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Observational Study

Abundance of *Enterobacteriaceae* in the colon mucosa in diverticular disease

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Abstract

AIM

To compare gut bacterial diversity and amount of *Enterobacteriaceae* in colonic mucosa between patients with and without diverticular disease (DD).

METHODS

Patients in a stable clinical condition with planned elective colonoscopy were included. Blood samples and colon mucosa biopsies were collected at the

colonoscopy. Study questionnaires including questions about gastrointestinal symptoms were completed by the patients and physicians. DNA from mucosa samples was isolated and the amount of *Enterobacteriaceae* was estimated using PCR assay. Terminal restriction fragment length polymorphism was applied to assess microbial diversity. Diversity was estimated by calculations of richness (number of terminal restriction fragments) and Shannon-Wiener and Simpson's indices.

RESULTS

A total of 51 patients were included, 16 patients with DD [68 (62-76) years] and 35 controls [62 (40-74) years] without any diverticula. Patients with DD had significantly higher levels of *Enterobacteriaceae* than those without DD ($P = 0.043$), and there was an inverse relationship between the amount of *Enterobacteriaceae* and the Simpson's index ($r_s = -0.361$, $P = 0.033$) and the Shannon-Wiener index ($r_s = -0.299$, $P = 0.081$). The Simpson's index ($P = 0.383$), Shannon-Wiener index ($P = 0.401$) or number of restrictions fragments ($P = 0.776$) did not differ between DD and controls. The majority of patients experienced gastrointestinal symptoms, and 22 patients (43.1%) fulfilled the criteria for irritable bowel syndrome, with no difference between the groups ($P = 0.212$). Demography, socioeconomic status, lifestyle habits, inflammatory biomarkers, or symptoms were not related to the amount of *Enterobacteriaceae* or bacterial diversity.

CONCLUSION

Patients with DD had higher amount of *Enterobacteriaceae* in the colon mucosa compared to patients without diverticula.

Key words: Bacterial diversity; Diverticular disease; *Enterobacteriaceae*; Gut microbiota; Irritable bowel syndrome

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Core tip: Colon mucosa biopsies were collected from consecutive patients ($n = 51$) at the time of elective colonoscopy. Patients were grouped into patients with diverticular disease (DD) ($n = 16$) and controls without any diverticula ($n = 35$). The amount of *Enterobacteriaceae* and bacterial diversity were analyzed. Patients with DD had significantly higher levels of *Enterobacteriaceae* than controls ($P = 0.043$). Bacterial diversity did not differ between groups. All but 8 patients exhibited some kind of gastrointestinal symptoms, and 22 patients (43.1%) fulfilled the criteria for irritable bowel syndrome, without difference between groups ($P = 0.212$). Demography, socioeconomic status, lifestyle habits, inflammatory parameters, or gastrointestinal symptoms did not affect the gut microbiota examined.

Linninge C, Roth B, Erlanson-Albertsson C, Molin G, Toth

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INTRODUCTION

Diverticular disease (DD) is a common gastrointestinal disease of unknown etiology. The symptoms of DD are similar with symptoms of irritable bowel syndrome (IBS)^[1], e.g., abdominal pain, bloating and altered bowel habits, and are present in 10%-25% of subjects^[2]. About 1.5%-4% of patients with DD develop diverticulitis at some time during their lives^[3,4]. An acute attack of diverticulitis may lead to chronic symptoms called post-diverticulitis IBS, in analogy with post-infectious IBS observed after an acute attack of gastroenteritis^[5,6]. The hypothesis behind IBS development is that low-grade inflammation and/or altered intestinal gut microbiota in DD may contribute to visceral hypersensitivity and dysmotility with ensuing symptoms^[7,8].

The gut microbiota is discussed as important for the etiology and pathophysiology in a wide range of diseases. Bacterial diversity is higher in lean compared to obese individuals, and in healthy states compared to unhealthy states, and some bacterial groups, e.g., *Enterobacteriaceae*, are associated with over-weight and inflammation^[9-11]. The family *Enterobacteriaceae* is commonly found in the gut ecosystem, where *Escherichia coli* is the most abundant species of the family^[9]. Low bacterial diversity and increased levels of *Enterobacteriaceae/Escherichia coli* have been linked to inflammatory bowel disease (IBD) in humans^[12-14]. The findings of abundance of *Enterobacteriaceae/Escherichia coli* in experimental animal models of intestinal inflammation^[15], and the ability of these bacteria to induce colitis^[16], have strengthened the hypothesis that these bacteria are of importance in the etiology of IBD.

Only a few studies have been performed regarding microbial composition in DD. Recently, lower amounts of *Enterobacteriaceae* were found in the colon mucosa of DD patients compared with healthy controls^[17], whereas higher amounts of *Akkermansia* and no difference in the *Escherichia coli* subgroup were found in feces in another DD cohort^[18].

The primary aim of the present study was to compare the level of the large Gram-negative bacterial family *Enterobacteriaceae* and gut bacterial diversity in colon mucosa between consecutive patients diagnosed with DD and patients with normal endoscopic findings. Secondary aims were to evaluate the influence of demography, socioeconomic status, lifestyle habits, inflammatory parameters and gastrointestinal symptoms on the gut microbiota.

MATERIALS AND METHODS

Study population and study design

All consecutive patients referred to elective colonoscopy at the Department of Endoscopy, Skåne University Hospital, Malmö, were invited to participate in the study. All patients were in a stable clinical condition, and no one suffered from any acute inflammation, such as diverticulitis. The only exclusion criteria were age of ≤ 18 years and inability to understand the Swedish language. The patients were informed in oral and written communications at the arrival to the Department the day of examination. If they agreed to participate, they had to complete a study questionnaire about demography, socioeconomic status, lifestyle habits, family history and medical history, the Visual Analog Scale for Irritable Bowel Syndrome (VAS-IBS), and a nutrition questionnaire to analyze dietary habits. The colonoscopy was performed according to clinical routines. Four different mucosa biopsies were obtained from the mid part of the colon descendens. Samples were stored at -80 °C until the gut microbiota was analyzed by quantitative polymerase chain reaction (qPCR) and terminal restriction fragment length polymorphism (T-RFLP). Blood samples were collected according to clinical routines and analyzed at the Department of Clinical Chemistry. A study protocol was completed by the physician about clinical findings and histopathological diagnoses. The patients were divided into two groups depending on the colonoscopy finding: patients with DD, and patients without any diverticula who served as controls.

Tissue sampling

The patients were examined by colonoscopy according to clinical routines after prior laxation with Laxabon® (potassium chloride and macrogol; BioPhausia, Stockholm, Sweden). At the end of the colonoscopy, when the clinical examination was completed, four different mucosa biopsies were obtained from intact, inter-diverticular mucosa in the mid part of the colon descendens. This location was chosen since the left colon is the region most often affected by diverticula and is more accessible than the right colon. The biopsies were immediately frozen in liquid nitrogen and kept frozen at -80 °C until analysis. Histopathological examination was performed on separate mucosa samples when IBD had to be excluded or verified.

Questionnaires

Study questionnaire: The questionnaire included questions on age, body mass index (BMI), family history, lifestyle habits, educational achievement, occupation, civil status, circumstances concerning delivery and breast-feeding, place of birth and moving patterns, and medical history. The patients had to answer whether they had been diagnosed with celiac disease, IBD, lactose intolerance, reflux or ulcer. They

were asked whether they experienced gastrointestinal symptoms which fulfilled the Rome IV criteria of functional dyspepsia or IBS^[19,20]. This questionnaire was in structure and design similar to questionnaires used by other large current population-based and on-going screening projects in Sweden (*i.e.*, LifeGene, EpiHealth, BIG-3, SCAPIS).

VAS-IBS: The VAS-IBS was used to investigate gastrointestinal complaints in the study groups. VAS-IBS is a validated, self-rating questionnaire for estimation of the most common gastrointestinal complaints experienced during the last 2 wk^[21]. This questionnaire has also been validated for estimation of symptoms over time^[22]. The five items measured in the VAS-IBS address the symptoms of abdominal pain, diarrhea, constipation, bloating and flatulence, and nausea and vomiting. These items were measured on a scale from 0-100, where 0 represented severe problems and 100 represented a complete lack of problems. Whether the patient suffered from symptoms or not, was defined as a score above the median values in healthy subjects^[22].

Food questionnaire: The questionnaire included questions about dietary intake each meal in the form of red meat, fish or vegetables, making it possible to estimate dietary patterns. The number of days per week for intake of juice, coffee/tea, milk, sour milk, muesli, berries and fruit, marmalade, bread, cheese, ham and egg at breakfast, or snack were filled in. The participants were asked whether the lunch and dinner were homemade, or whether the participant had a lunch or dinner at a restaurant or a frozen precooked meal.

Microbial analyses

DNA extraction: Three of the four mucosa samples, mean weight 15 ± 0.6 mg, were used for DNA extraction. DNA was isolated and purified by EZ1 Advanced XL (EZ1 DNA Tissue Kit and Bacteria Card; Qiagen, Hilden, Germany)^[10,23].

qPCR: The amount of *Enterobacteriaceae* was estimated using a qPCR assay according to Karlsson *et al.*^[10]. Primers used for the qPCR assay have been used and published previously^[24,25]. The detection limit was 10^2 genes/reaction. For standard curves, 10-fold dilution series of the target DNA were made in EB buffer (Qiagen). Number of bacteria was expressed as \log_{10} 16S rRNA genes/g feces.

Microbial diversity: T-RFLP was applied to assess the microbial diversity, as previously described^[26]. Thresholds for internal standard and terminal restriction fragments (T-RFs) were set to 5 and 15 fluorescence units, respectively.

Calculations: Microbial diversity was estimated by calculation of richness (number of T-RFs) and Shannon-

Wiener and Simpson's diversity indices as described by Karlsson *et al.*^[10], with the exception that T-RFs within 40-580 base pairs were included in the T-RFLP profile analysis and calculation. The diversity indices take into account both richness and evenness when considering the relative abundance of bacterial groups. Both indices are commonly used to assess microbial diversity^[27]. Samples below the limit of detection (in qPCR) were replaced by the limit of detection for statistical analysis.

Patient categorization

Depending on presence or absence of diverticula, the included patients were divided into patients with DD or control patients. The control patients either exhibited normal macroscopic endoscopic and microscopic histopathological findings or presence of benign polyps. The group categorization was performed independent of gastrointestinal symptoms. All patients with IBD or malignancy were excluded from the study. The diagnosis of IBD was set when the patients fulfilled the criteria for Crohn's disease, ulcerative colitis or microscopic colitis, *i.e.*, clinical and endoscopic findings in addition to inflammation at the histopathological examination, in accordance to the diagnoses criteria^[28].

Statistical analysis

The statistical calculations were performed using the SPSS software, version 24.0 (Armonk, NY, United States). Non-parametric tests were used because of the low number of participants in each group and the skewed distribution of the values of VAS-IBS. Comparisons of continuous variables between groups were performed by either Mann-Whitney *U* test or Kruskal-Wallis test. Fisher's exact test was used for dichotomous variables, and Spearman's correlation test was used for correlations between parameters. Values are presented in median and interquartile ranges (IQRs) or number and percentage. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

In total, 77 patients were invited to participate in the study. Nineteen patients denied to participate and 58 patients were included. Six patients were later excluded since they fulfilled the criteria for IBD, and one because of colon malignancy. Finally, 51 patients were included in the present study, 16 with DD and 35 controls without organic changes visible at the colonoscopy or at the histopathological examination ($n = 12$), except non-malignant polyps ($n = 23$). The reasons for referral to colonoscopy were presence of gastrointestinal symptoms which rendered a colonoscopy to exclude IBD, malignancy or DD ($n = 17$), follow-up after previous resection of polyps ($n = 17$), rectal bleeding ($n = 11$), screening for cancer due to heredity ($n = 4$), or perforation to the urinary tract ($n = 2$). Only one

subject in the DD group had a history of verified acute diverticulitis.

There was an equal sex distribution in the groups. Subjects without DD were slightly older than controls [68 (62-76) years vs 62 (40-74) years, $P = 0.072$], which may explain that more DD patients than controls had completed primary school as the highest education level. Age differences may also explain the lower degree of physical activity in the DD group. A few patients in both groups had been treated with antibiotics during the last 6 mo (Table 1). The moving patterns did not differ between groups. Sporadic cases of heart and lung diseases were found in both groups (data not shown).

Gastrointestinal symptoms

Altogether, 22 patients (43.1%) fulfilled the Rome IV criteria for IBS. The prevalence of functional dyspepsia, IBS, gastric ulcer, lactose intolerance and reflux was equally distributed between groups. Each symptom item estimated by the VAS-IBS questionnaire was present in about half of all patients examined. Only 4 patients in each group did not have any form of gastrointestinal symptoms (Table 2). There was a wide variety in symptom intensity within each group also. None of the items in VAS-IBS correlated with age (data not shown).

Dietary patterns

All the participants who completed the nutrition questionnaire ($n = 42$) started the day with a breakfast, which in the vast majority of cases consisted of coffee or tea, together with bread and/or muesli and milk products. Twenty-seven participants had homemade lunch, whereas ten participants had lunch at a restaurant or had precooked meals, and five participants never had any lunch. Thirty-three participants had dinner at home, whereas eight participants had regular dinner at a restaurant or did not have dinner. Those who had homemade lunch suffered from more gastrointestinal symptoms compared with those who did not eat lunch, had lunch at a restaurant or had precooked meals, although bloating and flatulence was the only item that reached statistical significance [52 (25-93) vs 88 (70-100), $P = 0.024$]. The difference could not be related to any differences in socioeconomic factors or smoking or alcohol habits (data not shown) or in age span [66 (50-76) vs 65 (59-72), $P = 0.851$]. When the patients were divided into three groups depending on lunch habits [(1) home-made lunch; (2) lunch at a restaurant or precooked meals; and (3) no lunch], those who had homemade lunch registered the most severe gastrointestinal symptoms on all the VAS scales, although the differences did not reach statistical significance (data not shown).

Microbiota and inflammatory biomarkers

Patients with DD had significantly higher levels of *Enterobacteriaceae* than patients without diverticula ($P = 0.043$; Table 3). Although patients with DD more

Table 1 Basal characteristics of the subjects *n* (%)

Characteristic	Diverticular disease, <i>n</i> = 16	No diverticula, <i>n</i> = 35	<i>P</i> value
Age in year	68 (62-76)	62 (40-74)	0.072
Sex, male/female	6/10	18/17	0.384
Body mass index in kg/m ²	27 (24-30)	25 (22-27)	0.136
Education			0.001
Primary school	9 (60.0)	7 (20.0)	
Secondary school	1 (6.7)	20 (57.1)	
Higher education	4 (26.7)	6 (17.1)	
Missing	1 (6.7)	2 (5.7)	
Occupation			0.332
Working/studying	4 (25.0)	15 (42.9)	
Retired	9 (56.3)	16 (45.7)	
Sick leave/disability	2 (12.5)	2 (5.7)	
Missing	1 (6.3)	2 (5.7)	
Civil status			0.376
Single/living alone	2 (12.5)	2 (5.7)	
Married/cohabitation	8 (50.0)	22 (62.9)	
Divorced/widowed	5 (31.3)	6 (17.1)	
Missing	1 (6.3)	5 (14.3)	
Physical activity			0.033
Mostly sitting	5 (31.3)	1 (2.9)	
Light activity	6 (37.5)	15 (42.9)	
Moderate but regular activity	3 (18.8)	14 (40.0)	
Regular activity	1 (6.3)	3 (8.6)	
Missing	1 (6.3)	2 (5.7)	
Smoking			0.668
Never smoked	4 (25.0)	13 (37.1)	
Former smokers	7 (43.8)	15 (42.9)	
Current smokers	4 (25.1)	5 (14.3)	
Missing	1 (6.3)	2 (5.7)	
Alcohol intake frequency			0.765
Never	4 (25.0)	4 (11.4)	
Once monthly or less	3 (18.8)	10 (28.6)	
2-4 times a month	3 (18.8)	9 (25.7)	
2-3 times a week	4 (25.0)	8 (22.9)	
≥ 4 times a week	1 (6.3)	2 (5.7)	
Missing	1 (6.3)	2 (5.7)	
Alcohol amount at each intake			0.231
1-2 glasses	7 (43.8)	20 (57.1)	
3-4 glasses	2 (12.5)	7 (20.0)	
≥ 5 glasses	2 (12.5)	1 (2.9)	
Missing	5 (31.3)	6 (17.2)	
Alcohol intake of 6 or more glasses			0.361
Never	9 (56.3)	15 (42.9)	
Once monthly or less	5 (31.3)	11 (31.4)	
Daily or several days a week	1 (6.3)	4 (11.4)	
Missing	1 (6.3)	5 (14.3)	
Antibiotic use last 6 mo	5 (31.3)	5 (14.3)	0.299
Probiotic use	2 (1.3)	2 (6.1)	0.701
Vaginal delivery	15 (93.8)	31 (88.6)	1

Values are presented as median (interquartile ranges), unless otherwise indicated. Differences between groups were calculated by Fisher's exact test or Mann-Whitney *U* test. *P* < 0.05 was considered statistically significant.

often had lower education and less physical activity, the different subgroups of these parameters did not affect the amount of *Enterobacteriaceae*, diversity indices of Shannon-Wiener or Simpson, or the number of T-RFs (*P* = 0.413, *P* = 0.803, *P* = 0.770, and *P* = 0.588, respectively, vs *P* = 0.684, *P* = 0.616, *P* = 0.745, and *P* = 0.316, respectively). There were no differences in any parameters between controls with and without polyps (data not shown).

There was an inverse correlation between the

amount of *Enterobacteriaceae* and Simpson's index (*rs* = -0.361, *P* = 0.033) and a tendency to correlation between *Enterobacteriaceae* and Shannon-Wiener index (*rs* = -0.299, *P* = 0.081). The Shannon-Wiener and Simpson's indices correlated with each other (*rs* = 0.947, *P* < 0.001) and number of T-RFs (*rs* = 0.917, *P* < 0.001 and *rs* = 0.772, *P* < 0.001, respectively).

Several of the patients had humoral inflammatory parameters above or beneath the reference values, i.e., plasma-C-reactive protein (CRP): < 3 mg/L; blood-

Table 2 Degree of symptoms based on Visual Analog Scale for Irritable Bowel

	Diverticular disease, <i>n</i> = 16	No diverticula, <i>n</i> = 35	<i>P</i> value	Symptom level, median	Symptom, <i>n</i> (%)	<i>P</i> value
VAS-IBS, median (IQR)						
Abdominal pain	81 (49-100)	84 (48-100)	0.759	95	21 (60)/9 (56)	1
Diarrhea	96 (61-100)	83 (50-100)	0.404	97	23 (66)/8 (50)	0.506
Constipation	95 (52-100)	98 (54-100)	0.613	91	14 (40)/7 (44)	1
Bloating and flatulence	75 (23-100)	61 (40-100)	0.711	85	22 (63)/9 (56)	0.749
Nausea and vomiting	93 (48-100)	97 (80-100)	0.347	98	17 (49)/9 (56)	0.756
Absence of any GI symptom	4 (25)	4 (11.4)	0.236			
GI comorbidities, <i>n</i> (%)						
Celiac disease	0	0				
Functional dyspepsia	5 (31.3)	8 (22.9)	0.509			
IBS	5 (31.3)	17 (48.6)	0.212			
Gastric ulcer	5 (31.3)	7 (20.0)	0.476			
Lactose intolerance	1 (6.3)	0	0.093			
Reflux	5 (31.3)	9 (25.7)	0.738			

Data are presented as median [interquartile range (IQR)] or number and percentages. Symptom number is the number in each group presenting with symptoms. The level of VAS-IBS used to differentiate between symptoms or not is defined as a score above the median values in healthy subjects (No 22). Mann-Whitney *U* test or Fisher's exact test. *P*-value < 0.05 was considered statistically significant. GI: Gastrointestinal; IBS: Irritable bowel syndrome; VAS-IBS: Visual Analog Scale for Irritable Bowel Syndrome.

Table 3 Mucosal count of Enterobacteriaceae and gut microbiota diversity and humoral inflammatory biomarkers

	Diverticular disease, <i>n</i> = 16	No diverticula, <i>n</i> = 35	<i>P</i> value
Enterobacteriaceae, log ₁₀ 16S rRNA genes/g	9.27 (7.34-10.04)	7.76 (7.13-8.76)	0.043
Shannon-Wiener index	2.02 (1.80-2.36)	2.30 (1.94-2.48)	0.401
Simpson's index	0.80 (0.75-0.86)	0.82 (0.76-0.88)	0.383
T-RF, <i>n</i>	17.0 (11.0-21.0)	17.0 (12.5-22.0)	0.776
P-CRP, mg/L	4.40 (1.38-5.80)	1.70 (0.60-6.00)	0.346
B-leukocytes, 10 ⁹ /L	8.40 (6.38-9.98)	8.10 (5.90-8.85)	0.466
B-thrombocytes, 10 ⁹ /L	289 (219-334)	219 (186-266)	0.149
P-albumin, g/L	36 (34-42)	36 (34-40)	0.819

Gut microbiota was analyzed in feces, and inflammatory biomarkers in blood or plasma. Data are presented as median (interquartile range). Mann-Whitney *U* test. *P*-value < 0.05 was considered statistically significant. B: Blood; P: Plasma; T-RF: Terminal restriction fragments.

leukocytes: $3.5-8.8 \times 10^9/L$; blood-thrombocytes $125-340 \times 10^9/L$; and plasma-albumin: 36-48 g/L. The level of inflammatory biomarkers did not differ between patients with or without DD (Table 3). Neither did presence nor absence of IBS affect the plasma levels of CRP (*P* = 0.194) and albumin (*P* = 0.902), or blood levels of leukocytes (*P* = 0.912) and thrombocytes (*P* = 0.509). There was no correlation between any of the inflammatory biomarkers and the level of *Enterobacteriaceae* or bacterial diversity (data not shown).

Neither the amount of *Enterobacteriaceae* nor the diversity indices correlated with age, BMI, or any items of the VAS-IBS (data not shown). When calculating differences between patients with and without any of the gastrointestinal symptoms, there were no differences in amount of *Enterobacteriaceae* or diversity indices (data not shown). Presence of IBS did not affect the amount of *Enterobacteriaceae* (*P* = 0.867), Shannon-Wiener index (*P* = 0.533), Simpson's index (*P* = 0.478), or number of T-RFs (*P* = 0.828).

There were no differences in the amount of

Enterobacteriaceae or the diversity indices between those who had a regular vs irregular breakfast intake of coffee/tea, dairy products, or cereals. The gut microbiota parameters examined were not influenced by intake of homemade lunch or dinner, smoking and alcohol habits, intake of probiotics and antibiotics, or movement patterns (data not shown).

DISCUSSION

In the present study examining symptomatic patients with elective colonoscopy, patients with DD had higher amount of *Enterobacteriaceae* compared with patients without diverticula, whereas the presence of gastrointestinal symptoms or IBS did not affect the amount of *Enterobacteriaceae*. Patients who had homemade lunch showed more symptoms of bloating and flatulence than those who did not have any lunch or had lunch at a restaurant/precooked meal. None of the studied lifestyle and socioeconomic parameters affected the amount of *Enterobacteriaceae* or bacterial diversity of the gut.

The present result of higher levels of *Enterobacteriaceae* in mucosa of DD is in opposite to the previous result of Barbara *et al.*^[17]. The differences may be explained by the different study design and different composition of the control group. The present study enrolled mainly symptomatic patients examined by colonoscopy to exclude organic diseases or patients with heredity for colon cancer. Barbara *et al.*^[17] used asymptomatic or symptomatic patients enrolled to colonoscopy in a screening program to exclude malignancy or as follow-up after polyp resections. Thus, the control group in Barbara *et al.*^[17] consisted of a smaller cohort ($n = 14$) of asymptomatic subjects, and a lower percentage of symptomatic DD, with sex and age differences between groups. The microbiota composition differed between mucosal biopsies and feces^[17]. We decided not to analyze fecal microbiota in our study, since there are greater differences between fecal and mucosal microbiota than between individual subjects, and it is considered more reliable to measure microbiota composition in mucosa than feces^[29]. The general composition estimated by microbial diversity may be more important to health than the levels of individual bacterial strains^[9,10,14].

Abundance of *Enterobacteriaceae/Escherichia coli* is associated with IBD, both in animal models and in humans^[12,13,15,16]. The gut microbiota generates biologically active small molecules, *e.g.*, amino acids, short-chain fatty acids, sugars and organic acids, which are presumed to affect the health of the host^[30]. Basic microbiome metabolism was altered in IBD, with reduced amino acid synthesis and carbohydrate metabolism and increased nutrient uptake. Furthermore, genes involved in pathogenesis processes such as secretion of enterotoxins, wall-degrading enzymes and cytokine production were over-represented in Crohn's disease^[13]. This would lead to tissue destruction and bacterial overgrowth, with structural and functional dysbiosis.

In the present study of DD, the abundance of *Enterobacteriaceae* in the colon mucosa at a distance from the diverticula could hypothetically reflect a low-grade inflammation in the bowel wall. The previous publication by Barabra *et al.*^[17] suggested chronic low-grade gut mucosa inflammation in DD, through histopathological examination. Such low-grade inflammation was not reflected in the humoral inflammatory system, confirmed by overall normal CRP and blood cells levels, but may be captured in mucosal biopsies^[17,31]. A low-grade inflammation may contribute to pain sensitization and visceral hypersensitivity and symptom development^[7,8], which contributes to the increased risk of IBS after acute diverticulitis^[6].

It remains unclear whether microbial changes are a cause or a consequence of DD. We do not know whether inflammation is a primary event, leading to weakening of the bowel wall and eventually to development of diverticula, or if inflammation is secondary to the presence of DD distant in the bowel with retention of luminal contents and bacterial overgrowth. Even if the

microbial changes are secondary, the dysbiosis may further accelerate the pathologic process and weakening of the bowel wall by mechanisms explained above^[13].

Microbial dysbiosis in combination with genetic, environmental, and psychosocial factors are proposed to be involved in the etiology of IBS^[20,32]. *Escherichia coli* was increased in Chinese IBS patients compared with controls, whereas no differences of these feces bacteria were found between IBS patients and healthy controls from other regions^[33]. This is in line with our present study, which did not show any correlations between gastrointestinal symptoms or IBS and *Enterobacteriaceae*.

Gastrointestinal symptoms without visible organic damages are called functional bowel disorders, where IBS is the most common of the disorders, with a prevalence of 10%-15% in the population^[20]. A great deal of the present patients suffered from IBS or IBS-like symptoms, whereas some patients experienced gastrointestinal symptoms without fulfilling the Rome IV criteria^[20]. Symptomatology is not enough to distinguish between different bowel disorders, as found in the present study. It has previously been shown that patients with IBS have as severe symptoms as those with organic changes, *i.e.*, primary Sjögren's syndrome and enteric dysmotility^[34]. A great symptomatic overlap between DD and IBS is described previously^[1], which further underlines that disease classification must be based on organic criteria and not on symptoms solely. Biomarkers for IBS and DD are lacking, but measurements of markers of dysbiosis, inflammatory cells in mucosa, and metabolomes may be able to distinguish IBS from DD in the future. Probiotic therapy is an efficient treatment of IBS^[35], whereas the evidence of efficiency in treatment of DD is insufficient.

Since this was a cross-sectional study, we do not know the reason for more symptoms being present in the group with homemade food. The reason may depend on patients with more severe symptoms avoiding visiting a restaurant, to have better control over their food intake.

The strength in the present pilot study is that we have analyzed mucosal biopsies instead of feces. The mucosa microbiota composition is anticipated to be more reliable than the feces composition. To compare another patient group with similar degree of symptoms seems more appropriate than to compare DD with healthy, non-symptomatic subjects. Further, we have considered food intake and other lifestyle habits affecting microbiota composition. The weakness is the small cohort size. Furthermore, since the patients were enrolled consecutively, there was no matching between cases and controls of, *e.g.*, age, sex or lifestyle habits. In a larger study, some of the demographic parameters and lifestyle habits could have shown statistically significant influence on the gut microbiota. We chose to initially perform this as a pilot trial with a limited amount of patients, as the methodology is very expensive. Since it now has been shown that there are differences

in DD according to the gut microbiota, it is important to continue with further studies and more extensive analyses. Since this was a cross-sectional study, we do not know whether the microbial alterations are primary in the development of diverticula or just secondary to DD, with retention of luminal content.

In this pilot study, patients with DD had higher amount of *Enterobacteriaceae* in the colon mucosa compared to patients without DD. Assessment of gut microbiota may distinguish DD from other patient groups and may be involved in etiology and pathophysiology of the disease. Gastrointestinal symptomatology seems to not be related to the amount of *Enterobacteriaceae* or to the bacterial diversity.

ARTICLE HIGHLIGHTS

Research background

Diverticular disease (DD) is a common gastrointestinal disease of unknown etiology. The symptoms of DD are similar with symptoms of irritable bowel syndrome (IBS). The gut microbiota is discussed as important for the etiology and pathophysiology in a wide range of diseases. Bacterial diversity is higher in lean compared to obese individuals, and in healthy states compared to unhealthy states, and some bacterial groups, *e.g.*, *Enterobacteriaceae*, are associated with over-weight and inflammation. The family *Enterobacteriaceae* is commonly found in the gut ecosystem, where *Escherichia coli* is the most abundant species of the family. Only a few studies have been performed regarding microbial composition in DD. Recently, lower amounts of *Enterobacteriaceae* were found in the colon mucosa of DD patients compared with healthy controls, whereas higher amounts of *Akkermansia* and no difference in the *Escherichia coli* subgroup were found in feces in another DD cohort. Thus, it is hypothesized that gut microbiota is involved in the etiology and pathophysiology of DD, but the few studies performed so far have shown inconclusive results.

Research motivation

Today, there is no efficient treatment option for DD, neither to prevent disease development nor to reduce the symptoms when the disease has been established, which renders a lot of suffering to the patients. To find out the etiology is crucial to be able to prevent and efficiently treat the disease. New knowledge within this disease field may point out the direction for future research.

Research objectives

The primary aim of the present study was to compare the level of the large Gram-negative bacterial family *Enterobacteriaceae* and gut bacterial diversity in colon mucosa between consecutive patients diagnosed with DD and patients with normal endoscopic findings. Secondary aims were to evaluate the influence of demography, socioeconomic status, lifestyle habits, inflammatory parameters and gastrointestinal symptoms on the gut microbiota. These objectives were possible to realize by the present study design. Further studies according to the same study design, but with larger patient cohorts, are important to perform to confirm the results.

Research methods

All consecutive patients referred to elective colonoscopy at the Department of Endoscopy, Skåne University Hospital, Malmö, were invited to participate in the study. If the patients agreed to participate, they had to complete a study questionnaire about demography, socioeconomic status, lifestyle habits, family history and medical history, the Visual Analog Scale for Irritable Bowel Syndrome (VAS-IBS), and a nutrition questionnaire to analyze dietary habits. The colonoscopy was performed according to clinical routines. Four different mucosa biopsies were obtained from the mid part of the colon descendens. Samples were stored at -80 °C until the gut microbiota was analyzed by quantitative polymerase chain reaction (qPCR) and terminal restriction fragment length polymorphism (T-RFLP). Blood samples were collected according

to clinical routines and analyzed at the Department of Clinical Chemistry. A study protocol was completed by the physician about clinical findings and histopathological diagnoses. The patients were divided into two groups depending on the colonoscopy finding: patients with DD, and patients without any diverticula who served as controls. Three of the four mucosa samples, mean weight 15 ± 0.6 mg, were used for DNA extraction. DNA was isolated and purified by EZ1 Advanced XL (EZ1 DNA Tissue Kit and Bacteria Card; Qiagen, Hilden, Germany). The amount of *Enterobacteriaceae* was estimated using a qPCR assay according to Karlsson *et al.* Primers used for the qPCR assay have been used and published previously. The detection limit was 10^2 genes/reaction. For standard curves, 10-fold dilution series of the target DNA were made in EB buffer (Qiagen). Number of bacteria was expressed as \log_{10} 16S rRNA genes/g feces. T-RFLP was applied to assess the microbial diversity, as previously described. Thresholds for internal standard and terminal restriction fragments (T-RFs) were set to 5 and 15 fluorescence units, respectively. Microbial diversity was estimated by calculation of richness (number of T-RFs) and Shannon-Wiener and Simpson's diversity indices as described by Karlsson *et al.*, with the exception that T-RFs within 40-580 base pairs were included in the T-RFLP profile analysis and calculation. The diversity indices take into account both richness and evenness when considering the relative abundance of bacterial groups. Both indices are commonly used to assess microbial diversity. Samples below the limit of detection (in qPCR) were replaced by the limit of detection for statistical analysis.

Research results

Finally, 51 patients were included in the present study, 16 with DD and 35 controls without organic changes visible at the colonoscopy or at the histopathological examination ($n = 12$), except non-malignant polyps ($n = 23$). The reasons for referral to colonoscopy were presence of gastrointestinal symptoms which rendered a colonoscopy to exclude inflammatory bowel disease, malignancy or DD ($n = 17$), follow-up after previous resection of polyps ($n = 17$), rectal bleeding ($n = 11$), screening for cancer due to heredity ($n = 4$), or perforation to the urinary tract ($n = 2$). Only one subject in the DD group had a history of verified acute diverticulitis. There was an equal sex distribution in the groups. Subjects without DD were slightly older than controls [68 (62-76) years vs 62 (40-74) years, $P = 0.072$]. Altogether, 22 patients (43.1%) fulfilled the Rome IV criteria for IBS. The prevalence of functional dyspepsia, IBS, gastric ulcer, lactose intolerance and reflux was equally distributed between groups. Each symptom item estimated by the VAS-IBS questionnaire was present in about half of all patients examined. Only 4 patients in each group did not have any form of gastrointestinal symptoms. There was a wide variety in symptom intensity within each group also. None of the items in VAS-IBS correlated with age. Those who had homemade lunch suffered from more gastrointestinal symptoms compared with those who did not eat lunch, had lunch at a restaurant or had precooked meals, although bloating and flatulence was the only item that reached statistical significance [52 (25-93) vs 88 (70-100), $P = 0.024$]. The difference could not be related to any differences in socioeconomic factors or smoking or alcohol habits or in age span [66 (50-76) vs 65 (59-72), $P = 0.851$]. Patients with DD had significantly higher levels of *Enterobacteriaceae* than patients without diverticula ($P = 0.043$). Although patients with DD more often had lower education and less physical activity, the different subgroups of these parameters did not affect the amount of *Enterobacteriaceae*, diversity indices of Shannon-Wiener or Simpson, or the number of T-RFs ($P = 0.413$, $P = 0.803$, $P = 0.770$, and $P = 0.588$, respectively, vs $P = 0.684$, $P = 0.616$, $P = 0.745$, and $P = 0.316$, respectively). There were no differences in any parameters between controls with and without polyps. There was an inverse correlation between the amount of *Enterobacteriaceae* and Simpson's index ($r_s = -0.361$, $P = 0.033$) and a tendency to correlation between *Enterobacteriaceae* and Shannon-Wiener index ($r_s = -0.299$, $P = 0.081$). The Shannon-Wiener and Simpson's indices correlated with each other ($r_s = 0.947$, $P < 0.001$) and number of T-RFs ($r_s = 0.917$, $P < 0.001$ and $r_s = 0.772$, $P < 0.001$, respectively). Several of the patients had humoral inflammatory parameters above or beneath the reference values, *i.e.* plasma-C-reactive protein (CRP): < 3 mg/L; blood-leucocytes: $3.5-8.8 \times 10^9/L$; blood-thrombocytes $125-340 \times 10^9/L$; and plasma-albumin: 36-48 g/L. The level of inflammatory biomarkers did not differ between patients with or without DD. Neither did presence nor absence of IBS affect the plasma levels of CRP ($P = 0.194$) and albumin ($P = 0.902$), or blood levels of leukocytes ($P = 0.912$) and thrombocytes ($P = 0.509$). There was no correlation between any of the inflammatory biomarkers and the level of *Enterobacteriaceae* or bacterial

diversity. Neither the amount of *Enterobacteriaceae* nor the diversity indices correlated with age, BMI, or any items of the VAS-IBS. When calculating differences between patients with and without any of the gastrointestinal symptoms, there were no differences in amount of *Enterobacteriaceae* or diversity indices (data not shown). Presence of IBS did not affect the amount of *Enterobacteriaceae* ($P = 0.867$), Shannon-Wiener index ($P = 0.533$), Simpson's index ($P = 0.478$), or number of T-RFs ($P = 0.828$). There were no differences in the amount of *Enterobacteriaceae* or the diversity indices between those who had a regular vs irregular breakfast intake of coffee/tea, dairy products, or cereals. The gut microbiota parameters examined were not influenced by intake of homemade lunch or dinner, smoking and alcohol habits, intake of probiotics and antibiotics, or movement patterns. The problems that remain to be solved are whether the difference in gut microbiota composition are primary events in the disease development or secondary to the DD. The causality to DD must still be defined.

Research conclusions

The new finding of the present study is the abundance of *Enterobacteriaceae* in colon mucosa in DD, and that this abundance was not related to age, BMI, socioeconomic parameters, gastrointestinal symptoms or lifestyle habits. Microbial diversity was not affected by DD or any other parameters measured. The new theory that this study proposes is that the composition of gut microbiota is involved in DD. The summarization of this study is that gut microbiota may be affected in patients with DD. This study is the first study where a clinical cohort of patients is consecutively enrolled during colonoscopy to analyze gut microbiota in colon mucosa, where the only difference between the groups compared is the presence or absence of colon diverticula. Previous studies have enrolled participants in screening programs or analyzed microbiota composition in feces. The authors also studied socioeconomic features and lifestyle habits in the cohort, to be able to adjust for confounders. The new hypotheses proposed are that gut microbiota is involved in DD and that demography, socioeconomic parameters and dietary habits may be of less importance for the microbiota than the presence or absence of colon diverticula. The new methods proposed are the enrolment of consecutive clinical patients in scientific trials, analyses of gut microbiota in mucosa instead of feces, analysis of microbial diversity to get a general reflection of the gut microbiota, analysis of the amount of *Enterobacteriaceae* or other bacteria by qPCR, and estimation of gastrointestinal symptoms by the VAS-IBS questionnaire. The new phenomenon found were that presence or absence of colon diverticula are more important for gut microbiota than demography, socioeconomic parameters, gastrointestinal symptoms, or lifestyle habits. Another new phenomenon was that patients with homemade lunch had more gastrointestinal symptoms than patients who did not eat lunch or had lunch at a restaurant. The authors confirmed the hypothesis that the amount of *Enterobacteriaceae* was affected by DD, but failed to confirm the hypothesis that overall bacterial diversity was influenced by colon diverticula. The authors also failed to confirm the hypotheses that demography, socioeconomic parameters, gastrointestinal symptoms and lifestyle habits were associated with gut microbiota composition. The major implication for clinical practice in the future is to consider dysbiosis in patients with DD. Tests to determine gut microbiota are available for clinical use, and should be considered in the management of these patients.

Research perspectives

The experience the authors have learnt from this study is that presence or absence of DD is more important for the gut microbiota composition than demography, socioeconomic parameters, gastrointestinal symptoms, and lifestyle habits. The authors have also learnt from this study that homemade food is not always the best for patients in the management of gastrointestinal symptoms. The authors must further study the importance of gut microbiota in DD. The authors should continue to include patients with DD in experiments to analyze gut microbiota composition to get larger cohorts, and to perform clinical trials to evaluate the effect of probiotics in symptom management of DD. The best method is to analyze gut microbiota in colon mucosa instead of feces. The VAS-IBS is also a useful tool to estimate gastrointestinal symptoms.

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