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INFLUENCE OF *LACTOBACILLUS RHAMNOSUS GG* METABOLITES ON GROWTH OF PERIODONTAL DISEASES BACTERIA

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ABSTRACT

Taking into consideration biochemical and physiological properties and previously obtained results, it was decided to determine the influence of *Lactobacillus rhamnosus GG* (*L.GG*) metabolites on *in vitro* growth of selected aerobic and anaerobic bacteria being the cause of inflammatory changes within parodontium and gingiva. To prevent possible *L.GG in vitro* invasion the bacteria were enclosed inside alginate microcapsules. The metabolites of *L.GG* immobilised in microcapsules displayed inhibitory effect on growth of some pathogenic anaerobic bacteria causing periodontitis – *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. Moreover the metabolites from *L.GG* immobilised in microcapsules inhibited the growth of *Actinomyces odontolyticus, Actinomyces naeslundii, Actinomyces israelii, Neisseria ssp.* and *Haemophilus influenzae*, bacteria causing gingivitis.

Key words: Lactobacillus rhamnosus GG, gingivitis, periodontitis

INTRODUCTION

Lactobacillus rhamnosus GG (*L.GG*) has significant influence on health state through the ability to proliferate and secrete antibacterial metabolites. Unlike other lactic acid bacteria , *L.GG* does not produce bacteriocins, but it produces nitric oxide and hydrogen peroxide, which support the activity of lactic acid [3,10]. It does not intensify fermentation processes since it is unable to decompose sucrose and fructose, what enables its application in buccal cavity. *The* environment of buccal cavity is microbiologically diverse. It facilitates the growth of aerobic and anaerobic bacteria being components of bacterial dental plaque, which is the fundamental etiological factor of caries and periodontitis. The significant role of *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Actinomyces odontolyticus, Actinomyces naeslundii, Actinomyces israelii, Neisseria, Haemophilus influenzae* was confirmed in etiopathology of periodontal diseases [13, 21]. The investigation of the influence of metabolites *in vitro* on growth of selected aerobic and anaerobic bacteria was the aim of the study.

MATERIALS AND METHODS

Strains. The lyophilisate of Lactobacillus rhamnosus GG ATCC 53103 (L.GG), Actinomyces israelii, Actinobacillus actinomycetemcomitans, Actinomyces israelii ATCC 10049 and Actinobacillus actinomycetemcomitans ATCC 33384 used in this study was obtained from London ATCC collection. Neisseria ssp and Haemophilus influenzae strains were isolated from buccal cavity ulcerations in patients of SPSK-1 Clinic Hospital in Szczecin. Actinomyces odontolyticus and Actinomyces naeslundii were obtained from Institute of Immunology and Experimental Therapy Polish Academy of Sciences in Wrocław. Porphyromonas gingivalis was obtained from Merck laboratory.

L.GG lyophilisate was suspended in MRS broth for Lactobacillus according to de Man, Rogosa and Sharpe, Oxoid (CM0359). The bacteria were incubated for 24h at 36,6°C, until the medium became turbid.

Immobilisation. The strain L.GG was immobilised in alginate microcapsules. Microcapsules were obtained by dropping polymer solution (with microorganisms) into substances causing gelation [4]. Hydrogel microcapsules were formed, with outer porous membrane (alginate and calcium ions complex), and their inside was filled with L. GG suspension. In order to obtain microcapsules having repeatable shape (fig. 1), in this study SEP 11S & SEP 21S, Model SEP 11_(ASCOR S.A.) syringe pump was used. Lyophilisation was used to enable prolonged storage of immobilised bacteria.

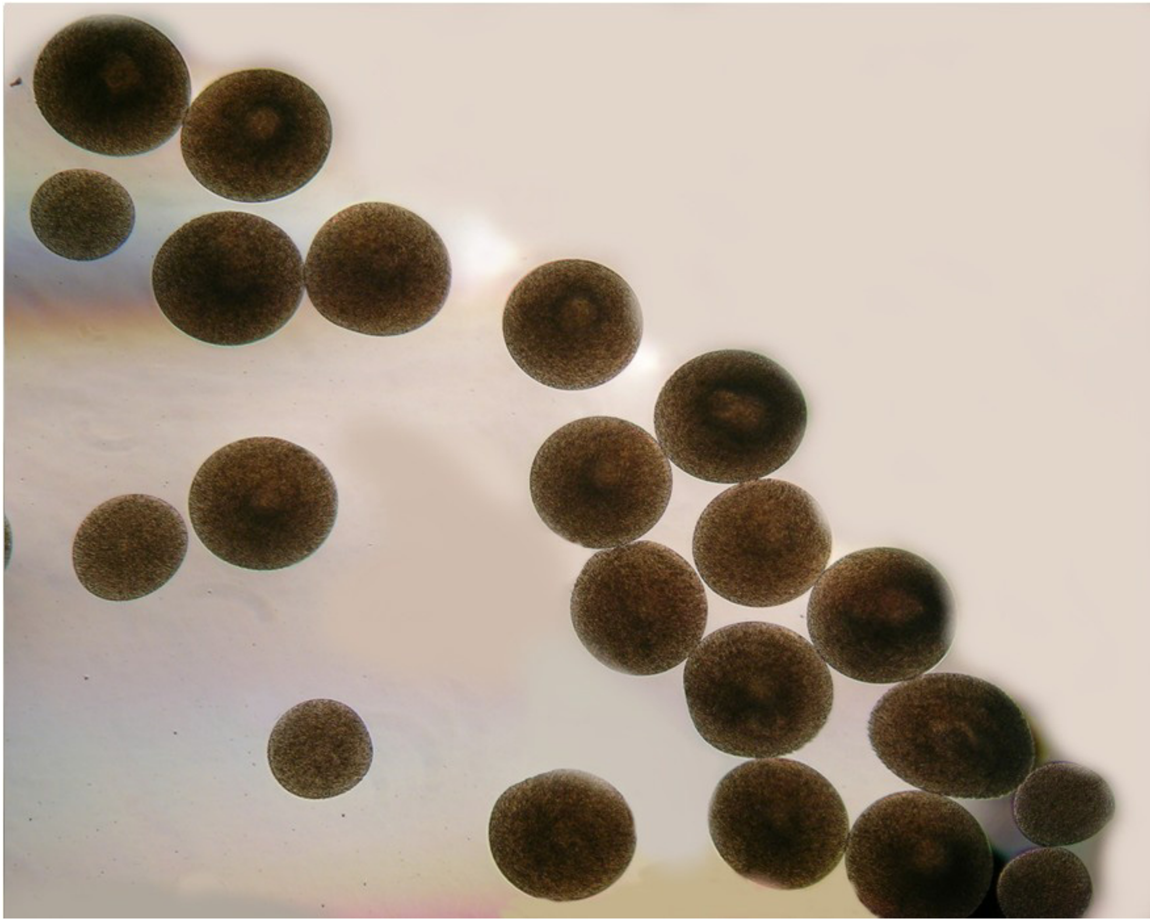

Fig.1. The photo of microcapsules containing *Lactobacillus rhamnosus GG* taken with Nikon Eclipse TE 2000 – S microscope. Magnification x40.

Microorganisms culture. *A. odontolyticus, A. naeslundii* and *A. israelii* lyophilisates were suspended in TSB (Tryptone Soya Broth, Oxoid). *A. actinomycetemcomitans* was cultured in liquid BHI medium (Brain Heart Infusion, Oxoid) with 5% of rabbit blood. Tubes with bacteria were placed in vacuum jars (Oxoid) intended for culturing bacteria requiring modified atmosphere. Inside the jars gas packs modyfying gases composition (Oxoid) were placed in order to obtain atmosphere containing 5% CO_2. The tubes containing pathogenic bacteria were incubated for 72h at 36,6°C.

P. gingivalis lyophilisate was suspended in liquid medium (Merck) provided with the lyophilisate. *P. gingivalis* is strictly anaerobic, thus for its culture jars and gas packs reducing O_2 (Oxoid) were used. The incubation at 36.6°C lasted 96h.

In solid selective media, enabling the growth of selected strains of pathogenic bacteria, four equal holes were cut using sterile cork borers. The volume of cut holes was 0.15ml. The holes were filled with MRS medium with microcapsules containing *L. GG*. The mass of microcapsules in each of the hole was 0.005mg. The plates containing microcapsules with *L. GG* were incubated at 36.6°C for 12h in order to saturate selective media for pathogenic bacteria with *L. GG* metabolites. Next, the dilutions of *Neisseria ssp, H. influenzae, A. odontolyticus, Actinomyces naeslundii, A. israelii, A. actinomycetemcomitans* and *P. gingivalis* were spread onto plates. 0.1ml of the suspension containing 3 x 10^3 of bacterial cells was spread onto solid media. The plates were incubated at 36.6°C for 12h in conditions adjusted for growth of pathogenic bacteria. In case of cultures of *A. odontolyticus, A. naeslundii, A. israelii, A. actinomycetemcomitans, P. gingivalis* the Oxoid jars and gas packs were used to modify the atmosphere. After incubation the inhibition zones around the cut in the media holes were measured.

Statistical analysis. To answer the question whether the difference between the mean values is statistically significant, i.e. it represents the difference within the population rather than random variation, Shapiro-Wilk test was used for the examination of the distribution consistency of variable tested in population with normal distribution. The results were statistically analysed with Statistica 7.1 software (Statsoft).

If the value of probability $\boldsymbol{p}$ falls below a pre-defined test significance level $\boldsymbol{\alpha}$, then the hypothesis of the consistence with the normal distribution is rejected. The Shapiro-Wilk test is preferable normality test since its more powerful comparing to other tests. In case of rejecting the zero hypothesis, in order to show the statistically significant differences in concentration between the groups, non-parametric Wilcoxon match-pairs ranks test is used for results in medical and biological studies, which is a non-parametric alternative of t-test for correlated samples [5, 16].

RESULTS

In the study the influence of *L. GG* metabolites *in vitro* on the growth of selected buccal cavity pathogenic bacteria, especially anaerobic bacteria flora, was determined. The effect of antagonistic activity of *L.GG* was measured in mm and the mean values of growth inhibition zones were calculated from measurements performed for one Petri dish. Significant influence of *L.GG* metabolites on the growth of the following microorganisms was shown: *Neisseria ssp*, *Haemophilus influenzae, A.odontolyticus, Actinomyces naeslundii, Actinomyces israelii, Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. The presence of inhibition zones around the holes containing microcapsules with *L.GG* was observed for all of the examined bacteria. The growth of *Haemophilus influenzae* was the most inhibited by *L.GG* metabolites.

In the fig. 2 a, b, c, d, e, f, and g the distribution of 30 mean values of measurements taken on single plate from the cultures of *Neisseria ssp*. [a], *Haemophilus influenzae* [b], *Actinomyces odontolyticus* [c], *Actinomyces naeslundii* [d], *Actinomyces israelii* [e], *Actinobacillus actinomycetemcomitans* [f] and *Porphyromonas gingivalis* [g] are presented. The values in vertical axis show the measured distance from the centre of growth inhibition zone in mm. The values presented in perimeter illustrate the sequence of measurements.

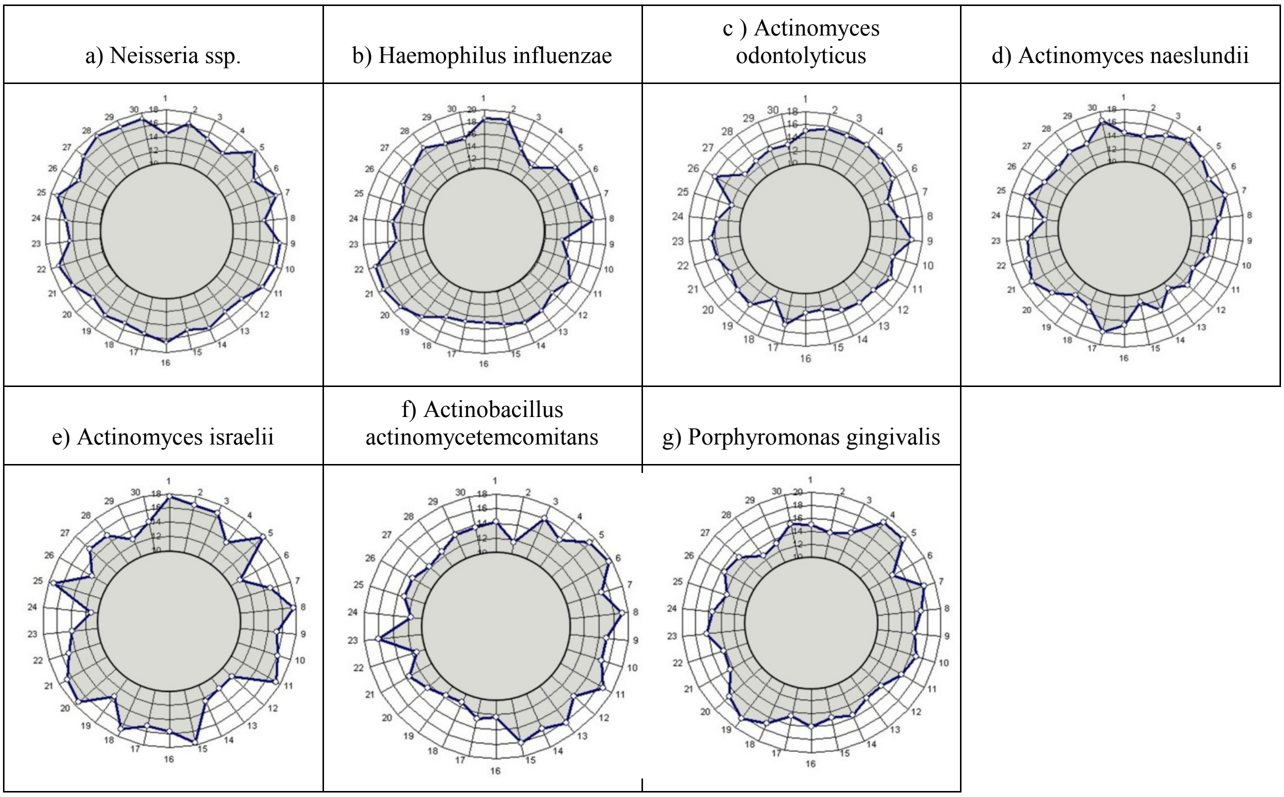

Fig. 2. Bacterial growth inhibition zones [mm].

The results were compared and the probability of occurrence of statistically significant differences was determined [5].

The non-parametric Wilcoxon match-pairs ranks test was used, with $p<0.05$ for the results obtained for microorganisms cultures. Statistically significant differences were shown for growth inhibition of *Neisseria ssp.* and *Actinomyces odontolyticus, Actinomyces israelii and Actinomyces naeslundii*. *Haemophilus influenzae* and *Actinomyces odontolyticus, Actinomyces naeslundii*, *Actinomyces israelii and Actinobacillus actinomycetemcomitans, Actinomyces odontolyticus* and *Porphyromonas gingivalis, Actinomyces naeslundii* and *Porphyromonas gingivalis.*

The results obtained, considering mean values of bacterial growth inhibition zones and standard deviations, are presented graphically in Fig. 3.

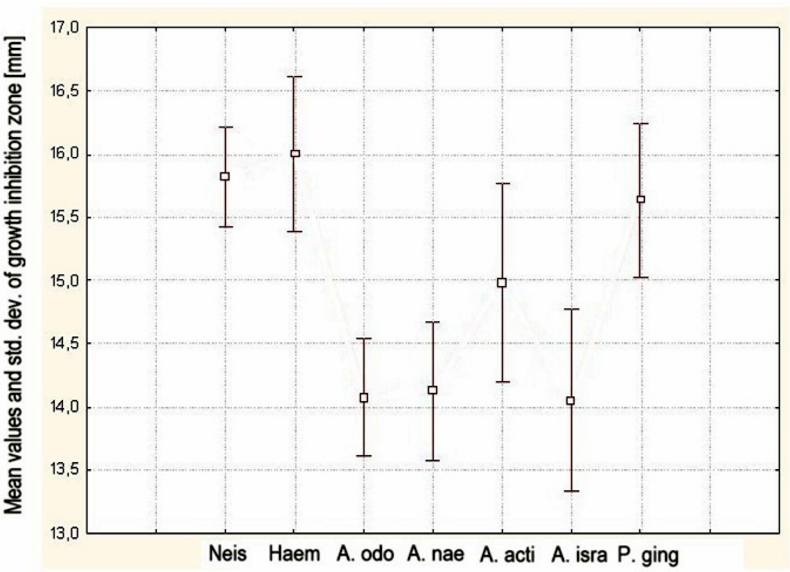

Fig. 3. Mean values and standard deviations for growth inhibition zones of bacteria being the cause of gingivitis and periodontitis. Neis - *Neisseria ssp,* Haem - *Haemophilus influenzae,* A. do. .*Actinomyces odontolyticus, A. nae* - *Actinomyces naeslundii*, A.acti - *Actinobacillus actinomycetemcomitans, A. isra* - *Actinomyces israelii,* P. ging - *Porphyromonas gingivalis.*

Lack of statistically significant differences was observed between growth inhibition of Neisseria ssp. and P. gingivalis, A. actinomycetemcomitans and H. influenzae, H. influenzae and P. gingivalis, A. odontolyticus and A. israelii, A. actinomycetemcomitans and A. naeslundii, A. naeslundii and A. actinomycetemcomitans, and between A. israelii, A. actinomycetemcomitans and P. gingivalis and A. israelii (Fig.4.).

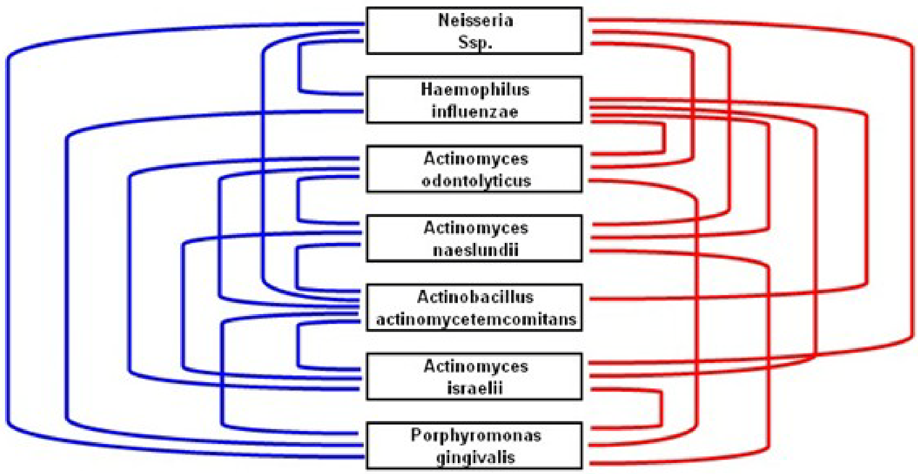

Fig. 4. Probability of occurrence of differences between growth inhibition zones of aerobic and anaerobic bacteria. Red lines show statistically significant differences, blue lines – lack of statistically significant differences between the groups.

L.GG metabolites show stronger, statistically significant, inhibition of the growth of aerobic than anaerobic bacteria. Also statistically significant variation in scale of growth inhibition zones of anaerobic bacteria was observed. *P. gingivalis* growth was stronger inhibited than that of Gram-positive rods of *Actinomyces* species. The differences in growth inhibition zones of *P. gingivalis and A. actinomycetemcomitans* were statistically insignificant.

DISCUSSION

For many years, in numerous studies on animals and humans, the antibacterial, antiviral or immunomodulating influence of *L.GG* rods was observed. Most of the research focused on attempts to treat infections within digestive gastrointestinal tract using *L.GG* [7, 8, 17, 18]. Besides local treatment, there are attempts to use *L.GG* rods in treatment of other systems than digestive. Disability to decompose sucrose and fructose is a biochemical feature of *L. GG* [15] and it was confirmed by own studies with API 50 CHL test showing lack of growth on these carbohydrates. Nase et al. [14] and Ahola et al. [2] observed in tests *in vitro* that application of this probiotic delays the growth of *Streptococcus mutans* [2, 14]. In periodontal diseases treatment the problem of chronic periodontitis caused by anaerobic microflora and immunity system disorder is still unresolved. It was decided to determine whether *L. GG* metabolites *in vitro* can inhibit the proliferation of mainly anaerobic bacteria, favourably modifying immunity. Placing *L. GG* inside microcapsules increased the chances of survival in the organism and favoured slow release of bacterial metabolites. Moreover it could protect patients with extreme lack of immunity against the possible *L. GG* invasion.The use of microcapsules for probiotic preparations protects bacteria against gastric acid and bile and affects their survival in alimentary tract [20]. If capsules containing *L.GG* were used in inflammatory changed gingival pockets the alginate would protect *L.GG* against direct contact with cellular membranes. The number of patients after transfusions of organs (kidneys, heart, liver, bone marrow), who often need a lifelong use of immunosuppressants, has increased in recent years. The presence of large numbers of viable *L.GG* could pose an additional threat of infection.

During the course of disease often serious tissue damage occur, together with exudation, oedema, bleeding from gums or teeth loss. Antibacterial factors present in saliva do not give significant protection against microorganisms causing perioodontitis because the bacteria locate in undergoing mineralisation plaque matrix and hardly accessible gingival pockets. Elimination of bacterial plaque may result in removal of causative agent of inflammation. So far there is no universal periodontitis treatment method which would prove to be highly effective [19]. In times of increasing resistance to antibiotics the research focused on finding natural counterparts having bacteriostatic and bactericidal effect. Similar studies were performed using alternative sources, i.e. propolis, plant extracts from e.g. *Cratoxylum formosum* ssp or oil of cloves, which exhibited activity against bacteria causing periodontal infections [1, 6, 9, 11, 12] Those bacteria were *A. actinomycetemcomitans, Capnocytophaga gingivalis, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia* and *Prevotella melaninogenica*. There are still no studies on using probiotic bacteria metabolites inhibiting growth of *A. odontolyticus, A. naeslundii, A. israelii, Neisseria ssp.* and *H. influenzae*, causing deep inflammation of gums.

In performed experiments, it was determined that the metabolites of *L.GG* immobilised in microcapsules showed *in vitro* inhibitory effect on growth of pathogenic anaerobic bacteria causing periodontitis. Those bacteria were *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis.* Moreover, the metabolites from *L.GG* immobilised in microcapsules inhibit the growth of *A. odontolyticus, A. naeslundii, A. israelii, Neisseria ssp.* and *H. influenzae,* the bacteria causing deep inflammatory changes in gingiva.

The treatment of acute inflammatory states is based on pathogen directed antibiotic therapy. Still chronic diseases, located deep in the tissues, caused by variable bacterial flora, firstly aerobic, then anaerobic, often together with immune system disorder, are of a major problem. With the lack of results from antibiotic therapy another methods are used, such as application of autovaccines, bacteriocins or, finally, exploitation of bacterial antagonism phenomenon by adding favourable bacteria to preparations or food.

CONCLUSIONS

1. Metabolites of *L.GG* showed *in vitro* inhibitory effect on growth of pathogenic anaerobic bacteria causing periodontitis - *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis.*
2. *L.GG* metabolites inhibited the growth of *Actinomyces odontolyticus, Actinomyces naeslundii, Actinomyces israelii, Neisseria ssp.* and *Haemophilus influenzae*, the bacteria causing deep inflammatory changes in gingiva.

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