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Relative homogeneity of oral bacterial flora in Crohn's disease compared to ulcerative colitis and its connections with antioxidant defense — preliminary report

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Abstract: Introduction: Interactions between oral microbiota and systemic diseases have been suggested. We aimed to examine the composition of oral microbiota with reference to antioxidative defense and its correlation with clinical state in Crohn's disease (CD) in comparison to ulcerative colitis (UC). Materials and Methods: Smears were taken from the buccal and tongue mucosa of patients with CD, UC and controls, and cultured with classical microbiology methods. Bacterial colonies were identified using matrix-assisted laser desorption/ionization (MALDI) with a time-of-flight analyzer (TOF). Blood morphology and C-reactive protein (CRP) were analyzed in the hospital laboratory. Antioxidative defense potential (FRAP) was determined using spectrophotometry in saliva and serum.

R e s u l t s: Oral microbiota in CD patients were characterized by lower diversity in terms of the isolated bacteria species compared to UC and this correlated with reduced FRAP in the oral cavity and intensified systemic inflammation. Oral microbiota composition in CD did not depend on the applied treatment. In CD patients, a negative correlation was observed between the FRAP value in saliva and serum and the CRP value in serum. Individual differences in the composition of oral microbiota suggest that different bacteria species may be involved in the induction of oxidative stress associated with a weakening of antioxidative defense in the oral cavity, manifested by ongoing systemic inflammation.
C o n c l u s i o n s: Analysis of both the state of the microbiota and antioxidative defense of the oral cavity, as well as their referencing to systemic inflammation may potentially prove helpful in routine diagnostic applications and in aiding a better understanding of CD and UC pathogenesis associated with oral microbiota.

Key words: Crohn's disease, ulcerative colitis, oral microbiota, antioxidant.

Introduction

Current developments in technology leading to research on the human microbiome indicate that a healthy oral cavity is colonized by bacteria, which thus far have been seen as etiological factors of individual local diseases, such as periodontitis, halitosis or dental caries [1, 2]. Presently, the idea of only selected, single bacteria species being etiological factors of diseases is being abandoned for the theory of ecology, explaining the role of associated microbiota in the disturbance of the microbiological equilibrium and the formation of individual pathological states. The role of interactions between the external environment and microorganisms and the immunologically susceptible host is emphasized, thanks to which interspecific shifts and dysbiosis occur, leading to the development of numerous diseases, such as non-specific enteritis, including Crohn's disease (CD) and ulcerative colitis (UC) [3, 4].

An altered microbiota of the oral cavity has been observed in CD and other systemic diseases, such as diabetes, bacteremia, endocarditis, cancers or autoimmune diseases [5–8]. Thus, it is important to find a relationship between oral bacteria diversity and other oral biomarkers and their impact on the pathophysiology of various diseases. Information obtained in this manner through the assessment of relationships with the remaining systemic biomarkers may be used to develop new algorithms. The use of biomarkers obtained using low invasiveness methods based on widely accessible material, that is saliva or oral cavity smears, may be applicable in early diagnostics or monitoring of the state of these diseases.

Based on our own study [9, 10], the production of reactive oxygen species (ROS) in patients with CD strictly depends on the inflammatory process in the organism and it may contribute to disturbance of the oxidative—antioxidative balance.

Disturbances in ROS levels within the oral cavity may be the result of changes occurring under impact from oral bacterial flora. This is caused when microorganisms

capable of producing a disease are absorbed by immunity cells of the host such as neutrophils, macrophages and fibroblasts, thus forming ROS and reactive nitrogen species (RNS).

Excessive ROS and RNS, without efficient repair systems, results in structural and functional changes to proteins, lipids and nucleic acids. Byproducts of these reactions penetrate into the blood, urea or saliva, and thus some may be used as disease predictors, reflecting intensification of the ongoing inflammation or dynamically developing oxidative stress (OxS) [11, 12]. Unlike other diseases no detailed studies into CD have published results of OxS among bacteria of the oral cavity microbiota in adults [13–15].

In response to elevated bacterial invasion, the immune system of the host commences elimination of microbes through increased OxS and elevated salivary antioxidant production. The role of antioxidants in health and disease and the mechanisms associated with inclusion/exclusion of antioxidants is not fully understood. Interactions between microorganisms and the patient and the resulting action of the antioxidative systems of the host depend on numerous factors, including the predominant bacteria species, and the antioxidative systems may act jointly or independently of the antioxidative systems of the host. At the same time, the role of extrinsic factors, such as diet, and nutritional and hygienic behavior, is also important.

Immune system cells, such as neutrophils, macrophages or fibroblasts found in the oral cavity, are capable of active production ROS or RNS to combat microorganisms and microorganisms may influence the efficiency of the antioxidative systems of the host. As an example, certain bacteria of the oral cavity, such as Enterococci, are capable of endogenous ROS production, which contributes to the growth of OxS markers in the saliva and makes the interpretation of salivary antioxidant activity more difficult than would be the case with sole measurement of salivary antioxidant indicators. These data indicate the importance of the assessment of the relationship between the microbiome and the immune system of the host. Worthy of determination is also the relationship between the variability of the indicators of OxS over time and the oral microbiota composition in patients with CD in comparison to UC. Biological rhythms associated with food consumed and their variation over time may impact upon the induction of OxS and nitrosative stress as a symptom of unstable signaling between the microbiome and the host and the result of oral microbiota composition variability. This is proven by the fact that the levels of oxidative stress indicators increase after a meal, which stimulates growth of certain pathogenic bacteria and increased metabolism (e.g., *S. mutans*), disturbing the proportions in the oral microbiome. Thus, the hypothesis we sought to verify concerns variability over time of OxS levels in saliva and its association with the composition of the oral microbiota, which will stimulate ROS/RNS production.

The presented study demonstrates the composition of the oral microbiota with reference to antioxidative defense elements and their correlation with clinical state, as well as systemic inflammation in CD in comparison to UC.

Materials and Methods

Study population

Consecutive adult patients with CD and UC were enrolled. The study population included 18 subjects with CD (age 26–64 years) and 13 with UC (age 26–67). CD and UC were diagnosed based on classic, endoscopic, histological and radiological criteria [16, 17]. The patients were recruited from the Division of Gastroenterology and Hepatology at the University Hospital in Kraków, Poland. The controls were represented by 5 healthy volunteers recruited from the hospital staff. The exclusion criteria were the following: any known systemic infection or disease, pregnancy or lactation, alcoholism, tobacco smoking, use of antibiotics, antioxidants (e.g., vitamins C, E) or anti-inflammatory drugs within the last 6 months, periodontal disease, presence of an oral mucosal inflammatory condition (e.g., aphthous, lichen planus, leukoplakia), removable orthodontic appliances, and symptoms of acute infectious illness (e.g., fever, sore throat).

The study was performed in accordance with the ethical principles of the Helsinki Declaration of 2008. Informed consent to the study procedure was obtained from all participants. The protocol was approved by the Bioethics Committee of Jagiellonian University in Kraków, Poland (KBET/200/B/2014). All individuals provided written informed consent to participate in the study.

Clinical assessment

Clinical assessment included the presence of comorbidities, cigarette-smoking habits, and medications. In patients with CD and UC, the following parameters were evaluated: disease duration, disease location, disease activity, complications, and past surgical procedures. Complications were defined as abscesses, fistulae, and stenoses resulting in post-obstructive symptoms. To determine the location of lesions in CD and UC patients, the Montreal classification was employed [18]. To assess CD and UC activity, the CD activity index and UC activity index were used [19, 20].

Saliva sampling

Saliva samples were collected from all participants using a Salivette® Cotton Swab system (Sarstedt AG & Co., Numbrecht, Germany). The fasted subject rinsed their

mouth with tap water for 30 s and expectorated it before saliva collection, placed a cotton swab in their mouth and chewed it for 3 min. to stimulate salivation. Salivettes with swabs saturated with saliva were centrifuged at 1000 rpm for 2 min. at 4°C. Clear saliva samples (about 1 ml) were immediately aliquoted to sterile 0.2 ml micro test tubes, Eppendorf type, and frozen at −80°C until assayed.

Laboratory tests

Fasting blood samples were collected from the antecubital vein in the morning. On the same day, the following laboratory parameters were determined: complete blood count, CRP, and iron and albumin concentrations in serum. Complete blood count was performed with a Sysmex XE-2100 hematology automated analyzer (Sysmex, Kobe, Germany). CRP, iron and albumin were assayed using a Modular P clinical chemistry analyzer (Roche Diagnostics, Mannheim, Germany). Additional blood samples were centrifuged at 3000 × *g* for 10 min at 4°C, and plasma samples were stored at −80°C for determination of FRAP and protein.

Total antioxidant potential (FRAP assay)

FRAP levels in plasma and saliva were measured according to the method of Benzie and Strain [21, 10]. The plasma and saliva samples were mixed with 3 ml reagent mixture containing acetate buffer (pH 3.6), 5 mM tripyridyltriazine in 40 mM HCl, and 20 mM ferric chloride. FRAP values were calculated by preparing an aqueous solution of known FeII ($FeSO_4 \cdot 7H_2O$) concentration in the range of 0–1.0 mM and a blank containing FRAP reagent mixture. Absorbance was assessed at a wavelength of 593 nm (FLUOstar Omega spectrophotometer; BMG Labtech). FRAP values were expressed as mM/mg of protein.

Concentrations of total protein in plasma and saliva were measured using the bicinchonic acid (BCA) method according to the manufacturer's instructions (Sigma-Aldrich, USA), as described previously [10, 15]. The BCA method entails reducing Cu2+ to Cu+, and Cu+ ions react in an alkaline medium with BCA, which gives it a violet color. Absorbance was read at a wavelength of 562 nm at 37°C (FLUOstar Omega spectrophotometer; BMG Labtech), and a calibration curve was plotted and a simple linear regression equation derived. Protein concentrations were expressed in mg/mL [10].

Microbiological methods

Smears from the mucosa of the cheek and the dorsal portion of the tongue were collected with a sterile cotton swab. The samples were transported within 4 hours to

the laboratory in 1 ml sterile physiological saline, pH 7.0 (PBS) at room temperature. The samples were disrupted by gentle vortexing and sonication for 30 s. Subsequently, serial dilutions of the starting solution were prepared in sterile physiological saline. Samples with the above dilutions were inoculated on plates with 10% blood agar and on selective media used to detect certain microorganism groups. The following selective media were subsequently used: CHROMagarTM chromogenic agar supporting growth of Candida and yeasts supporting gr agar for *Enterococci* (BD Enterococcosel Agar), and BD MacConkey II Agar selective medium intended for the Coli group bacteria (CCA); and chromogenic agar for isolation and differentiation of *Enterobacteriaceae*. The inoculated media were incubated in microaerophilic conditions in the presence of 5% CO_2 at a temperature of 37°C over a period of 24–48 hours. Based on their morphology, colonies of yeasts, *Enterococci*, *Enterobacteriaceae* and *Staphylococci* grown on selective media were counted. Distinct microorganism species were isolated and cultured on media dedicated to growth and differentiation of microorganisms, in order to obtain clear isolates for further identification. The preliminary identification was performed based on microscopic examination of preparations stained with the Gram method and on the differences in the results of biochemical identification (API Candida system; API 20 STREP for Streptococci and API Staph, by bioMérieux, France for Staphylococci). *Enterobacteriaceae* spp. cultured on the McConkey agar were identified using an API 20E set (bioMérieux, Lyon, France). Based on the coloration and morphology of yeast colonies on CHROMagarTM *Candida*, the isolates were identified as *Candida albicans*. The biochemical diagnosis was performed according to the manufacturer's instructions.

The bacteria species obtained in the tests were verified by means of mass spectrometry (MS) system MALDI with TOF (Bruker Daltonik, Germany). Prior to the identification, a preliminary extraction was performed (using ethanol and formic acid). Two microliters of extract were placed on a metal plate and left to dry at room temperature. The metal plate with applied samples was placed in the measurement chamber of the MALDI Biotyper apparatus and was subject to the action of a laser beam. Under the influence of desorption and bacterial protein ionization, the ionized peptides were accelerated in a strong electric field and the ion TOF was measured. Based on the obtained distribution of peptides according to the molecular weight, charge and variable time-of-flight, the MALDI-TOF MS system automatically generated spectrometric peak spectra corresponding to ions with variable weight to charge ratios and analyzed the amount, intensity and correlation of peaks, as well as comparing the tested spectrum with reference spectra of microorganisms. The automatic spectrum measurement and its comparative analysis with standard

spectra was performed with an Ultraflextreme mass spectrometer in combination with MALDI Biotyper 3.0 software (Bruker Daltonik, Germany) connected to the microorganism profile database. Based on this software, the spectral protein profile was determined for the selected microorganisms. The likelihood of correct identification in the MALDI Biotyper 3.0 software was expressed in the form of a point indicator: 2,300–3,000 reliable identification of a microorganism to the specific level; 2,000–2,299 reliable identification of a microorganism to the generic level and probable result of identification to the specific level; 1,700–1,999 reliable identification result to the generic level; 0–1,699 unreliable identification result.

Statistical analysis

Statistical analyzes were conducted using R software, version 3.5.2 [22]. Normality was assessed with the Shapiro-Wilk test. Analyzes were conducted at a 0.05 level of significance. Comparisons of qualitative variables in groups were conducted with the chi-squared test (with Yates' correction for 2 × 2 tables) or with Fisher's exact test (when low expected values occurred). Correlations between quantitative variables were assessed with Pearson's (in cases of normal distribution of both variables) or Spearman's (otherwise) correlation coefficients. Strength of association was assessed with the following schema: $|r| \geq 0.9$ — very strong, $0.7 \leq |r| < 0.9$ — strong, $0.5 \leq |r| < 0.7$ — moderate, $0.3 \leq |r| < 0.5$ — weak, $|r| < 0.3$ — very weak [23]. Multivariate analysis of the simultaneous impact of many independent variables on one quantitative dependent variable was conducted by means of linear regression. 95% confidence intervals were reported along with regression parameters. Comparisons of quantitative variables in more than two groups were conducted with ANOVA (in cases of normal distribution in each group) or with the Kruskal-Wallis test (otherwise). Fisher's LSD test (in cases of normal distribution in each group) or Dunn's test (otherwise) were used as post-hoc procedures.

Results

Demographic data and blood test results

Demographic data and results of blood morphology and biochemical tests in patients with CD and UC and in persons from the control group are listed in Table 1. No statistically significant differences were found among the examined parameters (Table 1).

Table 1. The results of blood tests in the studied groups of patients with 'Crohn's disease, patients with ulcerative colitis and healthy persons in the control group.

		Crohn's disease	Ulcerative colitis	Control	p*
Age (years)	Mean ± SD	40.18 ± 12.97	43.55 ± 13.65	55 ± 6.56	0.198
	Median	40	41	54	P
	Quartiles	28–48	33.5–49.5	51.5–58	
Gender	Female	6 (33.33%)	9 (69.23%)	3 (100%)	0.067
	Male	12 (66.67%)	4 (30.77%)	0	F
WBC [10^3/μL]	Mean ± SD	8.25 ± 3.67	9.32 ± 3.9	5.4 ± 1.78	0.27
	Median	7.9	9.44	5.36	P
	Quartiles	4.75–11.21	6.34–10.57	4.5–6.28	
RBC [10^6/μL]	Mean ± SD	4.22 ± 0.62	4.22 ± 0.66	4.83 ± 0.35	0.291
	Mediana	4.03	4.33	4.71	P
	Quartiles	3.91–4.85	3.76–4.76	4.63–4.96	
Hb [dL]	Mean ± SD	12.06 ± 1.7	11.93 ± 1.93	14.1 ± 0.56	0.156
	Median	12.7	11.2	14	P
	Quartiles	10.7–13.1	10.2–13.7	13.8–14.35	
Ht [%]	Mean ± SD	36.92 ± 4.72	36.35 ± 4.57	42.33 ± 1.56	0.133
	Median	37.3	35.7	42.5	P
	Quartiles	33.2–39.4	32.15–40.5	41.6–43.15	
MCV [fL]	Mean ± SD	88.34 ± 7.92	86.76 ± 7.11	87.97 ± 5.95	0.864
	Mediana	89.1	86.3	89.5	P
	Quartiles	84.4–93.2	83.2–88.1	85.45–91.25	
Microcytes [%]	Mean ± SD	4.56 ± 5.54	3.91 ± 2.58	–	0.384
	Median	1.7	3.25	–	NP
	Quartiles	1.03–5.62	1.88–5.95	–	
Macrocytes [%]	Mean ± SD	4.5 ± 2.22	4.73 ± 2.32	–	0.895
	Median	3.9	3.85	–	NP
	Quartiles	3.55–4.35	3.6–4.33	–	
PLT [10^3/μL]	Mean ± SD	342.35 ± 101.09	362.45 ± 120.81	204 ± 78.89	0.063
	Median	336	325	195	NP
	Quartiles	245–401	312–384	162.5–241	
Iron [μmol/l]	Mean ± SD	10.91 ± 7.92	6.7 ± 6.51	–	0.358
	Median	8.02	6.7	–	P
	Quartiles	5.71–12.65	4.4–9.01	–	
Albumin [g/l]	Mean ± SD	37.16 ± 6.18	39.67 ± 1.53	–	0.742
	Median	37	40	–	P
	Quartiles	33.6–42.55	39–40.5	–	
CRP [mg/l]	Mean ± SD	13.02 ± 18.33	9.04 ± 6.89	1 ± 0	0.057
	Median	4.83	6.91	1	NP
	Quartiles	2.84–9.2	4.77–13.72	1–1	

Results are mean ± SD; no statistically significant differences were observed when p >0.05.

Microbiological identification

Presented below are identification results of strains of bacteria isolated from the mucous membrane of the oral cavity of patients with CD and patients with UC, as well as bacteria isolates from the mucosa of the cheeks and tongue collected from persons in control groups (without the above diseases). The identification was carried out using biochemical methods and the MALDI-TOF MS technique. The majority of results obtained from biochemical identification and with the use of the MS technique were consistent. Ultimately, the data were represented as a MALDI-TOF identification result due to the higher probability of identification being consistent with the real identification (Table 2, Fig. 1).

Table 2. Identification of isolated bacterial strains in patients with Crohn's disease, ulcerative colitis and control group based on routine biochemical methods and MALDI-TOF MS technique.

	Crohn's disease (n = 18)	Ulcerative colitis (n = 13)	Control (n = 3)	p*
S. vestibularis	5 (27.78%)	2 (15.38%)	0	0.574 F
S. mitis	0	5 (38.46%)	1 (33.33%)	0.009 F
S. oralis	0	5 (38.46%)	2 (66.67%)	0.002 F
S. salivarius	2 (11.11%)	1 (7.69%)	2 (66.67%)	0.062 F
S. gordoni	2 (11.11%)	2 (15.38%)	0	1 F
S. sorbinus	0	1 (7.69%)	0	0.471 F
S. mutans	0	1 (7.69%)	0	0.471 F
S. downei	1 (5.56%)	0	0	1 F
S. parasanguinis	2 (11.11%)	4 (30.77%)	0	0.238 F
A. odontolyticus	2 (11.11%)	3 (23.08%)	0	0.771 F
A. oris	2 (11.11%)	1 (7.69%)	0	1 F
B. cereus	1 (5.56%)	0	0	1 F
L. fermentum	0	0	2 (66.67%)	0.005 F
N. flavescens/sicca	0	1 (7.69%)	0	0.471 F
R. dentocariosa	4 (22.22%)	8 (61.54%)	0	0.046 F
R. mucilaginosa	6 (33.33%)	8 (61.54%)	2 (66.67%)	0.276 F
A. defectiva	1 (5.56%)	0	0	1 F
C. albicans	1 (5.56%)	0	0	1 F

*chi-square test, F-Fisher exact test (low expected values in the table).

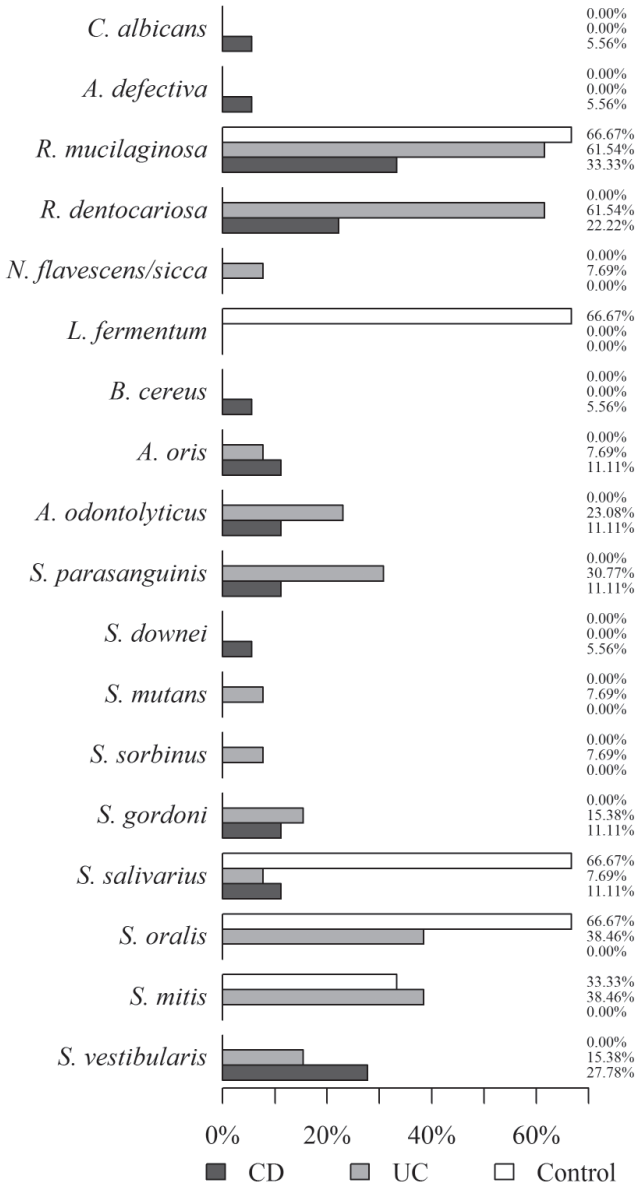

Fig. 1. Identification of isolated bacterial strains in patients with Crohn's disease (CD), ulcerative colitis (UC) and control group based on routine biochemical methods and MALDI-TOF MS technique.

In terms of the isolated species, the microbiota of patients with CD had the lowest diversity (Table 2, Fig. 1). Table 2 presents statistically significant differences between the isolated species of the studied groups. Significant differences between the groups

concerned the following species: *S. mitis* — this species was most common in the UC group and least common in the CD group; *S. oralis* was most common in the control group and the least common in CD. *L. fermentum* was only found in the control group, while *R. dentocariosa* was most common in the UC group and least common in the control group.

Total antioxidative potential (FRAP)

FRAP measured in the serum and in the saliva and the FRAP serum/saliva ratio in patients with CD, UC and in the control group differed statistically significantly between all three parameters ($p < 0.05$). *Post-hoc* analysis demonstrated that FRAP in saliva was significantly higher in the UC group than in the CD group; FRAP in the saliva was significantly higher in the control groups than UC, where it was significantly higher than in the CD group. The ratio of FRAP in serum to FRAP in saliva was significantly lower in UC and control groups than in the CD group. In patients with CD, FRAP in the serum was higher than FRAP in saliva (Table 3, Fig. 2).

Table 3. FRAP in saliva and serum and the ratio between FRAP in serum to FRAP in saliva in patients with Crohn's disease, ulcerative colitis and in the control group. Statistically significant differences between all three parameters ($p < 0.05$).

Parameter		Crohn's disease-A. (n = 18)	Ulcerative colitis-B. (n = 13)	Control-C. (n = 3)	p *
FRAP [mM/g protein] in saliva	Mean ± SD	0.04282 ± 0.00199	0.04556 ± 0.00245	0.04833 ± 0.00103	<0.001
	Median	0.04325	0.0463	0.0486	P
	Quartiles	0.0421–0.04385	0.0442–0.0473	0.0479–0.0489	C. B > A
FRAP [mM/g protein] in serum	Mean ± SD	0.06401 ± 0.00156	0.06607 ± 0.0024	0.06897 ± 0.00119	<0.001
	Median	0.06435	0.0667	0.0686	P
	Quartiles	0.06307–0.0648	0.064–0.0682	0.0683–0.06945	C > B > A
Serum/Saliva FRAP ratio	Mean ± SD	1.49764 ± 0.07977	1.45155 ± 0.03258	1.42702 ± 0.01466	0.007
	Median	1.48224	1.4406	1.42886	NP
	Quartiles	1.46979–1.49819	1.43154–1.47964	1.42019–1.43477	A > B. C

* P = normality of the distribution in groups, ANOVA + results of post-hoc analysis (LSD Fisher test); NP = no normality of distribution in groups, Kruskal-Wallis test and results of *post-hoc* analysis (Dunn's test).

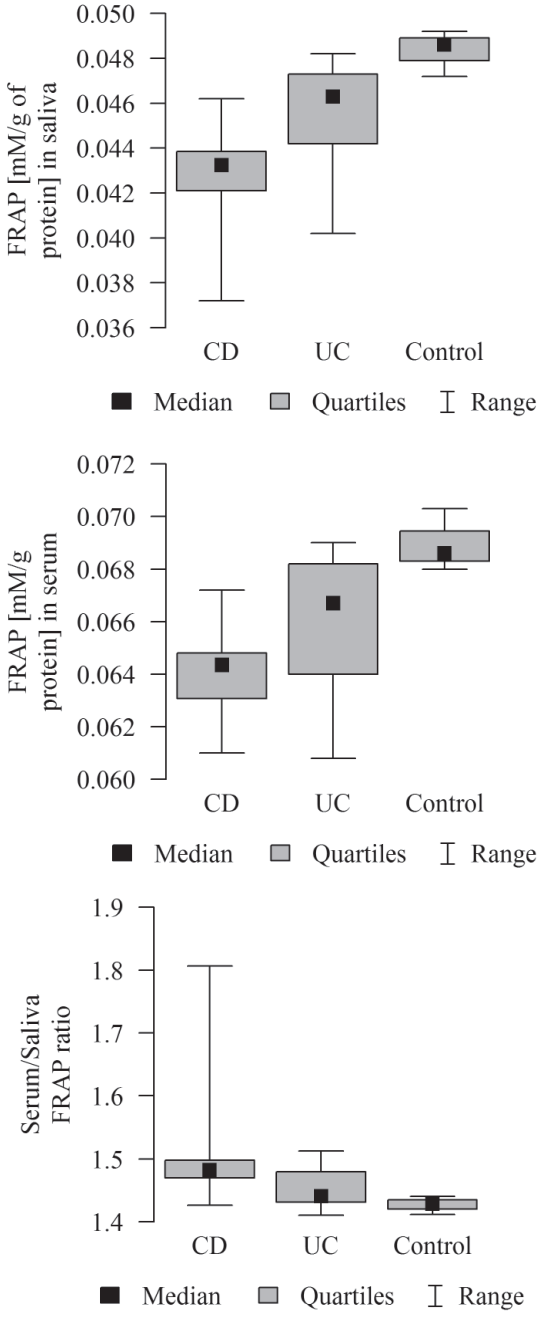

Fig. 2. FRAP in saliva and serum and the ratio between FRAP in serum to FRAP in saliva in patients with Crohn's disease (CD), ulcerative colitis (UC) and in the control group. Statistically significant differences between all three parameters ($p < 0.05$).

Relationship between total antioxidative potential and inflammation

The conducted study has demonstrated that CRP correlates significantly and negatively with the FRAP levels in saliva and serum ($p < 0.05$). Thus, the higher the CRP the lower the FRAP in both serum and saliva. In addition, CRP correlates significantly and positively with the ratio of FRAP in serum to FRAP in saliva ($p < 0.05$); thus, the higher the CRP the higher the ratio. Higher FRAP in serum than in saliva was observed (Table 4, Fig. 3).

Table 4. Relationships between serum CRP concentration and FRAP concentration in saliva and serum ($p < 0.05$) and FRAP ratio in serum / saliva in the studied groups.

Parameter	Correlations with CRP			
	Correlation coefficient	p *	Direction of correlation	Power of dependence
FRAP in saliva	–0.598	p = 0.001 NP	Negative	Medium
FRAP in serum	–0.643	p <0.001 NP	Negative	Medium
Serum/saliva	0.473	p = 0.011 NP	Positive	Weak

* P = normal distribution of both correlated variables, Pearson's correlation coefficient; NP = No normality of distribution of at least one of the correlated variables, Spearman's correlation coefficient.

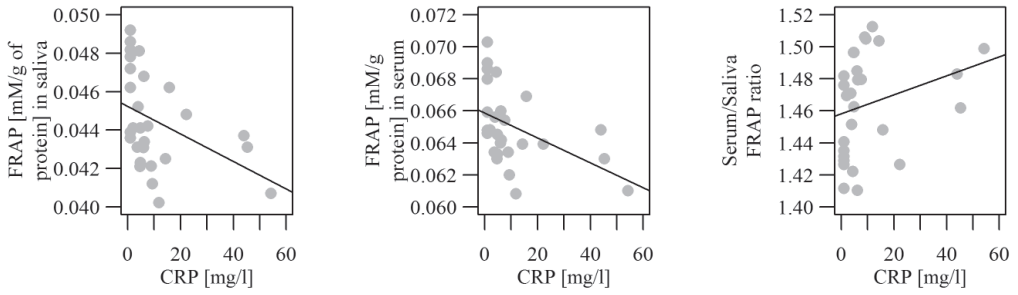

Fig. 3. Graphical presentations of relationships between CRP concentration in serum and FRAP concentrations in saliva and serum ($p < 0.05$) and FRAP ratio in serum/saliva in the studied groups.

Discussion

Modern research on the microbiome has produced evidence that oral cavity diseases have a multifactorial basis [24, 25]. However, evidence for the occurrence of predominant microorganism species in CD is fragmentary and this frequently leads to inconsistent results [26, 27] and also relates the obtained results to healthy persons who remain outside of the hospital environment. The purpose of the conducted

study was to identify the bacteria colonizing mucous membranes of patients with CD in comparison with persons with UC on the gastroenterology ward of the University Hospital in Kraków. An assessment of the diversity of the oral cavity flora in selected patient groups was performed, and the occurrence of relationships between antioxidative defense in the oral cavity and on the periphery and generalized inflammation was analyzed in selected patient groups.

Reduced antioxidative defense in the saliva of patients with CD was associated with the relatively lowest diversity of bacterial flora of the oral cavity and the strongest exhibition of inflammation in the periphery. On the other hand, the slightly higher microbe species richness of the flora in patients with UC (*Streptococcus salivarius* group, *Viridans* streptococci, *Lactobacillus*, *Actinomyces*, classified as pioneer species) was linked to a higher antioxidative potential (higher FRAP) compared with patients with CD and controls and to lower inflammation intensity (lower CRP than that in patients with CD). Similar results were observed by Docktor *et al.* in a group of children with CD [28]. However, these researchers used healthy persons from outside of the hospital environment as the control relative to patients with CD. The richness and diversity of the bacterial flora of healthy persons outside of a hospital environment is not surprising [28, 29].

Reduced diversity of the bacterial flora of the oral cavity in patients with CD compared to UC may be associated with a higher iron level in the CD group compared to patients with UC. As demonstrated in the observations of Lee *et al.*, iron metabolism is strictly linked to the composition of the intestinal flora of patients with inflammatory bowel disease [30]. This has been proven by the fact that oral administration of iron preparations to UC patients reduces the diversity of bacterial phylotypes and their metabolites in stool in comparison to intravenous iron administration [30, 31].

The factors determining the variability of the bacterial flora composition in patients with CD or UC remain largely unknown; however, a higher share and contribution of OxS or inflammation has been postulated as a modulator of the observed differences [32]. Microorganisms associated with OxS can be numerous, and only prospective research over time in selected patient groups will provide answers to the question as to whether and how the selected oral and intestinal microbiota species depend on oxidative defense, or whether ongoing systemic inflammation is associated with OxS. As an example, Jurczak *et al.* showed that the oxidative-antioxidative balance was disturbed in children with caries due to shifts in oral microbiota composition and, depending on the isolated bacteria species, the antioxidative systems of the host supported or inhibited bacterial antioxidative systems [15]. Thus, the total antioxidative potential is the resultant of the host's antioxidant action and antioxidants of the bacteria colonizing the oral cavity. This phenomenon may be explained by the fact that the bacteria that cause oral cavity diseases, e.g. caries,

present a strict relationship with environmental acidification, as well with OxS, which are processes associated with elevated responses of bacterial enzymatic systems (the NADH-Nox intracellular oxidase system, coded NOx), or with the host's enzymatic systems [33]. The growth of bacterial antioxidative systems, such as NADH oxidase, is accompanied by a decrease in the generation of ROS. Therefore, it constitutes a specific survival form for certain bacteria species, for which the presence of genes for antioxidative enzymes in their genomes may be considered a significant factor of their virulence [34]. Thus far, reactions to OxS have only been studied in some bacteria species; apart from *E. coli*, *Rothia mucilaginosa* also possesses several genes associated with OxS (thioredoxin, mycothione reductase, reductase and oxydoreductase), which are considerably elevated under the impact of oxidative or nitrosative stress and which constitute the regulation factor for the redox state in the oral microbiome-host system [35, 36]. However, no detailed studies have been published on the effects of OxS among bacteria from the oral microbiome of patients with CD.

Both the oral-intestinal microbiota, as well as the cells of the immune system cells of the host may induce ROS production and OxS. Moreover, potential systemic causes or extrinsic factors, i.e. diet, impact the variability of both observed systems, i.e. oxidation-reduction in saliva or changes in the oral microbiome, which should not be omitted in an analysis of the impact of the aforementioned factors on health or disease [37].

In order to assess the relationship between the oral microbiota or the oral/intestinal microbiota and OxS markers or inflammation indicators, further prospective research is planned assessing the impact of daily rhythms or external environmental conditions (such as hospital or non-hospital environments, as well as diet) on changes over time in the oral-intestinal microbiota at the intra-individual level.

It is known that even slight changes over time in oral microbiota composition are significantly related to the given host, in whom the elements of the immune system are widely differentiated and depend on health or disease condition. The vast majority of available studies on the microbiome in humans do not include individual populational differences determining intra-individual variability. It is also known that the oral-intestinal microbiome demonstrates considerable variability throughout an individual's life (for instance, at birth and in the subsequent hours the oral-intestinal microbiome of the child is subject to massive exposure to microorganisms originating from the external environment, e.g. breathing, breast-feeding and contact with parents and medical personnel). On the other hand, the permanent colonization of the oral cavity commences in the postpartum period. When a newborn child is only 24 hours old, pioneering microorganisms are being established which determine the composition of the oral-intestinal microbiome [38, 39]. Thus, all analyzes and conclusions concerning the oral-intestinal microbiota should be treated as a prospective study assessing the dynamics of these changes over time.

Our results are in line with those of Gonze *et al.*, which demonstrated that the microbiota may change between hosts even under the same environmental conditions, which means that intra-individual variability does not have to be associated with changes to the surrounding environmental conditions [40]. As presented in this study, the oral microbiota of a patient from a hospital environment may have a highly varied composition, expressed by the variability of colonization of specific ecological niches by selected microorganism species, and this may even be the case for patients on the same hospital ward, which is apparently associated with the colonization site of selected bacterial strains [41]. Thus, it appears valid to examine the microbiota of a given host with regards to their immune condition and the group of patients, to which the given person is qualified, as well as the changes over time in those persons depending on their exposure to environmental factors. Research has shown that the microbiota characteristic of a given individual host is a combination of the host's rich microbiota guaranteed by i.a. transmission of selected microorganism groups between individual hosts, and also the distribution of microorganisms in given ecological niches occupied by networks of microorganism species associated with the given host at a local level [42].

Moreover, research on the bacterial metagenome from the human microbiome has revealed that the unilateral approach to microbiome produces false results, as collection of single samples of material originating from persons from different environments and analysis of these samples in terms of correlations in the occurrence of selected microorganism species, e.g. *Rothia*, and co-occurrence of OxS in the given host has produced divergent results, i.e. 22.2% of such studies produced negative correlations, 0.1% observed positive correlations, and as many as 77.7% of cases were not linked to any significant correlation [32]. In our study, the analysis of the relationship turned out to be difficult due to the low number of examined persons, although the preliminary assessment favors a positive correlation associated with the occurrence of such species as *R. mucilaginosa* and *R. dentocariosa* with OxS and intensified inflammatory processes in the group of patients with non-specific inflammatory intestinal diseases, i.e. CD and UC.

Intensification of OxS associated with elevated ROS production does not have to be linked to overgrowth of bacterial flora, but it may be delayed over time [43]. Correlation profiles between selected microorganism species from the oral microbiota and OxS indicators and between inflammation markers appear to be unique characteristics of each species [44, 45]. A negative relationship was observed between e.g. *Streptococcus* and *Rothia*, and total antioxidative potential. The lower diversity of species which were predominant and characteristic for CD or UC correlated with OxS and inflammation markers much more frequently than did common species (such as *Viridans streptococci*, *Lactobacillus*, *Actinomyces*). As an example, *S. mitis* and *S. oralis* were among the less common species in the CD group, although they demonstrated

considerable correlations with impaired antioxidative defense (lower FRAP) in the UC and control groups. However, those correlations were not statistically significant in the examined UC and control groups. Although *S. mitis* and *S. oralis* are capable of inhibiting ROS *in vitro*, their activity may be inhibited by other microorganism species from the oral microbiota [46]. Naturally, it is highly likely that other bacteria species may impair the natural functions of *S. mitis* to inhibit ROS production, particularly in samples where *S. mitis* correlate with OxS indicators. Moreover, the oral microbiota is colonized by a range of bacteria species, which demonstrate variable capacity to produce or inhibit ROS, which constitutes their evolutionary adaptation increasing their survival chances in difficult environmental conditions.

Oral microbiota profiles with indicators of lowered antioxidative defense, reflecting intensification of OxS, differed between persons from the hospital environment, which may be explained by various factors. Firstly, the variable metabolic activity of bacteria depending on the environment from which they were originally isolated is not taken into account when we consider species colonizing different regions of the oral mucosa (surface of the tongue, cheeks, palate etc., associated with various functions), which may explain why the intra-individual correlation of determined bacterial models with the antioxidative defense markers is not uniform in separate individuals, even from the same patient group. Moreover, a study of the level of pure microorganism isolates may not fully mimic actual ROS production under the conditions of the oral cavity. Moreover, it has been demonstrated that selected bacteria species occurring in control subjects depend on the immune system of the host in different manners. Thus, only selected microorganisms possess the capacity to produce ROS, which means that the results vary within separate individuals.

Although highly active metabolic bacterial cells and those which retain relationships with the immune system of the host can be determined quantitatively, it appears that it is difficult to determine divergent species or those which live in metabolically inactive form or which attach to already formed ecosystems, changing their potential from being harmless to the host to being more pathogenic, stimulating ROS production under *in vivo* conditions. In our study, the appearance of specified bacteria species correlated positively or negatively with elements of antioxidative defense, and, what is more, with an intensified/lowered inflammatory reaction in the CD, UC and control groups. This suggests that individual differences in compensating mechanisms exist for given patient groups as well as individual differences between individuals within groups. Thus, we cannot provide one universal template according to which selected bacteria species are bound with ROS production and have a synergistic or inhibitory effect with the human antioxidative system in the oral cavity.

Undoubtedly, modern advances of technology such as proteomics and metabolomics have a decisive importance in the determination of inter-individual differences at the level of human microbiome, and they may provide valuable

information on the determination of which microorganisms are associated with OxS locally or induction of inflammation in the periphery, as well as of which bacteria determine the same metabolic function in the bodies of various hosts.

In the presented approach, the observations of the time-dependent dynamics of changes in oral microbiota composition may be of considerable importance.

In our future research we would like to verify the hypothesis concerning the impact of OxS and generalized inflammation on the composition of the oral-intestinal microbiota and the impact of the external conditions on its composition in a hospitalized group of patients with CD and other non-specific intestinal diseases, such as UC, which are our subject of interest.

In summary, assessment of the oral microbiota composition may provide information on the composition of the oral microbiome, which appears to be highly heterogeneous even in similar hospital conditions, i.e. the same hospital ward. Correlation analyses of individual samples from patients may provide ambiguous results. Thus, a study of changes in the dynamics of microbiota composition over time may enable estimation of the relationship of the selected bacteria species with the selected marker of OxS or the ongoing peripheral inflammation within the selected patient groups.

Such considerations must take into account the existence of relationships between bacteria and other bacteria species (synergistic effect; one species supports another one-coexistence) and between bacteria and biochemical markers. In general, analysis of correlation has demonstrated that there are specific bacterial profiles (and not individual bacteria species) which are linked to OxS in humans. Generation of OxS and the functioning of antioxidative defense elements in the oral cavity in humans should be subject to more detailed research, since relationship analyses do not clearly demonstrate that certain microorganism species cause inflammation while others do not.

The results of the present study underline the importance of oral cavity microbiota assessment in patients with CD, particularly in combination with other biochemical markers of the oral cavity or the system, such as CRP. The high costs of the test equipment required for assessment of the oral microbiome in hospitalized patients remain a limitation to the assessment, as in the case of DNA sequencing, which appears to be an adequate tool enabling analysis of a vast number of samples from a given hospital patient and their retrospective assessment over time for the purpose of enhanced diagnostics and monitoring of the state of patients and explaining the phenomena associated with the oral microbiome or hospital hazards associated with infections resistant to treatment.

Conflict of interest

None declared.

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Contribution statement

K.S. and W.K. conceived the idea of the research and designed it. D.O., D.C. and M.C.-G. carried out the literature research and clinical study. P.K., A.K., E.K. and W.K. performed analytical and microbiological examinations. K.S., W.K. and T.M. analyzed the data, K.S. and W.K. wrote the paper and revised the manuscript for final submission.

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