Introduction

Wheat, a valuable source of essential nutrients, is the most consumed grain product in the world and is widely used in the food industry. It provides a person with carbohydrates, fibre, protein, B vitamins, calcium, magnesium, phosphorus, potassium, zinc, and iron [1]. However, the consumption of cereals and their structural components is associated with some pathological conditions. Provoking components can be protein and non-protein components of cereals: gliadins, glutenins, fermented oligo-, di-, mono-saccharides, polysols, and plant protection factors. These abnormal reactions of the organism to ordinary food were recently grouped as “gluten-related diseases” (GRDs).

Gluten-related disorders

The term “gluten intolerance” includes three currently studied pathological conditions associated with food intolerance to cereals. These are celiac disease (CD), wheat allergy, and non-celiac gluten sensitivity (NCGS) [2, 3]. Gluten allergy is a classic demonstration of food allergy, which occurs due to wheat (not gluten) consumption, leading to the formation of hypersensitivity. IgE immunoglobulins play a crucial role in the development of gluten allergy [4]. The CD is a lifelong autoimmune disease in genetically predisposed individuals, which occurs with damage to the small intestine mucous membrane (T-cell-mediated enteropathy) in the background of gluten consumption (gluten is a collective

Features of the intestinal microbiome in patients with gluten-sensitive diseases who are on a gluten-free diet

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Abstract. Background. Intestinal dysbiosis is associated with violating both quantitative and qualitative intestinal microbiome (IM) composition. It accompanies different gastrointestinal disorders, including non-celiac gluten sensitivity (NCGS) and celiac disease (CD). A gluten-free diet is the only existing treatment for CD and NCGS. One of the questions of interest is the characteristics of the IM of patients with gluten-related diseases and its relationship with diet therapy. Materials and methods. The study included 25 adults, 14 (56%) with CD and 11 (44%) with NCGS; all were on an agladian diet. We determined the faecal content of the Bacteroidetes, Firmicutes, and Actinobacteria and the rest bacterial DNA (“other” indicator) using the real-time polymerase chain reaction. Results. The Bacteroidetes content was 28.4 (Q1-Q3 9.195–37.83) % in CD and 24.98 (Q1-Q3 9.615–30.597) % in NCGS patients, p = 0.565. Firmicutes content was 53.47 (Q1-Q3 49.98–56.798) % in CD patients and 53.0 (Q1-Q3 48.12–68.53) % in NCGS, p = 0.763. The Actinobacteria content was 4.8 (Q1-Q3 3.82–6.84) % in CD patients and 5.37 (Q1-Q3 4.09–7.77) % in NCGS, p = 0.572. There was a moderate positive correlation with the diet duration (p = 0.397, p = 0.03). The Firmicutes/Bacteroidetes ratio was 1.996 (Q1-Q3 1.35–6.85) in CD patients and 2.0 (Q1-Q3 1.65–7.58) in NCGS patients, p = 0.681. The content of “other” types of IM was 15.39 (Q1-Q3 7.44–22.33) % in CD patients and 13.78 (Q1-Q3 10.65–15.58) % in NCGS patients, p = 0.936. Conclusions. We found similar intestinal microbiome features in patients with celiac disease and non-celiac gluten sensitivity. The detected intestinal microbiome changes are most likely to be a consequence of dietary features in such patients, namely the side effect of maintaining an agladian diet.

Keywords: celiac disease; gluten-free diet; gluten intolerance without celiac disease; microbiome...
name of proteins contained in cereals — wheat, rye, barley) [3]. NCGS is a syndrome characterised by intestinal and extraintestinal symptoms associated with consuming cereal-based foods in people who do not have celiac disease and/or gluten allergy [5]. This means that NCGS is a diagnosis of exclusion. It is important to note that NCGS does not cause intestinal damage or sensitisation to wheat proteins, as it happens with celiac disease and gluten allergy, respectively.

**Epidemiology of gluten-related disorders**

The latest data shows that incidence is 1.1–1.7 % worldwide [6]. A wheat allergy occurs with a frequency of 0.2–1 % [7]. Due to the lack of diagnostic markers and population studies, the prevalence of NCGS studied insufficiently. The estimated prevalence of NCGS varies from 1 to 13 % of the population [8].

In Ukraine, epidemiologic studies on the prevalence of CD were not conducted, and the prevalence of CD and other GRDs was not studied. Before the Russian invasion, the Ukrainian Society of Celiac Disease accounted for 1600 persons that followed a gluten-free diet (GFD), including those with CD, NCGS, and wheat allergy.

**Gluten-free diet and microbiota**

GFD is a method of clinical nutrition that involves the limitation of gluten-containing food or its replacement with gluten-free ones. A GFD is only one treatment for people with CD [9].

A microbiome is a set of microorganisms that live on the surface and inside the human. Gastrointestinal (GI) bacteria are crucial for human health [10]. Intestinal dysbiosis accompanies many gastrointestinal disorders [11]. These include functional digestive disorders, organic diseases, and diseases associated with sensitivity to various foods, namely CD [12].

The intestinal microbiome (IM) features of patients with GRDs and their connection with the applied diet therapy are being actively investigated. In GRDs, a large number and variety of functional intestinal commensals decrease, and several pathobionts increase. Thus, the scientific data suggest CD is associated with intestinal dysbiosis with possible pathological potential [13].

The purpose was to investigate the features of the intestinal microbiome in patients with gluten-sensitive diseases who are on a gluten-free diet.

**Materials and methods**

The study included twenty-five people with gluten intolerance, of which 14 (56 %) had CD and 11 (44 %) had NCGS. The mean age of patients with CD was (M ± SD) (44.21 ± 15.01) years, including 12 (85.7 %) women and 2 (14.3 %) men. The mean age of patients with NCGS, including six women and four men, was (38.90 ± 11.63) years. The patients with CD and NCGS groups were comparable in age (p = 0.345) and sex (p = 0.192).

All patients did not take probiotics or antibiotics within six months before the study began.

The faecal collection took place within two weeks. Patients at home collected faecal analysis in disposable plastic containers delivered to the freezer as soon as possible. Samples were stored at −23 °C and delivered to the laboratory immediately after the collection stage.

In the process of research, we determined the content of the following types of bacteria in the faeces: Bacteroidetes, Firmicutes, Actinobacteria, other representatives of the intestinal microbiome (indicator “other,” which reflects the total percentage of all bacterial deoxyribose nucleic acid (DNA), except for the above), and calculated the Firmicutes/Bacteroidetes (F/B) ratio. The Diagen Genetic Laboratory, Kyiv, Ukraine, determined their content by real-time PCR. DNA was extracted from 1.5–2 g of aliquots of frozen stool by the phenol-chloroform method according to the protocol [14]. DNA was then eluted with elution buffer 200 µl. The amount and quality of DNA were measured using NanoDrop ND-8000 reagents (Thermo Scientific, USA). Samples with a DNA concentration of less than 20 ng were precipitated with ethanol for concentration or further purified according to quality standards. Bacterial DNA content was measured using a thermal cycler Rotor-Gene 6000 (QIAGEN, Germany) using DNA 16S rRNA primers specific for the respective types of bacteria. They are given in the Table 1.

The procedure for DNA detection by polymerase chain reaction (PCR) is described in more detail in the article [15].

Statistical processing was performed on a personal computer using EZR software v. 1.54 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [16], and, for charting, Office Excel 2016 (Microsoft Corporation, Redmond, Washington, USA). For parametric analysis, we used the two-sample t-test, and for non-parametric analysis, we used the Wilcoxon rank-sum test.

**Table 1 — Primers that were used to determine the content of the main types of intestinal microflora**

<table>
<thead>
<tr>
<th>Type of microorganisms</th>
<th>Direction</th>
<th>The nucleotide sequence (primer)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td>Forward</td>
<td>5'-CRAACAGGATTAGATACCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GTTAAAGGTTCTCCGAT-3'</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>Forward</td>
<td>5'-TCAAATCACAATTGACG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACCATGCAACCTGTC-3'</td>
</tr>
<tr>
<td><strong>Actinobacteria</strong></td>
<td>Forward</td>
<td>5'-TACGGCCGCAAGGCTA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCRTCCACCTCCCTGC-3'</td>
</tr>
<tr>
<td><strong>16S rRNA (“other”)</strong></td>
<td>Forward</td>
<td>5'-AACATCAAAGGATTGAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCCGTCAATTCTGTAGTT-3'</td>
</tr>
</tbody>
</table>
with the faecal content of Bacteroidetes, Firmicutes, their ratio (F/B), and Actinobacteria and did not correlate with the content of “other” types.

The content of Bacteroidetes was 28.4 (Q1-Q3 9.195–37.83) % in patients with CD and 24.98 (Q1-Q3 9.615–30.597) % in patients with NCGS, p = 0.565 (Fig. 1).

The graph shows no difference in this indicator between the groups of patients with gluten sensitivity (p = 0.565).

Thus, the Bacteroidetes content does not differ in patients on GFD.

The Firmicutes content was 53.47 (Q1-Q3 49.98–56.798) % in patients with CD and 53.0 (Q1-Q3 48.12–68.53) % in patients with NCGS, p = 0.763 (Fig. 2).

As shown in Fig. 2, there is no difference between the patients with gluten sensitivity groups in this indicator (p = 0.763).

The Actinobacteria content was 4.8 (Q1-Q3 3.82–6.84) % in CD patients and 5.37 (Q1-Q3 4.09–7.77) % in NCGS patients, p = 0.572 (Fig. 3). There is no difference between the groups of patients in this indicator.

We found a moderate positive correlation between Actinobacteria content and diet duration (p = 0.397, p = 0.03, Fig. 4).

The F/B ratio was 1.996 (Q1-Q3 1.35–6.85) in patients with CD and 2.0 (Q1-Q3 1.65–7.58) in patients with NCGS, p = 0.681 (Fig. 5).

As shown in Fig. 5, there is no difference between the groups of patients in this indicator as well (p = 0.681).

Notes: here and in Fig. 2, 3, 5, 6: bar = median, box = interquartile range, whiskers = values within 1.5 interquartile range from the 1st and 3rd quartiles, circles = outliers.

Results

The medians of the gluten-free diet duration were 9 (Q1-Q3 6–12) years in the CD group and 4 (Q1-Q3 3–5) years in the NCGS group; the difference is not significant (p = 0.087). Diet duration and the patient’s age correlated to analyse nonparametric data, we used the Mann-Whitney U-test. Spearman’s correlation test (two-sided critical region) was used to identify the relationship between the indicators.
The content of “other” types of intestinal microflora was 15.39 (Q1-Q3 10.65–15.58) % in CD patients and 13.78 (Q1-Q3 7.44–22.33) % in NCGS patients, p = 0.936 (Fig. 6).

As shown in Fig. 6, there were no differences (p > 0.9) between groups for this indicator.

In the last step, we run logistic regression to check the possible impact of the microbiome phyla on the diagnosis. The objective variable was the diagnosis, and the explanatory were the microbiome phyla. Running this regression, we did not find any significant influence. When adding the patient’s age and DD, the algorithm showed that only DD, adjusted for age and Firmicutes level, showed significant influence.

Discussion

Our study did not find a difference between the composition of the IM in patients with CD and NCGS. In our opinion, the features of IM in the examined patients indicate that the causal factor of the IM changes is the observance of a GFD.

The logistic regression algorithm showed that only DD, adjusted for age and Firmicutes level, significantly influenced the patient’s diagnosis. This means the patient’s diagnosis cannot be established based on IM indicators (we did not provide OR because this influence cannot be used in actual practice). Thus, overall IM composition in patients with CD and NCGS is not significantly different. We found a relatively small number of studies that compare CD and NCGS patients’ IM, and their findings are slightly controversial [17, 18]. A significant part of studies shows results similar to ours, although researchers in nearly all studies analyse IM on the genus, not phyla, level. For example, J.F. Garcia-Mazcorro et al. (2018) shows that Ruminococcaceae (Firmicutes) content is elevated in NCGS patients compared to healthy people [19], and many other studies showed that Firmicutes content is decreased in CD [17, 18]. In our study, the NCGS group had a slightly higher Firmicutes level than CD group, but the difference was insignificant. In our opinion, the significance was not achieved because the difference in genus content was insufficient to give the difference in phyla level. We think the IM similarity we saw in our groups is due to the influence of the GFD and high inter-individual variability [19].

Conclusions

We observed similar intestinal microbiome features in patients with celiac disease and non-celiac gluten sensitivity.

Intestinal microbiome constituents widely correlate with the time of a gluten-free diet. The detected intestinal microbiome changes are most likely to be a consequence of dietary interventions of such patients, namely the effect of maintaining a gluten-free diet.

References

Резюме. Актуальность. Кишковий дисбіоз пов’язаний із порушенням як кількісного, так і якісного складу мікробіоти кишечника, що призводить до шлунково-кишкових розладів. До них належать функціональні розлади травлення, органічні захворювання та особливості стани, якими вважаються реакції харчового несприйняття злакових, а саме непереносимість глютену без целіакії та целиакія. Безглютенова дієта (БГД) — це едійний лікувальний метод лікування целіакії та НГБЦ.

Особливості мікробіому кишечника в пацієнтів із глютенчутливими захворюваннями, які дотримуються безглютенової дієти.

Висновки. Автори виявили, що особливості мікробіому кишечника у пацієнтів із глютенчутливими захворюваннями, які дотримуються безглютенової дієти, не є наслідком особливостей харчування таких хворих, а саме побічним ефектом дотримання БГД. Біографії авторів відділення бактеріозу в системі комунікації мікроорганізмів в кишечнику, що відрізняє таке хворобу від целиакії.