

Abundance and Diversity of GI Microbiota Rather than IgG₄ Levels Correlate with Abdominal Inconvenience and Gut Permeability in Consumers Claiming Food Intolerances

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Abstract: Food intolerances are an increasing global health problem. Interactions between genetics and environmental changes such as microbial- and stress factors remain poorly understood.

Whereas the analyses of IgE mediated allergic responses is based on solid concepts, the roles of microbiota, gut permeability, and IgG antibodies remain widely unclear and are under fierce discussion for scientific relevance.

The present pilot study analyzes forty participants, under consultation of nutritional health professionals, for gastrointestinal discomfort and claimed food intolerances. Food frequency questionnaire addresses nutrition, lifestyle and present discomfort. Feces samples are analyzed for dominant microbiota using 16S rDNA based methods and the fecal marker Calprotectin. Blood samples are analyzed for IgG₄ levels.

The total microbial abundance significantly correlates with claimed discomfort ($R=-0.37$; $p=0.02$). The abundance and diversity of microbiota significantly correlates with low Calprotectin values ($R=-0.35$; $p=0.01$) and with higher abundance of *Faecalibacterium prausnitzii* ($R=0.78$; $p<0.01$) and *Akkermansia* ($R=0.82$; $p<0.01$). Participants with low discomfort show enhanced *Clostridium Cluster XIVa* ($p=0.008$). An increased diversity is also correlating with reduced antibodies against IgG₄ of egg white ($R=0.68$; $p<0.01$).

Data suggest an interaction of low gut permeability and reduced inflammation with an established microbial equilibrium. Self-reported abdominal inconvenience of participants relates mainly to characteristics of microbiota and gut permeability. Anti-inflammatory effects of *Faecalibacterium prausnitzii* or *Lactobacilli* and gut barrier functions of *Akkermansia* may have a key role in food intolerances. The role of IgG₄ linking food immune responses with intolerances remains unclear.

Keywords: Abdominal pain, *Akkermansia*, Calprotectin, DGGE, *Faecalibacterium prausnitzii*, IgG₄ ELISA, *Lactobacilli*.

INTRODUCTION

Food intolerances and food allergies are defined as “adverse reactions to food” [1]. They are a rapidly increasing problem in the western world and according to the World Allergy Organization (WAO) 220-250 million people are affected. The trend continues to rise, especially in children [2]. In Europe, 2-5% of people suffer from a food allergy, while intolerances are estimated at 20% [3]. Genetic aspects such as single nucleotide polymorphisms [4], environmental influences such as the discussed “hygiene hypothesis” [5], and stress from the social environment [6, 7] have been linked to intolerances. Recently aspects of gut microbiota and gut permeability are in discussion for their role in food intolerance [7].

An inappropriate tolerance to food antigen has been associated with mechanisms of apoptosis [8] or paralysis of T-cells [9] and failure in the production of regulatory T-cells

[10-12]. Among the food intolerances, an IgE-mediated allergy is well understood. Also mechanisms of lactose intolerance [13, 14], histamine intolerance [15], and fructose intolerance are well described [16]. In contrast many aspects [17] in the area of non IgE- mediated intolerances remain poorly understood [18-20]. Various food components, and also a number of natural and artificial food additives have been discussed for non-IgE mediated adverse reactions [21, 22]. Furthermore, the role of IgG antibodies in these intolerances remains highly controversial. IgG1 is formed as the initial response after contact with a new food antigen. With further exposure to the antigen, a change in the formation of IgG₄ antibodies seems to take place [23]. Following this change the amount of IgG₄ rises from 3% up to 50%. High IgG₄ concentrations may indicate an intense conflict of the immune system with food antigens. The synthesized IgG₄ antibodies are suspected to sensitise cells, but may also block the IgE mediated allergic response [24]. Actual untreated allergies show increased IgG₄ levels in 50% of cases. However, the identification of the triggering food is difficult as the symptoms often stop after hours or days. In many of these IgG₄ mediated immune responses a disturbed intestinal permeability, leading to a constant

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exposure of the immune system to antigenic structures, has been postulated as a reason of the food intolerance.

An important role was assigned for IgG₄ antibodies in immune-mediated pathological mechanisms in patients with a delayed non-IgE cow's milk allergy. These patients show a significantly higher concentration of beta-lactoglobulin-specific IgG₄ antibodies compared to cow's milk tolerant (asymptomatic) patients [25]. Obese children show significantly higher IgG antibody levels against food antigens than children of normal weight. In overweight people IgG antibodies may be associated with increased atherosclerosis risk and concomitantly increased CRP (C-reactive protein) levels [26]. Other publications indicate high IgG₄ values testing an elimination diet on patients with irritable bowel symptoms. The work of Zar *et al.*, (2005) also shows a positive influence on psychological symptoms [27-29]. It is suggested that the determination of IgG or IgG₄ antibodies can be used as marker for distinction between healthy and sick patients, for both food intolerance and allergy [30]. Despite this, the presence of food specific IgG/IgG₄ antibodies shall always be considered when common causes such as lactose intolerance, fructose malabsorption, celiac disease, or IgE mediated food allergies can be excluded [25].

In addition to gut permeability, individual gastrointestinal microbiota and their involvement in inflammatory and metabolic reactions of the gut may play a role in intolerances. The groups of *Faecalibacterium prausnitzii*, *Akkermansia* and *Lactobacilli* are associated with inflammatory immune regulation and the gut barrier [31-35]. *Faecalibacterium prausnitzii* is named as one of the main butyrate producers [33], often seen in conjunction with other bacteria producing acetate or propionate as substrates for butyrate production [36]. Also epigenetic effects of butyrate producing microbiota in the regulation of inflammatory genes for example Toll-like receptors (TLR) and short chain fatty acid (SCFA) receptors have been described, also by our group [37, 38]. *Lactobacilli* are associated with anti-inflammatory effects [39, 40]. Other researchers describe *Lactobacilli* as pro-inflammatory for example in type 2 diabetes [41-43]. *Akkermansia* are known to feed on mucus and might indicate inflammatory processes [34].

The present study was designed as a pilot study to establish parameters and inclusion criteria for a subsequent clinical study. Because of the broad nature of factors for food intolerance or gut inconvenience, inclusion criteria for the pilot study was self-reported abdominal discomfort of participants consulting with health nutritional experts. The present study aims to analyze possible interactions between questionnaire based symptoms, gut microbiota, Calprotectin as inflammatory and gut permeability parameter and IgG₄ levels. Convincing evidence for interactions between gut microbiota and claimed food intolerances and a possible involvement of specific IgG levels in certain food ingredients is discussed.

METHODS

Study Participants; in- and Exclusion Criteria

All study participants gave written consent for using blood and stool samples and data obtained from food

frequency questionnaires (FFQ) for the analyzes presented here. Clearance was given by the local ethical committee with acceptance number 31/2009 Top 8.

We studied a population of 40 participants, visiting health nutritional experts for gastrointestinal inconvenience. Inclusion criteria were: 21 years of age or more, both genders, present abdominal inconvenience with a suspicion of food intolerance, currently consulting with health nutritional experts. Exclusion criteria were: diagnosed inflammatory bowel disease, established IgE mediated food allergies, lactose intolerance, pregnancy, BMI > 30 kg/m² and diagnosed metabolic- and malignant disease. 37 participants (aged 46 ± 14.2 years; 29 female, 8 male) fulfil all criteria for an inclusion in the pilot study.

The FFQ, was adapted from EPIC questionnaires and forms addressing food intolerances [44] and focuses on the consumption frequency of foods and portion size. In addition to dietary habits, questions about lifestyle (*i.e.* smoking, alcohol consumption, and physical activity), medical relevant influences (*i.e.* vitamin and other supplements), BMI and age at enrolment were included. The questionnaire also asks for any history of potential gastrointestinal diseases, use of antibiotics, probiotics, prebiotics, as well as discomfort and proven or even self-reported (non-established) intolerances and allergies. Inconvenience is assessed in self-reporting, addressing present abdominal pain. The evaluation of the nutritional habits follows the recommendations of the OEGE (Austrian Society for Nutrition).

Fecal Sample Collection, Processing, and Analysis

After collection, study participants immediately stored their stool samples at -20°C. Fecal samples were transported frozen to the laboratory and immediately restored at -20°C. The DNA of 200mg stool sample was extracted using the QIAamp[®] DNA Stool Mini Kit (QIAGEN) following the manufacturer's protocol. DNA concentration and quality was determined with a Pico100 (Picodrop, UK) and agarose gel-electrophoresis. DNA was stored at -20°C until analysis.

The total bacterial diversity was assessed by DGGE (denaturing gradient gel electrophoresis) using the primer set 341f-GC 5'-CCT ACG GGA GGC AGC AG-3' and 518r 5'-ATT ACC GCG GCT GCT GG-3' according to Remely *et al.*, (2013) [41], primers for all bacteria with GC clamp are used on a 30% to 55% gel.

Microbiota is analyzed using 16S rDNA based TaqMan qPCR and SYBR Green qPCR in StepOnePlus[™] Real-Time PCR System (Applied Biosystems[®]) focusing on dominant bacterial groups: all bacteria, *Clostridium cluster XIVa*, *Archaea*, *Bacteroides*, *Bifidobacteria*, *Akkermansia*, *Lactobacilli* and *Faecalibacterium prausnitzii* (Table 1).

Primer and probes are checked for specificity with the Probematch function of the ribosomal database project 10 (<http://rdp.cme.msu.edu/>). The mixture of PCR reactions and serial DNA dilution of typically strains are prepared according to Pirker *et al.*, 2012 [45].

The Bühlmann Calprotectin ELISA kit is used for quantitative determination of human Calprotectin (MRP8/14; S100A8/S100A9) extracted from stool samples. After a brief

Table 1. Primer sequences of qPCR and conditions.

Target Organism	Primer/Probe	Sequence (5' - 3')	Conc. [pmol/μL]	Reference
All Bacteria	Fwd primer	ACT CCT ACG GGA GGC AG	10	[46]
	Rev primer	GAC TAC CAG GGT ATC TAA TCC	10	
	Probe	(6-FAM)-TGC CAG CAG CCG CGG TAA TAC-(BHQ-1)	2	
<i>Clostridium cluster IV</i> (<i>Ruminococcaceae</i>)	Fwd primer	GCA CAA GCA GTG GAG T	4	[47]
	Rev primer	CTT CCT CCG TTT TGT CAA	4	
	Probe	(6-FAM)-AGG GTT GCG CTC GTT-(BHQ-1)	2	
<i>Cluster XIVa</i> (<i>Lachnospiraceae</i>)	Fwd primer	GCA GTG GGG AAT ATT GCA	5	[48]
	Rev primer	CTT TGA GTT TCA TTC TTG CGA A	5	
	Probe	(6-FAM)-AAA TGA CGG TAC CTG ACT AA-(BHQ-1)	1,5	
<i>Bacteroidetes</i>	Fwd primer	GAG AGG AAG GTC CCC CAC	3	[49]
	Rev primer	CGC TAC TTG GCT GGT TCA G	3	
	Probe	(6-FAM)-CCA TTG ACC AAT ATT CCT CAC TGC TGC CT-(BHQ-1)	1	
<i>Lactobacilli</i>	Fwd primer	AGC AGT SGG GAA TCT TCC A	4	[50]
	Rev primer	ATT YCA CCG CTA CAC ATG	4	
<i>Akkermansia</i>	Fwd primer	CAGCACGTGAAGGTGGGGAC	2,5	[51]
	Rev primer	CCTTGC GGTTGGCTTCAGAT	2,5	
<i>Faecalibacterium prausnitzii</i>	Fwd primer	GGA GGA AGA AGG TCT TCG G	0.25	[52]
	Rev primer	AAT TCC GCC TAC CTC TGC ACT	0.25	

extraction step using a stool sample volume fraction and 49 volume fractions of extraction buffer, Calprotectin is selectively determined by sandwich ELISA. The wells of the microtiter plate are coated with a capture monoclonal antibody (mAb), which is highly specific to polymeric and heterodimeric Calprotectin.

The actual concentrations of the standards A-E are 4, 12, 40, 120, and 240ng/μl Calprotectin. During extraction the sample is diluted 1:50 and the extract diluted again 1:50, resulting in a total dilution of 1:2500. Both dilution steps were included in the standard concentration and are given as follows: 10, 30, 100, 300 and 600 μl/g Calprotectin.

The optical density of the solution, measured at a wavelength of 450nm using FLUOstar Optima, is directly proportional to the Calprotectin concentration of the sample. After entering the standard concentrations, the FLUOstar Optima software (version 1.30R2) automatically calculates the concentrations of Calprotectin [53].

Blood Sample Processing, IgG

Blood samples were collected in Li-Heparin tubes (Sarstedt, Germany). The blood samples were stored at -8°C. IgG₄ levels were also analyzed according ELISA following

the manufacturer's protocol. The Immunolab IgG₄ Screen Nutritional 20 ELISA[®] test kit is based on the principle of the enzyme immunoassay (EIA). After spectrophotometric analysis, Immunolab offers a simple evaluation of all its food intolerance screening tests. The definition of intolerance classes 1-4 follows the standard concentrations suggested by the manufacturer: Standard 1: 0.35 U/ml, Standard 2: 0.70 U/ml, Standard 3: 3.5 U/ml and Standard 4: 17.5 U/ml [54].

Statistical Analysis

Normal distribution (Kolmogorov-Smirnov-test) and descriptive statistics were tested with IBM SPSS Statistics 20. All correlations (Spearman), 2 sample t-test and linear fit were analyzed using Origin Pro 8. P-values <0.05 were taken as statistically significant. All data is shown as mean ± SD.

RESULTS

Nutrition

For 37 participants all data could be obtained. The average of age is 46±14.2 years. 29 of the participants are female, 8 are male. The evaluation of nutritional habits follows the recommendations of the OEGE (Austrian Society

for Nutrition) and where the diet is arranged in the 4 major food groups: milk and dairy products, fruit and vegetables, meat and meat products, and cereals.

With regards to milk and milk products 32% of the participants consume there commended 2-3 servings per day. 67% under eat milk and dairy products. Those participants show a tendency of reduced levels of *Lactobacilli* and *Bifidobacteria* in stool ($p=0.07$). 40% of these participants showed high class of IgG4 levels for “cow’s milk” without diagnosed intolerance, and stated an inconvenience after dairy product consumption. Even in absence of a diagnosed milk intolerance, discomfort increased, if the IgG4 levels are high or *Lactobacilli* and *Bifidobacteria* levels are low. Participants reporting a proven lactose intolerance were not included in the study.

In fruit and vegetables, 54% comply with the recommended “5 a day”. Within these 54%, 10% reported a fructose intolerance when asked. It is remarkable that these participants consume even more vegetables, to reach the “5 a day”. From those who do not achieve the “5 a day” 71% indicate discomfort after the consumption of fruit.

Cereals should be consumed up to 4-5 times a day, more than half of them in the form of whole grains. Only 27% of all 37 participants reach the recommendations and report no intolerance to cereals. 10% show intolerances (class 4) against the antigen of wheat flour and rye flour according to the IgG4 ELISA. From the remaining 73% only 11% show an intolerance.

Up to 3 servings (300-450g) meat or meat products are recommended per week. Only 30% report eating meat or meat products 3 times a week or less. 36% of them have no intolerance against pork meat, according to the IgG4 ELISA. The remaining 70% consume meat more than three times a week. 7% of them are revealed in IgG4 class three.

76% of all participants reported consumption of dietary supplements. The most frequently mentioned food supplements are: vitamin C, vitamin D, calcium, magnesium, and multivitamins. In conclusion the nutrition of all participants showed typical characteristics of a central European diet.

When asked about physical exercise, 60% replied with frequencies between once a day to several times a day, 11% exercise 4-6 times a week, 24% 1-3 times a week, and 5% only one to two times a month. In sports (exercise with sweating over 30 minutes) 54% follow the recommended two to three sport units per week or more. 16% practice sports only one or two times a month and 30% never do any sports.

Discomfort

46% of all participants address abdominal gastrointestinal discomfort in the FFQ, remaining 54% report no present discomfort, but abdominal pain in the last year. 19% of the study participants state fructose intolerance, 8% gluten intolerance, and 22% of participants state histamine intolerance in the FFQ. The absence of inconvenience is correlated with higher abundance of total microbiota ($R=-0.37$; $p=0.02$) as well as with diversity of microbiota.

No correlations are observed between groups reporting discomfort and no discomfort and IgG4 levels. However, participants reporting comfort/ discomfort convenience or inconvenience differ significantly in abundance of main butyrate producers *Faecalibacterium prausnitzii* ($p=0.049$) and *Clostridium cluster XIVa* ($p=0.008$; Fig. 1).

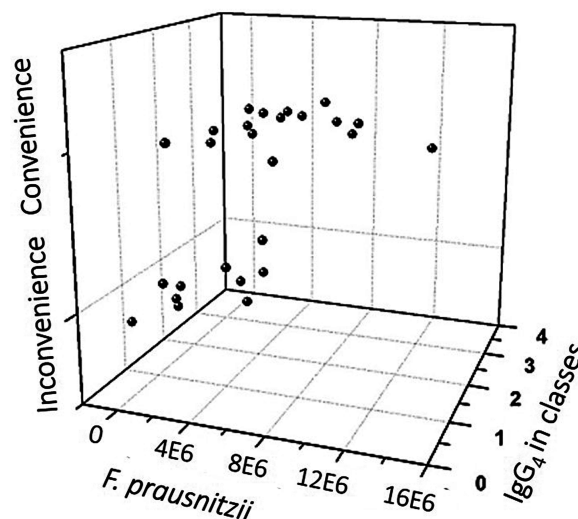


Fig. (1). 3D correlation model of *Faecalibacterium prausnitzii* in copies, IgG4 in classes and convenience/inconvenience.

Analysis of GI Microbiota

Fecal microbiota abundance is analyzed with 16S rDNA for all bacteria, *Clostridium cluster XIVa*, *Archaea*, *Bacteroides*, *Bifidobacteria*, *Akkermansia*, *Lactobacilli*, and *Faecalibacterium prausnitzii*. Participants with high all bacterial abundance show reduced values of Calprotectin ($R=-0.35$; $p=0.01$; Fig. 2). The total bacterial abundance is correlated with *Faecalibacterium prausnitzii* ($R=0.78$; $p<0.01$) as well as with *Akkermansia* ($R=0.82$; $p<0.01$) and *Lactobacilli* ($R=0.80$; $p<0.01$). In addition participants with higher abundance of all bacteria report decreased inconvenience ($R=-0.37$; $p=0.02$; Fig. 3). Therefore, a higher abundance of all bacteria lowers the abdominal pain in comparison to participants with low total bacterial amount.

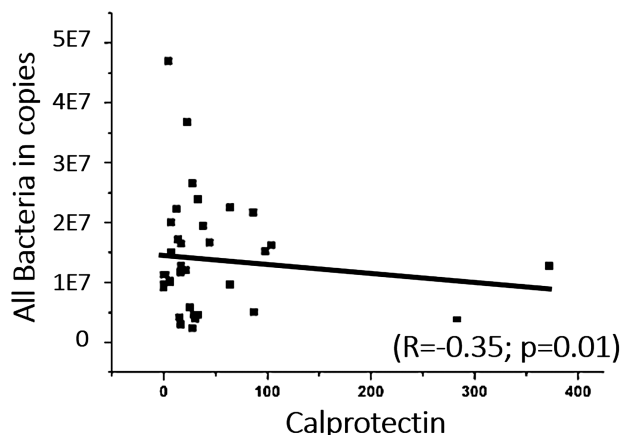


Fig. (2). Regression chart with linear fit between Calprotectin in $\mu\text{g/g}$ feces and all bacteria in copies ($R=-0.35$; $p=0.01$).

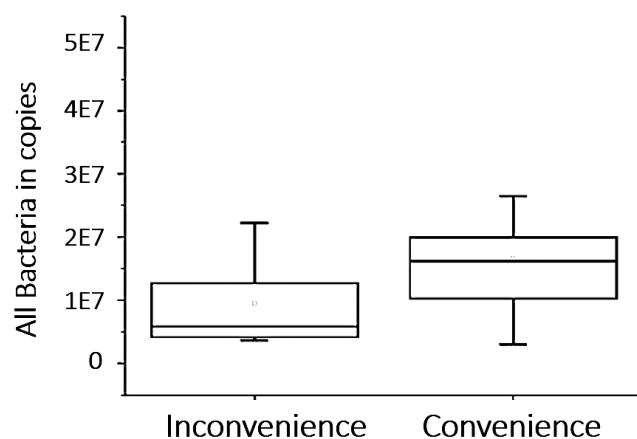


Fig. (3). Boxplot of self-reported convenience and inconvenience compared to all bacteria in copies; Box range 25, 27 Perc; Whisker indicates outliers; □ indicates mean; x indicates maximum and minimum data range.

Diversity is assessed by PCR-DGGE analyzes. As shown in Fig. 4, DGGE diversity has a band variety of 18.65 ± 4.04 bands, with a minimum of 12 and a maximum of 27 bands. In previous reports from our group, the analysis of dominant bacteria and DGGE correlates significantly with 454 pyrosequencing and the abundance of *Faecalibacterium prausnitzii* [55]. In accordance with the report we show a correlation between *Faecalibacterium prausnitzii* and diversity of DGGE bands ($R=0.37$; $p=0.02$, Fig. 5).

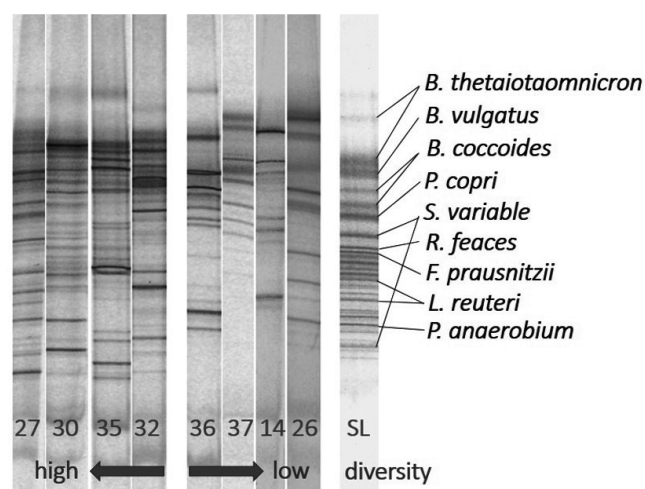


Fig. (4). DGGE image of PCR amplified all bacteria in a 30-50% gel; comparison of 8 band patterns from 4 participants with low (lane 36, 37, 14, 26) and 4 participants with high (lane 27,30,32,35) diversity; SL: standard lane with 9 common commensal bacteria.

Calprotectin

Calprotectin is analyzed in feces as a marker for inflammation but also as cause for possible changes in gut permeability and concurrent minor inflammation. Calprotectin

values vary between 3 and 371.7 μg Calprotectin/g feces, with a mean level of 45.7 ± 74.3 μg Calprotectin/g feces, and a median of 25.3 μg Calprotectin/g feces. A negative correlation is shown between the general abundance of all bacteria and Calprotectin ($R = -0.35$; $p=0.01$, Fig. 1). However, no correlation is indicated for Calprotectin values between specific aspects of microbiota or IgG₄ values, but those patients with a high diversity and bacterial abundance, often addressed as balanced microbial equilibrium, show low Calprotectin values.

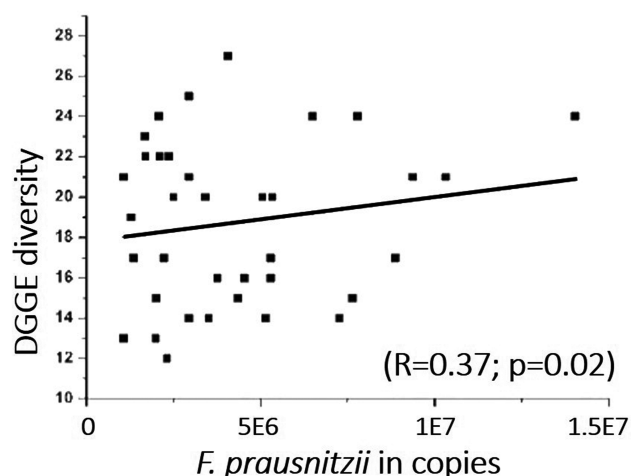


Fig. (5). Regression chart with linear correlation between *F. prausnitzii* in copies and DGGE diversity in band counts ($R=0.37$; $p=0.02$).

IgG₄ Levels

IgG₄ levels are analyzed against egg white, cow's milk, cod, wheat flour, rye flour, barley flour, orange, banana, kiwi, strawberry, celery, soy bean, carrot, tomato, hazelnut, peanut, curry, pepper, sesame, and pork (meat). These antigens are classified into four categories (class 1-4) according to method section [54].

5% of tested participants were grouped in class 1, 17% class 2, 36% class 3 and 42% class 4 as highest detected class. The foods with the highest IgG₄ class 4 are: egg white, cow's milk, wheat flour, rye flour, barley flour, banana, kiwi, and hazelnut. For egg white 27% are grouped in class 4 and 22% had no IgG levels. For cow's milk 38% show class 4 and 33% were not classified in one class. For wheat flour and rye flour 8% of the participants are classified in class 4, and 35% of the participants were grouped in no class in both. For barley flour 3% of the participants show the highest class 4. For banana 5%, for kiwi and hazelnut 11% of the participants are classified in class 4. The lowest IgG₄ classes have been detected for strawberry, celery, and soy bean. There has been no food class in which no IgG₄ values have been measured.

A correlation can be seen between egg white and wheat flour ($R=0.43$; $p<0.01$), as well as rye- ($R=0.40$; $p=0.014$) and barley flour ($R=0.60$; $p<0.01$). In addition a positive correlation is found between cow's milk and the collective of grainflours ($R=0.41$; $p=0.012$). No correlation is seen for

general IgG₄ levels with abundance or diversity of microbiota or Calprotectin values, however IgG₄ levels for egg white decrease with increasing DGGE bands ($R=0.68$; $p<0.01$) (Fig. 6). Wheat flour, rye flour, and barley flour correlate well: The p-value for the correlation between wheat flour and rye flour is <0.01 ($R=0.73$). For the correlation between wheat flour and barley flour the p-value is <0.01 ($R=0.48$) (Fig. 7).

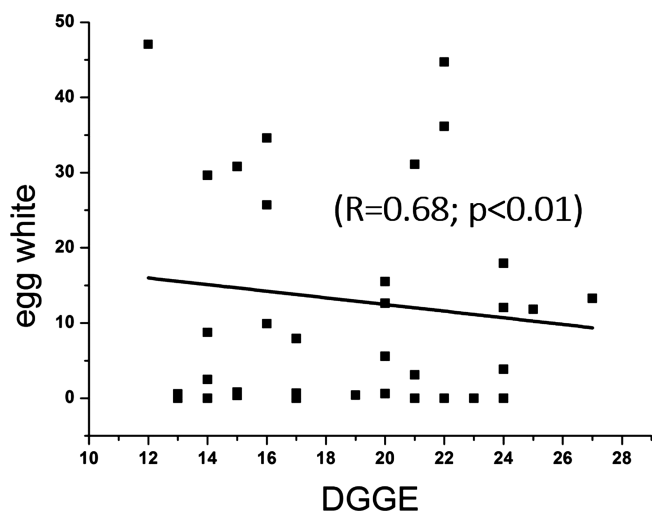


Fig. (6). Regression chart with linear fit between DGGE diversity in band counts and IgG₄ egg white in U/ml ($R=0.68$; $p<0.01$).

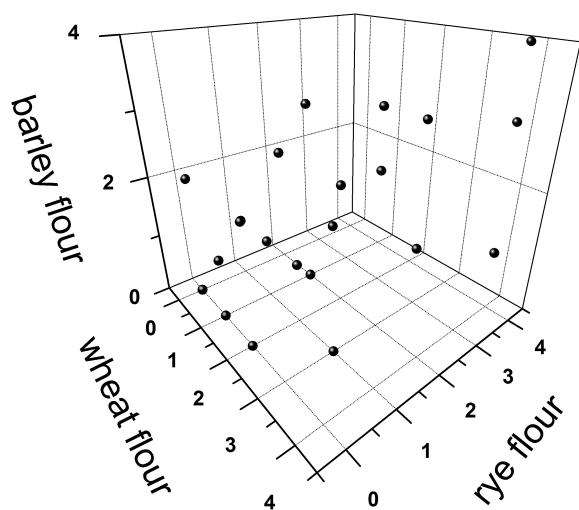


Fig. (7). 3D correlation model of wheat, rye, and barley flour IgG₄ in classes.

DISCUSSION

The present pilot study analyses interactions of gastrointestinal microbiota, gut permeability, nutrition, IgG₄ levels, and their impact or correlation on food intolerances and well-being. High total bacteria abundance is correlated with high amounts of *F. prausnitzii*, *Akkermansia*, low Calprotectin values, decreased inconvenience, and less abdominal pain. Bacterial diversity and *F. prausnitzii* abundance are positively correlated.

67% of participants did not reach recommended intake of milk and dairy products. These participants show a tendency of reduced *Lactobacilli* and *Bifidobacteria* levels in their stool ($p=0.07$). 40% of these participants showed high class of IgG₄ levels for “cow’s milk” and stated inconvenience after dairy product consumption. Even in absence of diagnosed milk intolerance, discomfort increased, if the IgG₄ levels are high or *Lactobacilli* and *Bifidobacteria* levels are low.

Post consumption discomfort was not the reason to consume fewer vegetables or whole grain products. The group with a low fruit consumption had a 3-fold higher self-reported fructose intolerance rate. 11% of participants had IgG₄ Class 4 levels for wheat-, flour-, and rye flour.

A population of 40 participants, visiting dietary health experts, was included in the pilot study with the objectives to find improved criteria and markers for a future study.

The analytical strategy to include the analysis of major commensal gastro intestinal microbiota groups in addition to food intolerances, IgG₄ levels and inconvenience, resulted in significant correlations: Several studies show that focusing only on IgG₄ levels and nutritional behavior does not explain intolerances [56, 57].

Strategies to lower in particular inconvenience in gluten sensitive persons, are following an prebiotic approach, addressing gastro intestinal microbiota in intolerances [58]. This prebiotic intervention may increase abundance of SCFA producers such as *F. prausnitzii*, and decreases inconvenience. Lopez-Siles *et al.*, (2012) [59] showed the importance of strain specificity in analyzing the role of *F. prausnitzii*. To show correlations of microbiota and intolerances including non-dominant microbiota or specific strains, 454 analysis may be important in future studies.

The inflammatory and intestine permeability marker Calprotectin did not correlate with IgG₄ levels, but correlated significantly with microbiota abundance and diversity in this study. To improve analysis of intestinal permeability in the different subareas of the intestine the sucrose, lactulose/mannitol, sucralose diffusion test should be considered as an additional important tool [60] for gut permeability.

The questionnaire used in the present study was carefully adapted from the EPIC study, multiple related questions were used to validate responses [44]. Health experts interviewed every participant personally in a face-to-face meeting. However an international validation is desirable.

Whereas correlations between microbiota and discomfort and Calprotectin were found in the present study the use of IgG₄ antibodies as marker for food intolerance could not be encouraged, as also discussed before by Steven O. Stapel 2008 [61]. Significant correlation is seen between IgG₄ titers of egg white and wheat, rye and barley flour ($R=0.43$; $p<0.01$; $R=0.40$; $p=0.014$; $R=0.60$; $p<0.01$). Zar *et al.*, [29] reported significantly IgG₄ titers correlation between wheat, beef, and lamb in IBS patients before. IgG₄ levels for egg white decrease with increasing DGGE bands ($R=0.68$; $p<0.01$). The diversity analyzed by DGGE shows a band variety of 18.65 ± 4.04 bands. This method has been proven to show correlations with 454 based pyrosequencing results

[36, 41]. As reported before, the gut microbiota shows individual characteristics depending on lifestyle, ethnicity, and nutrition [40, 54, 55]. In accordance with recent publications [53, 56] patients with a high abundance of all bacteria also show a significant higher abundance of *Faecalibacterium prausnitzii* ($R=0.78$; $p<0.01$). A tendency can be observed between the number of *Lactobacilli* and *Bifidobacteria* and the consumption of milk products ($p=0.07$) as already reported by Shiby *et al.*, (2013) [57].

An important aspect may be the high correlations between IgG₄ titers in the groups of wheat, rye, and barley flour ($R=0.72$; $p<0.01$; $R=0.48$; $p<0.01$) as this may indicate an interaction of their protein structures with immune responses. For Gliadin both IgE and IgA antibodies are known [59, 60]. The study by Constantin *et al.*, (2005) suggests that wheat includes antigens/epitopes which are mainly recognized by CD patients. These results suggest that food antigens affect the quality of the pathological immune response in the gut [61].

In subjects with high abundance of butyrate producing bacteria (*Faecalibacterium prausnitzii* ($p=0.049$), *Clostridium Cluster XIVa* ($p=0.008$)) absence of discomfort/convenience was significantly higher, despite high IgG₄ levels or nutrition.

Faecalibacterium prausnitzii, one of the main butyrate producers [32] with effect on epigenetic regulation of inflammatory genes [36], is discussed as an indicator for high gene counts [62]. The groups of *Lactobacilli*, *Faecalibacterium prausnitzii*, and *Akkermansia* have been widely discussed for their role in inflammatory immune regulation as well as in mucus and gut barrier associated aspects of microbiota [31-34]. *Akkermansia*, a mucin consuming and degrading species [33], discussed as a marker for gut wall permeability, shows also a trend for a correlation with convenience. Calprotectin, a marker for inflammation, discussed for gut permeability [62] correlates with the general abundance of all bacteria ($R=-0.35$; $p=0.01$). Participants with present pain (46%) show significantly higher Calprotectin values ($R=0.89$; $p>0.01$). However, those patients with a high diversity and abundance, seen as patients with balanced microbial equilibrium, showed typically low Calprotectin values (not significant). Recently an association between changes in microbiota, influences on the expression of γ -Aminobutyric acid receptors and pain was reported. For specific *Lactobacilli* strains increased expression of the receptor and reduced pain was mentioned. γ -Aminobutyric acid has a relaxing effect on gut smooth muscles [58]. In our results we observe a trend between enhanced abundance of IgG₄ and increased abundance of *Lactobacilli* ($p=0.05$). It could be hypothesized that characteristics of microbiota decide on the perception of abdominal inconvenience independently from immune reactivity addressing food compounds.

In conclusion an interaction between IgG₄ levels and single aspects of gastrointestinal microbiota and gut permeability can be suggested by the present results.

CONCLUSION

An interaction between IgG₄ levels and single aspects of gastrointestinal microbiota and gut permeability can be suggested by the present results.

The correlation of gut permeability and enhanced immune system response to food antigens needs to be observed. Presumably analysis of GI-microbiota can be a way to an individualized intervention in situations of gastro-intestinal discomfort linked to intolerances.

CONFLICT OF INTEREST

We hereby assure that the manuscript a) is an original work, b) has not been previously published whole or in part, and c) is not under consideration for publication elsewhere. All authors have disclosed any actual or potential competing interests regarding the submitted article and the nature of those interests. All authors a) have read the manuscript, b) agree the work is ready for submission to a journal, and c) accept responsibility for the manuscript's contents.

ACKNOWLEDGEMENTS

The work was supported by the Austrian Research Promotion Agency (FFG) and the Austrian Science Fund (FWF).

LIST OF ABBREVIATIONS

CRP	=	C-reactive protein
F. prausnitzii	=	<i>Faecalibacterium prausnitzii</i>
FFQ	=	Food frequency questionnaire
ÖGE	=	Austrian Society for nutrition
SCFA	=	Short-chain fatty acid
TLR	=	Toll-like receptors
WAO	=	World Allergy Organization

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