$ CFU/ml) (median-range) +	1 (0-5)	1 (0-5)	0.976
Obligate anaerobes (%) ++	1.27±1.06	1.40±0.87	0.688

+ Mann Whitney U
++ Student t-test, p<0,05

4.3.2. *L. reuteri* in subgingival samples

The presence of *L. reuteri* was not detected at the baseline in any of the patients in both groups. After the commencing of the *L. reuteri* containing lozenges (Prodentis®) by patients, *L. reuteri* was detected in 6 and 11 patients in the SRP + Probiotic group on day 21 and 90, respectively. *L. reuteri* counts in the subgingival sample is presented in Table 22. The microorganism was not detected in any of the 20 patients at days 180 and 360.

Table 22. *L. reuteri* levels in subgingival samples.

	SRP + Probiotic (Group I) (n=20)		
Level of detection	**$<10^3$**	**10^3-10^5**	**$>10^5$**
Day 0	0	0	0
Day 21	4	2	0
Day 90	2	9	0
Day 180	0	0	0
Day 360	0	0	0

5. DISCUSSION

Periodontal diseases primarily occur when there is an imbalance between the pathogenic and beneficial species on one side and host factors and environmental factors on the other side (152). As a biofilm-mediated disease, periodontal disease is inherently difficult to treat. One of the greatest challenges in treatment arises from the fact that there is no way to eliminate bacteria from the oral cavity, so bacteria will always be present in the periodontal environment. In addition, the bacteria within the biofilm are more resistant to antimicrobial agents and various components of the host response (153).

The prevention and treatment of periodontal infections is primarily based on the reduction or the elimination of the number of pathogens in the oral biofilm (42), which can be achieved by increasing the oral hygiene of the patients. However, achieving optimal plaque control is difficult since it hast to be performed meticulously. SRP has become the ‘‘gold standard’’ of nonsurgical treatment of periodontitis, with multiple clinical studies demonstrating that it effectively reduces the microbial load (154). During SRP, the dentist or periodontist removes manually the pathogenic biofilm. Although a thousand fold reduction in bacteria can be achieved immediately after SRP, no less than one week later, the initial number of bacteria is reached again by recolonization of the periodontal pockets (6, 155). However, a shift occurs in the composition of the newly formed biofilm in which there should be less periodontal pathogens. In the last years, different therapeutic strategies have been proposed to improve the results of SRP and hence to avoid the need of periodontal surgical interventions in some patients with advanced periodontitis. Three treatment approaches including staged debridement with quadrant or sextant, full-mouth SRP and full mouth disinfection are proposed. However, two systematic reviews combining the results of a number of randomized controlled trials concluded that all three approaches are effective and may be recommended for nonsurgical periodontal therapy (156, 157). For successful periodontal therapy the thoroughness of root debridement and the patients’ standard of oral hygiene are critical factors rather than the treatment modality (158).

Therefore, patients included in this study were scheduled with a 1-week time interval between the two sessions of SRP. Although healing may continue for a period of 9-12 months following SRP, data show that most of the healing completes at 3 months (4, 159). Therefore examination visits were chosen at day 90, 180 and 360 in the present study.

Several factors have been described which increase the risk for periodontitis including smoking. There are numerous mechanisms by which smoking mat affect host-parasite interaction in the oral cavity. On one hand, smoking diminishes both cell-mediated and hummoral immune response and on the other hand it favors infection with microbial pathogens and impairs antimicrobial therapy. Smoking can increase bacterial adherence to epithelial cells. Once colonized, deep pockets may offer an especially favorable environment for the growth of anaerobic periodontal pathogens. In summary, cigarette smoking appear to trigger a cycle of impaired immune responses and subgingival infection with periodontal pathogens leading to greater severity of periodontal disease (160). Therefore smokers were excluded from this study.

Even though there is a shift in the composition of the newly formed biofilm in which there should be less periodontal pathogens, unfortunately complete elimination of periodontal pathogens is not possible (161). The use of antiseptics and antibiotics has been advocated for many years, however current knowledge reveals that these adjunctive therapies have only a temporary effect on the oral biofilm. Also in oral microbiology, a steep increase in the development of antibiotic resistance has been documented (152). The highly complex microbiota of the mouth contains a wide variety of bacterial species but not all of them are harmful. Some microorganisms of the oral ecosystem are harmless and ‘beneficial’ to the host. These beneficial microbes could represent the future of medicine. Antibiotic usage destroys harmful bacteria together with the good bacteria that protect and help to fight for infection. Probiotics, on the other hand, re-populate the beneficial bacteria, which can eliminate pathogenic bacteria and fight against infection. Oral administration of probiotics may also benefit oral health by preventing the growth of harmful microbiota or by modulating mucosal immunity in the oral cavity (162). Maintaining the natural flora or re-gaining healthy

microflora by eliminating pathogenic bacteria and/or by increasing the host-defense system has become popular in the 20th century. Therefore prominence has been given to probiotic therapy.

The effects of probiotic applications on systemic health have been described in numerous in vitro and in vivo studies (163), (164), (111). Commonly, for the concept of bacteriotherapy and use of health-beneficial microorganisms to heal diseases or support immune function, most of the species ascribed as having probiotic properties belong to the genera *Lactobacillus* and *Bifidobacterium* (165). Promising study results from different fields of healthcare resulted in the introduction of probiotics for oral application (61), (16), (166).

Oral application of probiotics have been evaluated primarily in the management of dental caries (167), (133), (66), (168). Other several studies on the effects of probiotics in different fields of oral healthcare include halitosis (169), (136), (137) and oral candidiasis (167), (126). Only few studies have evaluated probiotic application from the periodontal health perspective.

The commensal bacteria of the indigenous oral flora are important in regulating the host defense and protecting against exogenous pathogens. Within an established flora these exogenous pathogens have difficulty in surviving and competing in the indigenous ecosystem (170). Considering the application of probiotics for periodontal disease, disruption of the established flora by total removal of plaque seems to be crucial for re-establishing the equilibrium and enhancing the replacement of indigenous microbiota (16).

The subgingival microflora is affected by supragingival plaque quantity, composition and rate of accumulation (6, 171). Therefore meticulous plaque control and maintenance is crucial for successful periodontal treatment. Therefore every patient included in this study was given oral hygiene instructions one week prior to the study and maintained stable through the study period.

Oral hygiene levels and accumulation of plaque deposits were evaluated by PI (147). One week prior to the study period, patients included in the study were instructed to brush their teeth by the modified Bass method and to use interdental brushes. Every patient was regularly checked for oral health instruction reinforcement at day 35, 60, 90,180 and 360.

Studies that have recorded the changes in different clinical parameters have demonstrated that the major changes occur during the initial 1–3 months after completion of the nonsurgical periodontal treatment (159, 172). Subsequently, up to 12 months, some additional healing and maturation of the periodontal tissues may occur, as evidenced by some further minor improvements in the clinical parameters. These studies confirm the clinical parameters observed in this study.

In both groups PI, GI and BoP were significantly reduced within each treatment group over the 360 days.

Mean baseline PI scores in SRP + ProDentis® and SRP + placebo groups were detected 2.29±0.28 and 2.30±0.41, respectively. Intra- and inter-group comparisons showed statistically significant differences for PI reduction ($p< 0.05$) at all evaluation periods. Intra-group comparisons of PI showed that all patients in both groups provided optimal oral hygiene level. At the end of day 360, PI reductions were found 0.73 and 1.39, in favor of the probiotic lozenge administered group. These findings are in accordance with previous probiotic studies (17, 132, 146) which demonstrated anti-plaque effects of *L.reuteri* containing lozenges. Krasse et al. (17) conducted a study to assess if the probiotic *L.reuteri* could be effective in the treatment of gingivitis and further to evaluate the influence of the probiotic on plaque and the *lactobacilli* population in the saliva. In their study, *L.reuteri* was efficacious in reducing both gingivitis and plaque in patients with moderate to severe gingivitis. Vivekananda et al. (132) demonstrated the plaque inhibition, anti-inflammatory and anti-microbial effects of L.reuteri Prodentis during non-surgical therapy and the maintenance phase of periodontal treatment. Thirty non-smoking patients with CP were included in the study. The study period was 42 days and the participants took L.reuteri tablets or the

corresponding placebo tablets twice daily from day 21 to day 42 in a split-mouth design protocol. At day 42 PI was significantly reduced in the SRP + Prodentis group to 0.76. In another study Vicario et al. (146) assessed the clinical effect of *L.reuteri* Prodentis as a probiotic agent in the treatment of initial to moderate CP. Periodontal clinical parameters were improved in the test group after 30-day intervention. The mean PI (%) at baseline was 69.5 for the test group and 62.9 for the control group. At visit 2, after use of the probiotic agent, the mean PI was 52.5 for the test group and 67.4 for the control group. The change in PI between visit 1 and visit 2 for the test group was statistically significant ($p = 0.009$), demonstrating a reduction of the plaque index after the use of the probiotic agent; however, the control group demonstrated a non-statistically significant increase in PI after the use of the placebo tablets. On the other hand Hallström et al. (23) reported no significant effect of *L. reuteri* containing lozenge administration on plaque accumulation in an experimental gingivitis model. Teughels et al. (22) could not demonstrate any significant results in terms of PI reduction for the SRP + Probiotic group. Even though outcome measures were consistently lower in the SRP + Probiotic group, only on a few occasions, these differences were statistically significant between both treatment groups. Also Iniesta et al. (21) could not demonstrate any clinical impact of *L. reuteri* containing probiotic tablets.

The inflammatory status of the gingiva was evaluated with GI and BoP scores. Statistically significant reductions were observed in both groups at day 21, 90, 180 and 360. Meticulous plaque and oral hygiene together with SRP lead to a reduction in bleeding tendency and inflammation of the periodontium (153, 159, 173, 174). In the SRP + Prodentis group, both the GI and BoP score were statistically significant reduced. Inter-group comparison revealed statistically significant differences in favor of the active group, indicating a lasting adjunctive effect of the probiotic lozenges till the end of the study at day 360. These results are similar to other studies that reported reductions in GI and BoP scores after probiotic application (14, 17, 18, 137). Twetman et al. (14) investigated the clinical effect of a chewing gum containing probiotic bacteria on gingival inflammation and the levels of selected inflammatory mediators in GCF in patients with gingivitis. The chewing gums contained two strains of *L.reuteri*: ATCC 55730 and ATCC PTA 5289 ($1x10^8$ CFU/gum, respectively). The authors found that the

pro-inflammatory cytokines IL-1β, TNFα, and IL-8 in GCF were reduced by active probiotic treatment. This may be the proof of a principle for a probiotic approach combating inflammation in the oral cavity and the findings from Twetman et al. (14) are confirmed in the present study as evidenced by the reduced GI and BoP scores. However, there are also contradictory studies that showed no clinical effects of probiotic application (23, 139). These studies were either in healthy or experimental gingivitis patients with no mechanical therapy performed. In order to be effective in the subgingival area, it is of importance to mechanically disrupt the mature biofilm so the therapeutic agent can be effective. Therefore SRP prior to probiotic administration may result in more effective outcomes.

The reduction in probing pocket depth is the result of both a gain in clinical attachment level and a recession of the marginal gingival tissues (173, 175). The gingival recession results from the reduction in swelling of the marginal gingival tissue. The inflamed tissue with its inflammatory cell infiltrate and the increased numbers of capillaries present in the gingival connective tissue is gradually replaced by a more collagen-rich tissue (176). These changes are accompanied by a gradual shrinkage of the tissue in an apical direction and towards the root surface. The interface between the root surface and the former pocket epithelium is partially transformed into a long junctional epithelium (177, 178). Both, the presence of the long junctional epithelium and the increased content in collagen fibers in the gingival connective tissue result in the gain in clinical attachment level, i.e. an increased resistance of the tissues against the penetration of a periodontal probe.

At the start of this study, individual acrylic stents with grooves, used as reference points, were prepared for each patient in order to standardize probe position and angulation. There was a significant improvement of PD in the SRP + Prodentis group, which could possibly due to the significant reduction in the PI and GI scores. In the test and control groups a PD reduction of 1.20 mm and 0.76 mm at day 21, and a reduction of 1.44 and 0.85 at day 90 was observed. Shimauchi et al. (144) evaluated the effect of probiotic intervention on the periodontal condition of subjects without severe periodontitis. A total of 66 volunteers received *L. salivarius* WB21 containing tablets

with xylitol or xylitol alone. They reported a significant improvement of the PI and PD in the probiotic group after an 8-week intervention. Vivekananda et al. (132) reported maximum PD reduction in those receiving SRP + Probiotic treatment and this reduction (1.31 mm) was more than twice the sum of the SRP alone reduction added to the Prodentis alone reduction (0.49 mm), which suggests a synergistic effect. In another similar design study by Teughels et al. (22), application of *L.reuteri* lozenges as an adjunct to SRP resulted in a faster PD reduction initially. Significant lower mean PD in the SRP + Probiotic group (-1.41 mm) for deep pockets was observed when compared to the SRP group (-1.39 mm). Moderate pockets tended to be lower in the SRP + Probiotic group at 12 weeks when compared to SRP alone. For moderate and deep pockets, the SRP + Probiotic group showed significantly larger PD reductions when compared to the SRP group.

This is accordance with the results of this study. Significantly greater PD reductions were observed in the test group compared to the control group from day 0 to days 21, 90, 180 and 360 ($p<0.05$). The results remained stable over the entire study period. This may be attributed to the effect of the mechanical debridement with the optimal supragingival plaque control of the patients together with the strict recall visits scheduled.

The attachment gain occurring in the periodontal tissues following nonsurgical therapy should ideally be evaluated together with PD reduction. Changes in the attachment levels can be either determined with clinical attachment level (CAL) or RAL measurements (179). The clinical attachment level is measured from a clinical landmark such as the cemento-enamel junction to the tip of the probe during probing. However repeated measurements of attachment level from the cemento-enamel junction have shown to inherit errors and not to be reliable. The stents allow guidance for repeated measurements and angulation of the probe. To minimize errors between different measurement intervals, individual occlusal acrylic stents with grooves were used to measure the attachment gain in this study.

Significant improvement of attachment gain for the SRP + Prodentis group was observed at day 90 (1,18 mm), day 180 (1,67 mm) and day 360 (1,39 mm). Attachment gain for SRP+ Placebo group was 0,79 mm at day 90, 0,66 mm at day 180 and 0,53 mm at day 360. Vivekananda et al. (132) detected an attachment gain of 1.09 mm in the SRP+ Prodentis group and 0.29 mm in the SRP+ Placebo group. Teughels et al. (22) reported an attachment gain of 1.42 mm in the test and 1.01 mm in the control group in moderate periodontal pockets. In deep pockets gain was reported 1.47 mm for test and 0.67 mm for the control group. These findings are in accordance with the attachment gain observed in this study.

Limited data is available about the appropriate probiotic dosing regiments and only few dose-comparison studies have been undertaken (180). In this study *L.reuteri* containing tablets were prescribed twice a day for 3 weeks. This rationale is based on the study of Twetman et al. (14), where a dose-response relationship or a threshold level seemed to appear, but it would be too early to propose any clinical recommendations at this stage (146). The evaluation of previous studies and the manufacturer's recommendation were chosen for the dosage in this study.

Parallel to the clinical evaluations microbiological parameters were assessed by subgingival plaque sampling. Although saliva samples may give reliable results in patients with periodontal inflammation, it is more relevant to study microbial composition in the gingival crevice (123). *Lactobacilli* are rarely detected in subgingival samples and they could not be found in any of the patients with CP in a study carried out by Köll-Klais et al. (181), demonstrating that the subgingival region is not a common habitat for *lactobacilli.* However, this study evaluated the presence of *L.reuteri* in subgingival plaque samples. Eight out of twenty patients in the active group showed *L.reuteri* colony formation by culture method, indicating that even though not permanent, a colonization is possible after 3 week administration of the Prodentis® lozenges.

Microbial evaluation was performed by culture method. Culturing techniques have been the classic diagnostic method to detect bacterial species residing in the subgingival microflora (182). However, limitations such as difficulty in recovering cultivable species in low numbers together with stringent requirements, such as the need for experienced personnel, time and relatively high cost, have led to the development of different non-cultural methods, mainly based on immune-diagnosis and nucleic acid-based detection methods (183). Real-time quantitative polymerase chain reaction (PCR) with species-specific and sensitive primers provides a very specific and sensitive method for an accurate detection of target microorganisms (182). However, detection of *L.reuteri* DSM17938 or ATCC PTA5289 without cross-amplification of other Lactobacillus species (81) or the differentiation between *L.reuteri* strains in multi-species samples (184), may not be possible. Another limitation of this study was that, even though we could detect *L.reuteri* in microbial samples, the differentiation between the two strains used was not possible.

The PCR method can detect both, viable and non-viable bacteria. Thus the diagnostic importance of PCR is immeasurable. If the non-viable microorganisms are detected following antimicrobial therapy, then the effectiveness of the antimicrobial agent is difficult to assess, i.e. prognostic value of PCR is compromised because it detects even non-viable organisms (185). Furthermore, culturing method is still considered the gold standard in periodontal microbiology and remains an important means of characterizing the subgingival microbiota (182).

Several pathogenic microorganisms including red and orange complex bacteria have been found to colonize in deep pockets (PD ≥ 5mm) (25, 32, 186, 187). Review of the literature reveals that studies assessing the pathogenicity of periodontal disease from subgingival plaque samples for microbiological analysis were obtained from periodontal sites with PD ≥ 5mm (188, 189). In light of this knowledge all subgingival samples from CP patients were taken from single rooted teeth at sites with PD ≥ 5mm and GI ≥ 2.

In this study, TVC ($x10^5$ CFU/ml) and proportions of obligate anaerobes were decreased in both groups when a comparison is performed from baseline to days 21, 90, and 180 ($p<0.05$). At day 360, both microbiological values returned to their original baseline levels ($p>0.05$). Intergroup analysis of TVC ($x10^5$ CFU/ml) and proportions of obligate anaerobes revealed statistically significant difference in favor of SRP + Probiotic group at days 21, 90, and 180 ($p<0.05$). A substantial decrease in TVC ($x10^5$ CFU/ml) and proportions of obligate anaerobes were observed at day 90 ($p<0.05$). Proportions of obligate anaerobes in SRP + Probiotic group continued to decrease significantly more than SRP + Placebo group between day 21 to 90 ($p<0.05$). It may be speculated that the administered probiotic might perform a cumulative burst effect. On the other hand, it has been stated that, the most obvious changes in the total microbiota occurred in the first 3 months, and remained considerably stable for the next 3 months (149) that is consistent with our microbiological findings. A previous study has shown that the bacterial recolonization occurs after 3 months (3) In this study, the recolonization was observed at day 180 and continued up to day 360. This result can be attributed to the adjunctive usage of probiotics since there is a statistically significant difference between the groups in terms of microbiological parameters in favor of SRP + Probiotic group at days 21, 90, and 180 ($p<0.05$). Although identification of specific obligate anaerobic strains was not performed in this study, statistically significant reduction in percentage of obligate anaerobes was observed at days 21, 90, and 180 ($p<0.05$). This result is consistent with Vivekananda et al. (132), Iniesta et al. (21) and Teughels et al. (22). These studies demonstrated that probiotics were useful in the elimination of specific obligate anaerobes.

Review of literature reveals very few studies that aim to analyze *L. reuteri* in the subgingival microbiological samples. Stamatova & Meurman (123) reported that *Lactobacilli* are rarely detected in subgingival samples since subgingival region is not a common habitat for this strain and they could not be found in any of the patients with chronic periodontitis (140). Iniesta et al. (21) detected the presence of *L. reuteri* in subgingival plaque samples in gingivitis patients by PCR at baseline and 8-weeks. The finding related to the detection of *L. reuteri* at baseline was attributed to the lack of adequate specificity of PCR and cross-amplification of the primer with other

Lactobacillus species. At baseline, no *L. reuteri* was detected in any of the patients in both groups. At days 21 and 90, *L. reuteri* was detected in 6 and 11 patients, respectively, which demonstrates the colonization of *L. reuteri* in the subgingival region. However, a proper statistical analysis could not be performed due to the fluctuations in the levels of *L. reuteri* in TVC from one patient to another. Disruption of the biofilm with mechanical intervention changes the microbiota from obligate anaerobes to facultative anaerobes and *L. reuteri* colonizes the subgingival region. Culturing method used by Caglar et al. (151) was selected for the detection of *L. reuteri.* The subgingival microbiological samples were not frozen or preserved, and cultured as soon as they were obtained. The immediate culturing without any waiting period might account for the demonstration of the target bacteria. *L. reuteri* was not detected at days 180 and 360. Krasse et al. (17) reported that, the occurrence of *L. reuteri* in saliva decreased after the end of the intervention period, indicating that no permanent colonization had occurred and that oral persistence of *L. reuteri* was only temporary. *L. reuteri* was not detected in all patients. Environmental factors and pre-established microbiota might effect the colonization (190). In biofilm-like communities, maybe probiotics should be continuously administered for permanent colonization and more beneficial effects. However, further studies are warranted to clarify this issue.

The present randomized controlled clinical trial demonstrated the significant adjunctive effect of probiotic containing lozenges in chronic periodontitis patients at days 21, 90 and 180 in terms of clinical and microbiological parameters. Although these lozenges retard the recolonization up to 6 months, at one year pre-treatment microbiota was observed. Considering the clinical and microbiological outcomes of probiotic lozenges, this agent could be proposed as a beneficial adjunctive alternative in the non-surgical treatment of patients with chronic periodontitis.

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7. APPENDIX

APPENDIX 1

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

Araştırmanın Adı / Protokol Numarası:

Araştırmanın Konusu:

Kronik Periodontitisli Hastalarda Başlangıç Periodontal Tedaviye Ek Olarak Probiyotik İçeren Strip Kullanımının (Prodentis) Klinik ve Mikrobiyolojik Olarak Uzun Dönem 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 striplerin klinik ve mikrobiyolojik olarak uzun dönem etkinliğinin karşılaştırmalı olarak değerlendirilmesidir.

Araştırmanın Süresi: : 01.04.2012 01.10.2012 tarihleri arasında yapılacaktır.

Araştırmaya Katılan Gönüllü Sayısı:45

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 3 tek köklü sondalanabilir cep derinliği ≥5, gingival indeks ≥ 2 olan dişe sahip 40 hasta seçilerek yapılacaktır.

Çalışmaya dahil edilecek bireylerin seçilmesi;

Yeditepe Üniversitesi Dişhekimliği Fakültesi Periodontoloji Anabilimdalı'na başvuran bireyler arasında aşağıdaki kriterler doğrultusunda bireyler seçilecektir.

1) Hastaların sistemik olarak sağlıklı olmaları,
2) Çalışmadan 6 ay öncesine kadar periodontal tedavi görmemiş ve periodonsiyumu etkileyecek ilaç kullanmamış olmaları
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 mikrobiyolojik örnekler, probiyotikler ve kullanılacak striplerle ilgili detaylı bilgiler verilerek sözlü ve yazılı onamları alınacaktır. Onamları alınan hastalara ağız hijyen eğitimi, uygun diş fırçası seçimi, diş ipi ve/ veya arayüz fırçası seçimi ve kullanımı öğretilecektir. Diş fırçalarken Modifiye Bass tekniğinin kullanımı anlatılacak ve günde iki kez, sabah ve akşam olmak üzere dişlerin bu teknikte fırçalanmasını takiben arayüz temizliği yapılması istenecektir.

Araştırmaya dahil edilen hastaların periodontal tedavileri tek bir hekim tarafından yapılacaktır. Başlangıç tedavisinden önce ağız hijyen eğitimi verilen hastalar 1 hafta sonra kontrole çağırılacak ve yeterli düzeyde ağız hijyenini sağlayan hastalar rastgele 20'şer 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 hastalardan daha önce tespit edilmiş sondalanabilir cep derinliği ≥5 mm ve gingival indeks ≥2 olan dişe sahip iki bölgeden steril paper pointlerle mikrobiyolojik örnekler 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 mikrobiyolojik örnekleri alınan ve klinik ölçümleri yapıldıktan sonra diş yüzeyi temizliği ve kök yüzeyi düzleştirmesi işlemi 1 hafta arayla toplam 2 seans olarak uygulanacaktır. Bu işlemler ultrasonik cihazlarla (piezon® OEM Built- in Kit, EMS, Switzerland) ve Gracey küretlerle (Gracey, SG 3/4, 5/6, 7/8, 11/12, 13 / 14 minifive, SAS $^{3}/_{4}$,Hu – Friedy, USA) gerçekleştirilecektir. Tur ucuna takılan kıl fırça, lastik kon ve temizleme patları ile dişler cilalanacaktır. Bu dönemde hastaların öğretilen mikrobiyal dental plak uzaklaştıma yöntemleri doğru uygulayıp uygulamadıkları da kontrol edilerek gerekli düzeltmeler yapılacaktır. Başlangıç periodontal tedavi dahilinde, oklüzal travmaya neden olacak erken temas noktaları saptanıp, bu alanlar ortadan kaldırılacaktır, çürük dişler mevcutsa, tedavileri gerçekleştiricilecektir. Ayrıca endodontik konsültasyon sonrasında tespit edilen devital dişler tedavi edilecektir. Çekim yapılacak dişler araştırmaya dahil edilmeyecektir.

1.gruba diş yüzeyi temizliği ve kök yüzeyi düzleştirmesi ile beraber *Lactobacillus reuteri (Prodentis)* içeren probiyotik strip 3 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) strip 3 hafta boyunca sabah ve akşam birer tane olmak üzere günde 2 kez kullandırılacaktır. 3. hafta, 3.ay, 6. ay ve 1. sene klinik ve mikrobiyolojik örneklemeler tekrarlanacaktır.

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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. ay, 6. ay ve 1. senede yapılacaktır. Bu işlemler sırasında, muayene sondu ve 0.4 mm çapında 15 mm'lik periodontal sonda (*Universitiy of North Carolina PCPUNC15, Hu-Friedy Ins. Co., ABD*) kullanılacaktır. Periodontal sondanın doğru yerleştirilebilmesi ve tüm ölçüm dönemlerinde hataların en aza indirgenmesi amacıyla sabit rehber noktaları bulunan hastaya özel akrilik stentler yapılacaktır. Bu stentler üst ve altçene için ayrı ayrı dişlerin oklüzal yüzlerini ve kuronal 1/3 ünü kaplayacak şekilde hazırlatılacaktır.

Plak indeksine göre;

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

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

2- Dişeti bölgesinde gözle görülebilen ince ve orta düzeyde mikrobiyal dental plak vardır, interdental bölge tamamen dolmamıştır.

3- Dişeti kenarında, dişeti oluğu içerisinde ve komşu diş yüzeyinde fazla miktarda mikrobiyal dental plak vardır, interdental bölge tamamen dolmuştur.

Gingival indeks

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

0- Normal dişeti

1- Dişetinde hafif iltihap gözlenmektedir, hafif renk değişimleri ve ödem vardır, ancak sondalamada kanama yoktur.

2- Orta derecede iltihap görülür, dişetinde kırmızılık, ödem ve parlaklık vardır, sondalamada kanama mevcuttur.

3- Şiddetli iltihap, belirgin kırmızılık ve ödem vardır, ülserasyon olabilir. Spontan kanamaya eğilim söz konusudur.

Sondalamada kanama

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

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.

Rölatif Ataşman Seviyesi

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

Mikrobiyolojik Kültür Yöntemi

Mikrobiyolojik örnekler her hastanın önceden tayin edilmiş sondalanabilir cep derinliği ≥5, gingival indeks≥2 olan periodontal cep bölgelerinden tedavi öncesi ve tedavi sonrası 3. hafta, 3. ay, 6. ay ve 1. senede alınacaktır. Örneğin alınacağı bölgedeki diş yüzeyinden supragingival plak sond ve gaz tampon yardımı ile uzaklaştırılıp diş yüzeyi hava spreyi ile kurutulacaktır. Kanamanın olmamasına dikkat edilerek steril 30 numaralı paper point(*Meta Biomed Co., Korea*.) periodontal cep içerisine hafif direnç hissedilene kadar yerleştirilip 10 sn beklenecektir. Alınan subgingival mikrobiyolojik örnek aseptik koşullarda 4,5 ml phosphate- buffered saline (*phosphate-buffered saline, PBS tablet*, *Medicago AB, Uppsala İsveç*) içeren tüplere aktarılacaktır. Homojen dağılım sağlamak amacıyla tüpler 30 sn süreyle vorteks karıştırıcıda karıştırılacak ve aynı tampon içerisinde on katlı sulandırmalar yapılacaktır. Uygun sulandırmalardan (10^{-1}, 10^{-2} ,…..10^{-6}) 0.1 ml'lik 2 ayrı hacim alınarak % 0.0005 hemin (*Sigma 33H0829, Sigma Chemical Co., ABD*), %0.00005 menadion (*Sigma 123H2617, Sigma Chemical Co., ABD.*) ve %5 oranında koyun kanı ile zenginleştirilmiş trypticase soy agar dökülen 2 petri kutusuna steril yavrulu tüp yardımıyla homojen olarak yayılacaktır. Birinci besiyeri anaerop koşullarda (Gas Pak Jar) (*Oxoid Ltd., İngiltere*.) 37ºC'de 7-10 gün, diğeri ise %5 CO_2 içeren ortamda (CO_2 Gen) (*Oxoid, CO_2 Gen, Oxoid Ltd., İngiltere*) 37 ºC'de 5 gün bekletilecektir.Besiyerlerinde üreyen mikroorganizmaların kolonileri sayılacak, oksijene karşı durumlarına göre fakültatif anaerop ve zorunlu anaerop olmak üzere 2 grup mikroorganizmanın 1 ml'deki sayısı ve oranı kaydedilecektir.

APPENDIX 1

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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. Pınar Merve Tekçe 0532-6003080

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.

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APPENDIX 2

YEDİTEPE ÜNİVERSİTESİ
HASTANESİ

22/02/2012

SAYI: B.30.2.YTÜ.0.70.10.00-001/331

KONU: Yeditepe Üniversitesi Diş Hekimliği Fakültesi Prof.Dr.Selçuk Yılmaz ve Dt. Pınar Merve Tekçe'nin çalışması Hk ;

Sn.Prof.Dr.Selçuk YILMAZ,

Sorumlu Araştırmacılığını gerçekleştireceğiniz "Kronik Periodontisli Hastalarda Başlangıç Periodontal Tedaviye Ek Oalrak Probiyotik İçeren Strip Kullanımının Klinik ve Mikrobiyolojik Olarak Uzun Dönem Etkinliğinin İncelenmesi " 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,

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Bilimsel Komite Başkanı
Yeditepe Üniversitesi Hastanesi

Prof. Dr. Canan AYKUT BİNGÖL
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KARAR BİLGİLERİ	**Karar No:** 164	**Tarih:14/02/2012**
	Prof.Dr.R.Selçuk Yılmaz ve Dt. Pınar Merve Tekçe sorumluluğunda yapılması tasarlanan ve yukarıda başvuru bilgileri verilen klinik araştırma başvuru dosyası ve ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş, gerçekleştirilmesinde etik bir sakınca bulunmadığına toplantıya katılan değerlendirme kurulu üyelerinin oy çokluğu ile karar verilmiştir.	

DEĞERLENDİRME KOMİTESİ BİLGİLERİ

ÇALIŞMA ESASI	Klinik Araştırmalar Hakkında Yönetmelik, İyi Klinik Uygulamaları Kılavuzu, Yeditepe Üniversitesi Tıp Fakültesi, Klinik Araştırmalar Değerlendirme Komitesi Kuruluş ve Çalışma Esasları.

DEĞERLENDİRME KURUL BAŞKANI UNVANI/ADI/SOYADI: Prof. Dr. R. Serdar ALPAN

DEĞERLENDİRME KOMİTESİ ÜYELERİ

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		İlişki *		Katılım **		İmza
Prof. Dr. R. Serdar Alpan	Farmakoloji	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. M. Reha Cengizlier	Pediatri	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. S. Sami Kartı	Hematoloji	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Prof. Dr. Serdar Öztezcan	Biyokimya	YÜTF	E ☒	K ☐	E ☐	H ☐	E ☐	H ☐	
Yrd. Doç. Dr. Baki Ekçi	Genel Cerrahi	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Doç Dr. Ferda Özkan	Patoloji	YÜTF	E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	
Prof.Dr. Nural Bekiroğlu	Biyoistatistik	MÜTF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Esra Can Say	Diş Has. Ted.	YÜDF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Meriç Köksal	Eczacılık	YÜEF	E ☐	K ☒	E ☐	H ☒	E ☐	H ☐	
Prof. Dr. Ali Rıza Okur	Hukuk	YÜHF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Doç. Dr. Başar Atalay	Beyin Cerrahi	YÜTF	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	
Yrd.Doç.Dr.Esin Öztürk Işık	Biyomedikal Müh.	YÜMF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Doç.Dr.Nesrin Sarıman	Göğüs Hastalıkları	MÜTF	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	
Bilge Firuzbay	Sivil Üye		E ☐	K ☒	E ☐	H ☐	E ☐	H ☐	

* : Araştırma ile İlişki
** : Toplantıda Bulunma

Önemli Not: Çalışmanızın Klinik Araştırmalar Değerlendirme Komitesi tarafından onaylanan protokole göre yürütülmesi ve çalışma protokolündeki değişiklilerin kurulumuza bildirilmesi gerekmektedir.

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KURUL ADI	**YEDİTEPE ÜNİVERSİTESİ TIP FAKÜLTESİ KLİNİK ARAŞTIRMALAR DEĞERLENDİRME KOMİTESİ**
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BAŞVURU BİLGİLERİ	ARAŞTIRMANIN AÇIK ADI	Kronik Periodontisli Hastalarda Başlangıç Periodontal Tedaviye Ek Olarak Probiyotik İçeren Strip Kullanımının Klinik ve Mikrobiyolojik Olarak Uzun Dönem Etkinliğinin İncelenmesi.			
	ARAŞTIRMA PROTOKOLÜNÜN KODU				
	EUDRACT NUMARASI				
	SORUMLU ARAŞTIRMACI ÜNVANI/ADI/SOYADI	Prof.Dr.Selçuk Yılmaz ve Dt. Pınar Merve Tekçe			
	SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	Periodontoloji			
	KOORDİNATÖRÜN ÜNVANI/ADI/SOYADI				
	KOORDİNATÖRÜN UZMANLIK ALANI				
	ARAŞTIRMA MERKEZİ	YEDİTEPE ÜNİVERSİTESİ DİŞ HEKİMLİĞİ FAKÜLTESİ			
	ARAŞTIRMA MERKEZİNİN AÇIK ADRESİ	YEDİTEPE ÜNİVERSİTESİ DİŞ HEKİMLİĞİ FAKÜLTESİ			
	DESTEKLEYİCİ VE AÇIK ADRESİ				
	DESTEKLEYİCİNİN YASAL TEMSİLCİSİ VE ADRESİ				
	UZMANLIK TEZİ/AKADEMİK AMAÇLI	UZMANLIK TEZİ ☒ (Doktora Tezi)	AKADEMİK AMAÇLI ☐		
	ARAŞTIRMANIN FAZI VE TÜRÜ	FAZ 1 ☐			
		FAZ 2 ☐			
		FAZ 3 ☐			
		FAZ 4 ☐			
		BE/BY ☐			
		DİĞER ☐	Diğer ise belirtiniz:		
		İLAÇ DIŞI ARAŞTIRMA ☐	Belirtiniz:		
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☒	ÇOK MERKEZLİ ☐	ULUSAL ☒	ULUSLARARASI ☐

	Belge Adı	Tarihi	Versiyon Numarası	Dili
DEĞERLENDİRİLEN BELGELER	ARAŞTIRMA PROTOKOLÜ			Türkçe ☒ İngilizce ☐ Diğer ☐
	ARAŞTIRMA BROŞÜRÜ			Türkçe ☐ İngilizce ☐ Diğer ☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU			Türkçe ☒ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU			Türkçe ☒ İngilizce ☐ Diğer ☐

	Belge Adı		Açıklama
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	HASTA KARTI/GÜNLÜKLERİ	☐	
	İLAN	☐	
	YILLIK BİLDİRİM	☐	

7. CURRICULUM VITAE

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European Federation of Periodontology

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Turkish Dental Association