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Virulence profiles of microbial strains isolated from patients with periodontal lesions

Radita Jivoinovici [1], Irina Gheorghe [2], Marcela Popa [2], Luminița Mărușescu [2], Carmen Curutiu [2], Mariana Carmen Chifiriuc [2], Veronica Lazar [2], Ecaterina Ionescu [1], Ruxandra Bartok [1], Ileana Suciu [1], Mihaela Chirila [1], Ioana Suciu [1,*]

[1] University of Medicine and Pharmacy "Carol Davila", Faculty of Dental Medicine, Bucharest, Romania
[2] Department of Microbiology, Faculty of Biology, University of Bucharest, Research Institute of the University of Bucharest-ICUB, Life, Environment and Earth Sciences, Bucharest, Romania

*corresponding author e-mail address:***joa_suciu@yahoo.com***

ABSTRACT

Periodontitis represents a chronic inflammatory disease of polymicrobial etiology that could lead to the destruction of bones and tissues that support the teeth. The investigation of the microbial composition of root canal or the gingival sulcus in 42 patients with periodontal diseases revealed the presence of 125 bacterial and yeast strains that were implicated in various forms of periodontal diseases such as: *Streptococcus sp., Micrococcus sp., Gemella sp., Enterococcus sp., Staphylococcus sp., Lactobacillus sp., Bifidobacterium sp., Aerococcus sp., Actinomyces sp., Leuconostoc sp., Fusobacterium sp., Prevotella sp., Pantoea sp., Pasteurella sp., Aeromonas sp., Propionibacterium sp., Veillonella sp.* The isolates exhibited different levels of attachment to eukaryotic cells, the results showing that 95% of the analyzed strains from root canal and 70% of the isolates from gingival sulcus were able to adhere, with an adherence index ranging from 5 to 100%.The microbial strains isolated from the periodontal lesions expressed virulence factors that enable them to colonize and damage the host tissues (i.e. ability to adhere to inert and cellular substratum, pore forming toxins, proteases, DN-ase and siderophore-like molecules).

Keywords: *periodontal lesions, microbial composition, periodontal diseases, yeast strains, bacterial strains, virulence factors.*

1. INTRODUCTION

Bacterial communities that colonize the human body are intimately linked with host physiology, immunity, metabolism, and nutrition [1-3]. As entrance gate, the mouth is colonized by a bacterial consortium with high taxonomic richness and phylogenetic diversity [4,5]. The microbial composition of the plaque biofilm has a critical role in oral health. Disruption of plaque homeostasis can stimulate tissue destruction and inflammation, leading to infections such as dental caries, gingivitis, and periodontitis [6]. A comprehensive and systematic profiling of the oral biofilm is necessary, not only to understand microbial associations with localized infections, but also because the oral microbiome has long been known as a reservoir for infections at other body sites [7]. Bacterial species normally found in the oral cavity have been associated with distal infections in the lungs, heart, brain, and liver, either reflecting their involvement in opportunistic infections as a result of systemic changes in the body, or perhaps suggesting a causative link [8-10]. Around 700 - 1000 bacterial species were described in the composition of dental plaque reaching densities of 10^8 bacterial cells/mg, many of them being uncultivable and associated in polymicrobial communities. The literature of the last decades had shown that almost all forms of the periodontal disease are consequences of the chronic, nonspecific or specific bacterial infections [11,12]. The sites colonized by these different microorganisms are diverse, ranging from the no shedding tooth surface to the continually shedding epithelium covering the mucosal surfaces. The microorganisms colonizing these surfaces are not present in a free-floating planktonic state. Rather, they are present as a biofilm—a "community of microorganisms attached to a surface" [13]. While the communities found on soft tissues often comprise a single microbial species, the most prevalent oral biofilm, dental plaque, exists as a complex multispecies entity attached to the tooth surface. Therefore, the plaque consortia can be a mirror and possibly even a monitor of oral and non-oral health and disease. Periodontal diseases result from a polymicrobial infection of the subgingival crevice. A lot of primary players in the disease process have been identified and their virulence factors were well characterized. The importance of the biofilm in plaque colonization and bacterial interactions and the impact of these interactions on expression or inhibition of specific virulence factors is not fully understood [14]. What is realized, however, is that given the right combination of bacteria, indigenous colonizers may become opportunistic pathogens. In this context the purpose of this study was the investigation of the microbial composition of the root canal and gingival sulcus in Romanian patients with periodontitis, and to reveal the cell-associated and soluble virulence microbial factors.

2. EXPERIMENTAL SECTION

2.1. Isolation and identification of the microbial strains.

A total of 42 patients from Endodontics and Periodontics Clinic from the Faculty of Dental Medicine and Pharmacy " Carol Davila" from Bucharest with pathology of periodontal tissues confirmed by clinical and radiographic examinations consented to participate in our investigation. Samples from each patient were placed in thioglycolate broth. The predominant cultivable microbiota was investigated by classical microbiological methods which included cultivation on enriched non selective media (Brucella blood agar), differential and selective media (MacConkey agar, Slanetz Bartley, Manitol Salt agar, Cetrimide agar). The microbial colonies were examined microscopically to establish the morphology and Gram stain affinity. Conventional biochemical tests (oxidase and catalase) were used to select the appropriate API microtube system [API Staph, API Strep, API 20E, API NE, API 20A] for the identification of the microbial isolates.

2.2. The evaluation of the adherence capacity to the cellular substratum.

This microbial virulence parameter was assessed by the Cravioto's adapted method. The Hep-2 cell line was cultivated for 24 hours at 37^0 C in MEM (Eagle Minimal Essential Medium) supplemented with 10% fetal bovine serum (Gibco BRL). 1 ml of microbial suspension with a density corresponding to the 0.5 Mac Farland standard, prepared in PBS using a 18-24 hours culture of was added over the cellular monolayer. The inoculated plates were incubated at 37_0 C for 2 hours, allowing the bacterial cells to adhere to the cellular substratum. After incubation, the cellular monolayer was rinsed with PBS, fixed with methanol for 5 minutes and colored with 1% Giemsa solution for 20 minutes. After staining, the wells were washed with tap water, dried at room temperature and microscopic examined with the 1000x immersion objective in order to establish the adherence *patterns*(localized, diffuse or aggregative) of the microbial isolates and to determine the adherence index (expressed as the ratio between the number of eukaryotic cells exhibiting adhered microbial cells per 100 cells counted on the microscopic field).

2.3.Assessment of the adherence capacity to the inert substratum.

The adherence capacity to the inert substratum of the microbial strains was determinate by quantifying the production of slime factor using the microtiter plate method [15]. The microbial suspensions corresponding to 0.5 Mac Farland density were seeded in broth media, distributed in 96 multiwell plates, then incubated in anaerobic condition at 37°C for 48 h. Visible microbial biofilms, formed at the bottom of wells, were washed three times, fixed with cold methanol for 5 minutes, colored with 1% violet crystal for 15 minutes, washed again with tap water and resuspended in 33% acetic acid. Broth media was used as a negative control. The biofilm formation was quantified by the observation of the color intensity of the obtained suspension that was proportional with the number of microbial cells adhered to the inert substratum and estimated as absent (0), weak (+), moderate (++) or strong (+++). All assays were done in triplicate.

2.4. Study of the soluble virulence factors production.

Culture media containing specific enzymatic substrates were used to highlight the expression of differentmetabolic enzymes: hemolysins, amylases, caseinases, gelatinases, aesculin hydrolysis, DN-ase, as previously reported [16]. Briefly, 18 h microbial culture was spotted onto agar plates with specific enzymatic substrata, i.e. 5% sheep blood (hemolytic activity), 1% starch (amylase activity), 1% casein and 0.4% gelatine (proteolytic activity), 1% aesculin (esculinase activity), 0.2% DNA (DN-ase production). Enzyme production was detected after 48-72h of incubation at 37°C, by macroscopic observations of specific modifications of the media around the culture spot (hemolysis, precipitation, halo).

3. RESULTS SECTION

Microbiological analysis in periodontitis represents an important tool inmonitoring the outcomes of antimicrobial treatments. The strains associated with periodontal diseases are Gram-negative anaerobic bacteria, Gram positive strains and also was revealed in our study yeasts. Microbial characterization of the 42 root canal and gingival sulcus samples from this study revealed the presence of 125 bacterial strains that were implicated in various forms of periodontal disease as*Streptococcus sp. 23% (*from which *Streptococcus oralis* represented 27,5%, *Streptococcus equinus 7%, Streptococcus mitis 21%, Streptococcus salivarius sp. salivarius 24%, Streptococcus intermedius 14%, Streptococcus acidominimus 10%), Micrococcus sp. 4%, Gemella sp. 7% (Gemella morbilorum* 66% from the analyzed species*), Enterococcus sp. 3%, Staphylococcus sp. 14% (Staphylococcus epidermidis 33%, Staphylococcus aureus 11%, Staphylococcus saccharolyticus 5,5%, Staphylococcus xylosus* 44%, *Staphylococcus capitis* 5,5%), *Lactobacillus sp*. 5%,

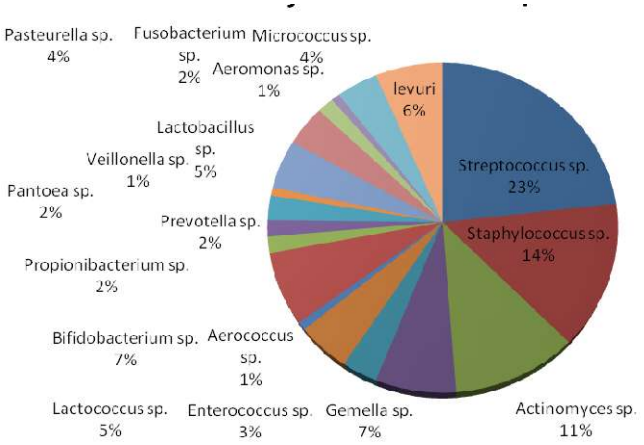

Figure 1. The distribution of the species in analyzed samples.

Bifidobacterium sp. 7%, *Aerococcus sp*. 1%, *Actinomyces sp.* 11% (72% *Actinomyces neaslundii*, and the rest *Actinomyces israelii*), *Leuconostoc sp*., 5%, *Fusobacterium sp.* 2%, *Prevotella sp.* 2%,

Pantoea sp. 3 2%, *Pasteurella sp.* 4%, *Aeromonas sp.* 1%, *Propionibacterium sp.* 2%, *Veillonella sp.* 1% and yeasts 6% (figure 1).

Many of the periodontal pathogens have surface structures necessary for attachment, including fimbriae, capsules and lipopolysaccharides, which facilitate the adhesion of the bacteria to a surface but also the co-aggregation with other plaque organisms, and are highly regulated to respond to environmental changes [17].

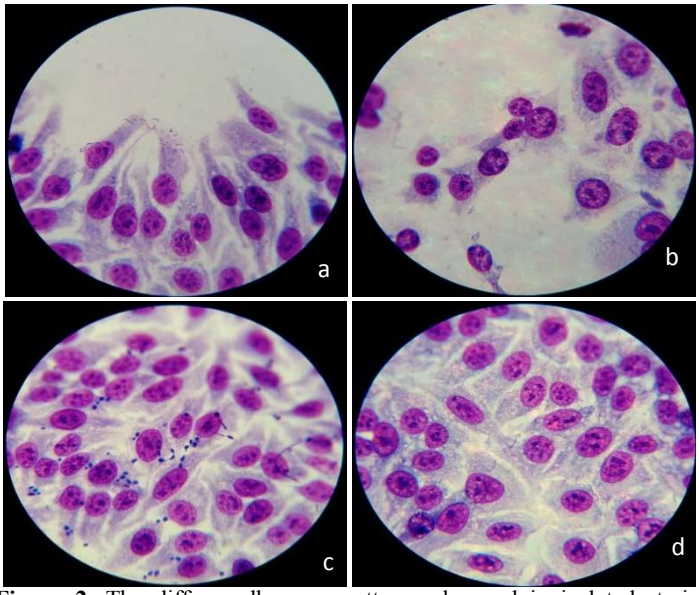

Figure 2. The diffuse adherence patterns observed in isolated strains (Giemsa, x1000).

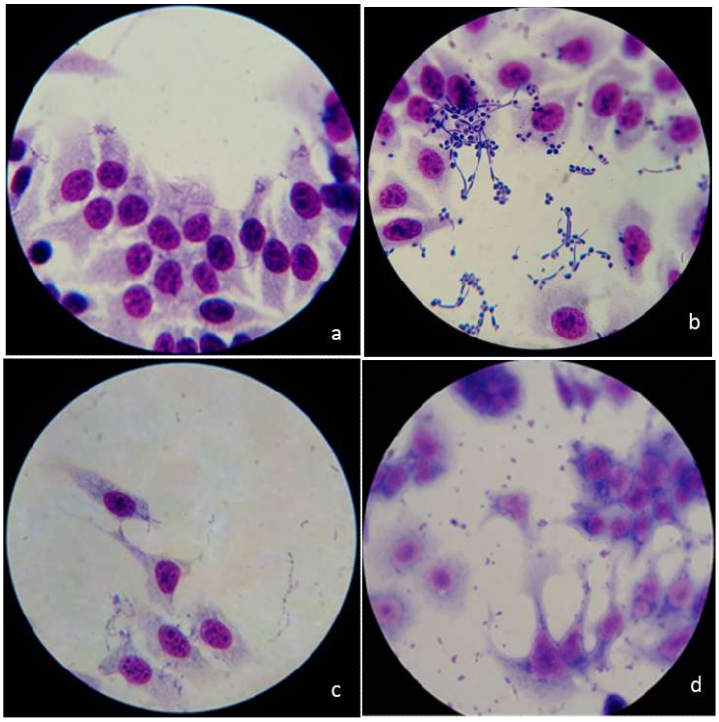

Figure 3. The localized adherence patterns observed in isolated strains (Giemsa, x1000).

In order to revealed the presence of the surface attachment structures, the bacterial strains isolated from root canal and gingival sulcus samples were tested for their ability to adhere to cellular substratum represented by eukaryotic cells belonging to Hep-2 cell line. The isolates exhibited different levels of attachment to eukaryotic cells, the results showing that 95% of the analyzed strains from root canal and 70% of the isolates from gingival sulcus were able to adhere, with an adherence index ranging from 5 to 100% similar to others studies [18,19]. The adherence *patterns* observed were diverse: diffuse (figure 2), localized (figure 3) and localized-aggregative (figure 4).

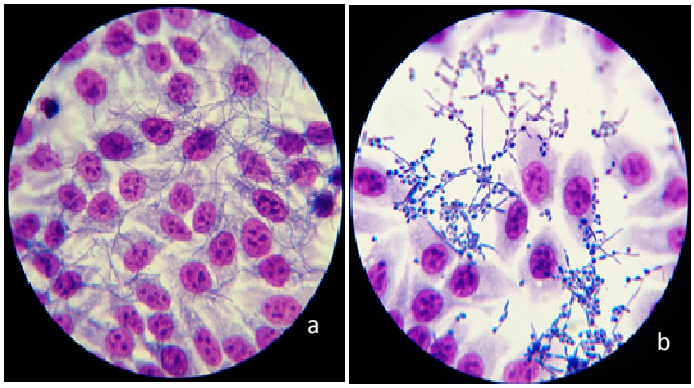

Figure 4. The localized-aggregative adherence patterns observed in isolated strains (Giemsa, x1000).

The microbial species must first adhere to a substrate in order to colonize a periodontal site, or to other bacteria that had previously adhered to that surface by co-aggregation and co-adhesion [20,21]. The term of *slime* was used initially by Christensen et al. (1982) referring to the glycocalix produced by the strongly adherent strains of *Staphylococcus epidermidis* isolated from the infected surface of medical implants, and subsequently, to other microbial species, producing hydrophilic exopolysaccharides for facilitating the adherence to inert abiotic surfaces. *Slime* represents an indicator of the resistance and survival capacity of the bacterial strains in the external environment, but also a virulence factor, in case of an infection of a host organism, by blocking the phagocytosis. In our study the results showed that the *slime* factor production, a necessary element in the colonization of subgingival bacteria, was present in 70% of the analyzed strains; these results demonstrate their potential to adhere, an essential required step for pathogenesis identified for many other pathogens [14].

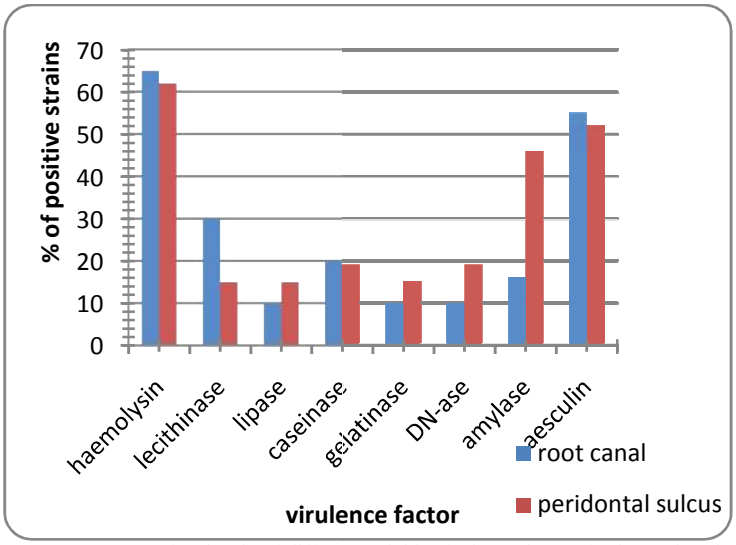

Figure 5. Expression levels of different soluble virulence factors in the analyzed strains.

Many pathogenic bacteria produce extracellular molecules that have tissue-damaging effects. Some of the diverse armamentarium of products from pathogenic bacteria serve as virulence factors in the pathogenesis of disease by facilitating the spread of bacteria or toxins through tissues [22]. Our results revealed that the investigated strains showed the capacity to produce pore forming toxins, including haemolysins (65% of the strains from root canal and 62% of the isolates from periodontal sulcus) –and lecithinases (30% of the strains from root canal and 15% of the isolates from periodontal sulcus), to affect the host cell membrane by acting on membrane lipids, suggesting the implication of these enzymes in host invasion- lipases (10 % versus 15%); proteases, i.e. caseinase (20 vs. 19%) and gelatinase (10 vs. 15%); amylase (16 vs. 46%); DN-ase (10 vs. 19%) and the production of siderophore-like molecules resulted from the *in vitro* hydrolysis of aesculin which are implicated in the regulation of multiplication rate as well as in the virulence factors expression (55 versus 52%) (figure 5).

4. CONCLUSIONS

The strains isolated from samples taken from patients with periodontal lesions exhibited a wide range of cell-associated and soluble virulence factor. The virulence profile of the isolates evaluated by the ability to adhere to cellular substratum and by production of soluble enzymatic factors (i.e. pore forming toxins, including haemolysins, lecithinases and lipases; proteases: caseinase and gelatinase; amylase; DN-ase and the production of siderophore-like molecules resulted from the *in vitro* hydrolysis of aesculin) showed that the periodontal bacteria possessed factors that enables them to colonize and damage the host tissues. Microbiological analysis and antimicrobial susceptibility testing should ideally form the basis for selecting the optimal antimicrobial treatment.

5. REFERENCES

[1] Cho I., Blaser M.J., The human microbiome: at the interface of health and disease, *Nat Rev Genet*., 13, 4, 260–270, **2012**.

[2]Madupu R., Szpakowski S., Nelson K.E., Microbiome in human health and disease, *Sci Prog.,*96, 2, 153–170, **2013**.

[3]Pflughoeft K.J., Versalovic J., Human microbiome in health and disease, *Annu Rev Pathol*., 7, 99–122, **2011**.

[4]Dewhirst F.E., Chen T., Izard J., Paster B.J., Tanner A.C., Yu W.H., Lakshmanan A., Wade W.G., The human oral microbiome, *J Bacteriol.,*192, 19, 5002–5017, **2010**.

[5] Stearns J.C., Lynch M.D., Senadheera D.B., Tenenbaum H.C., Goldberg M.B., Cvitkovitch D.G., Croitoru K., Moreno-Hagelsieb G., Neufeld J.D., Bacterial biogeography of the human digestive tract. *Sci Rep*., 170, **2012**.

[6] Marsh P.D., Dental plaque as a biofilm and some microbial community implications for health and disease, *BMC Oral Health*., 6,(Suppl 1), S14, **2006**.

[7]Shangase S.L., Mohangi G.U., Hassam-Essa S., Wood N.H., The association between periodontitis and systemic health: an overview, *SADJ*., 68, 1,10–12, **2013**.

[8] Parahitiyawa N.B., Jin L.J., Leung W.K., Yam W.C., Samaranayake L.P., Microbiology of odontogenic bacteremia: beyond endocarditis, *Clin Microbiol Rev*., 22, 1, 46–64, **2009**.

[9]Li X., Kolltveit K.M., Tronstad L., Olsen I., Systemic diseases caused by oral infection, *Clin Microbiol Rev*., 13, 4, 547–558, **2000**.

[10] Galimanas V., Hall M.W., Singh N., Lynch M.D.J., Goldberg M., Tenenbaum H., D.G., J.D., Dilani Senadheera B. Bacterial community composition of chronic periodontitis and novel oral sampling sites for detecting disease indicators**,** *Microbiome,* 2, 32, **2014**.

[11] Antipa C., Dascalu L., Chifiriuc M.C., Lazar V., Bleotu C., Ruța S.M., Isolation, identification and antibiotic susceptibility profiles in bacterial strains isolated from periodontal lesions, Annals *of Biological Research*, 5, 3, 22-26, **2014**.

[12]Moore W. E. C., Moore L. V. H., The bacteria of periodontal disease. *Periodontol.*, 1994, 5, 66–77, **2000**.

[13] O'Toole G., Kaplan H. B., Kolter R., Biofilm formation as microbial development, *Annu. Rev. Microbiol*., 54,49–79, **2000**.

[14] Guthmiller J.M. and Novak K.F, Chapter 8, Periodontal Diseases in Polymicrobial Diseases, Brogden K.A., Guthmiller J.M., editors. Washington (DC), ASM Press, **2002**.

[15] Christensen G.D., Simpson W.A., Younger J.J, Baddour L.M., Barrett F.F., Melton D.M., Beachey E.H., Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices, *J. Clin. Microbiol*, 22, 996–1006, **1985**.

[16] Israil A.M., Delcaru C., Chifiriuc M.C., Impact of different parameters upon the expression of certain virulence factors of nonhalophilic and halophilic Vibrio strains, *Romanian Biotechnological Letters,* 14, 4, 4545-4559, **2009**.

[17] Kato S., Kowashi Y., and Demuth D.R., Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin, *Microb Pathog*.,32, 1-13, **2002**.

[18] Antipa C., Popa M., Măruțescu L., Bleotu C., Lazar V., Bertesteanu S., Grigore R., Bezirtzoglou E., Ruța S.M., Virulence Profiles of Bacterial Strains Isolated From Periodontal Lesions, *Romanian Biotechnological Letters,* 20,4, 10662- 10669, **2015**.

[19] Cristea A.D., Măruțescu L., Chifiriuc M.C., Lazăr V., Suciu I., Iliescu A., Chirilă M.,Virulence profiles of microbial strains isolated from patients with chronic apical lesions*, Proc. Rom. Acad*., Series B, 16,1, 25–31, **2014**.

[20] Marsh PD, Bradshaw DJ Dental plaque as a biofilm. *J Ind Microbiol*. 15, 3, 169-75, **1995**.

[21] Lazar V., Chifiriuc M.C., Bucur M., Burlibaşa M., Sfeatcu R., Stanciu G., Savu B., Traistaru T., Cernat R., Suciu I., Investigation of dental-plaque formers biofilms by optic and confocal laser scanning microscopy and microbiological tools. *Rev Med ChirSoc Med Nat Iasi,* 112, 3, 812-20, **2008**.

[22] Hynes W.L. and Walton S.L., Hyaluronidases of Gram-positive bacteria, *FEMS Microbiology Letters,*183, 2, 201–207, **2000**.