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The Janus Face of Cereals: Wheat-Derived Prebiotics Counteract the Detrimental Effect of Gluten on Metabolic Homeostasis in Mice Fed a High-Fat/High-Sucrose Diet

*Marta Olivares, Julie Rodriguez, Sarah A. Pötgens, Audrey M. Neyrinck, Patrice D. Cani, Laure B. Bindels, and Nathalie M. Delzenne**

Scope: Cereals are important sources of carbohydrates, but also contain nutrients that could impact adiposity. The contribution of gluten to obesity and the effects of prebiotics—arabinoxylo-oligosaccharides (AXOS) and fructo-oligosaccharides (FOS)—that can be extracted from gluten-containing cereals are analyzed.

Methods and results: Mice are fed a control diet, Western diet (WD, consisting of high fat/high sucrose), or WD with 5% gluten. Prebiotics are tested in the WD with gluten. Gluten does not increase body weight and has a minor effect on ileal inflammation. Gluten decreases the expression of browning markers in the fat and increases the triglycerides synthesis in the muscle. AXOS decreases body weight and adiposity in fat pads muscle and liver. AXOS promotes gluten cleavage by the induction of prolyl endopeptidase that is translated into a reduction of gluten immunogenic peptides. Gluten has minor effects on cecal microbiota composition, whereas prebiotics increased *Bifidobacterium*, *Butyricicoccus*, *Prevotella*, and *Parasutterella*, which are all negatively correlated to the cecal content of gluten peptides.

Conclusion: While gluten may affect metabolic homeostasis, these effects are lessened when gluten is consumed along with cereal-derived fibers. If confirmed in humans, the authors bring new arguments to eat fiber-rich cereals to promote a healthy diet.

1. Introduction

Wheat gluten is a mixture of storage proteins predominantly made of equal parts of glutenins and gliadins. Similar storage proteins exist in the rye, barley, and oats, and are collectively referred to as gluten. Gluten entered our diet about 10 000 years ago with the introduction of agriculture and cereals farming. Today, cereals are an essential food source and more than 50% of the caloric intake worldwide is derived from the consumption of grains.[1] Consequently, the human diet is heavily based on gluten-containing foods and, in the case of the dietary pattern known as Western diet (WD), the daily gluten intake is estimated between 5 and 20 g per day.[2]

Gluten is rich in proline residues that confer resistance to proteolysis and lead to the generation of peptides with biological activity.[3] In some genetically predisposed individuals, gluten triggers an autoimmune-based enteropathy known as celiac disease.[4] Breaking down gluten into smaller peptides can reduce its immunogenicity. Hence, the oral supplementation with proline specific enzymes, like prolyl endopeptidase (PEP) or dipeptidyl peptidase 4 (DPP-4), can be a potential therapy.[5–8] However, this approach remains in the experimental phase and currently, the only treatment for celiac patients consists of a life-long gluten-free diet.

Besides, the contribution of gluten in other conditions has started to emerge. In subjects suffering from non-celiac gluten sensitivity, the adherence to a gluten-free diet ameliorates the symptomatology, and such dietary restriction also reduces the risk of type 1 diabetes.[9,10] Recent data obtained in animal studies show that gluten or gliadin, when added to a high-fat diet, increases adiposity and impairs glucose homeostasis.[11–13] Moreover, this was accompanied by alterations in the composition and activity of the gut microbiota.[13]

Lately, the tie between gluten and the onset of type 2 diabetes was analyzed by combining three prospective cohort studies that together add almost 200 000 subjects.[14] Surprisingly, the highest gluten intake was associated with lower disease risk.[14]

Dr. M. Olivares, Dr. J. Rodriguez, S. A. Pötgens, Dr. A. M. Neyrinck, Prof. P. D. Cani, Prof. L. B. Bindels, Prof. N. M. Delzenne
Metabolism and Nutrition Research Group, Louvain Drug Research Institute
Université catholique de Louvain (UCLouvain)
B-1200 Brussels, Belgium
E-mail: nathalie.delzenne@uclouvain.be
Prof. P. D. Cani
Walloon Excellence in Life sciences and BIOtechnology (WELBIO)
Louvain Drug Research Institute
UCLouvain, B-1200 Brussels, Belgium

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The proposed explanation is that limiting cereal intake to avoid gluten also compromises the intake of fibers and other phytochemicals present in cereals and that have a beneficial effect on health.[14] Note that, for example, a kernel of wheat has gluten in the starchy endosperm, but also dietary fibers in the endosperm and bran layers.[15,16] Accordingly, when healthy subjects followed a low-gluten diet (2 g per day) or high-gluten diet (18 g per day), the adherence to the first regimen was associated with a drop in *Bifidobacterium* and a lower intake of arabinose, xylo-, and fructo-oligosaccharides (FOS).[17] In fact, the coexistence of gluten and these components in the cereals raises difficulties to identify the culprit molecule of new disorders related to cereals. For instance, in non-celiac gluten sensitivity, the intake of fructan, rather than gluten, has been identified as responsible for the gastric symptoms.[18]

We and others have previously demonstrated the interest of fibers from wheat and cereals (arabinoxylans and fructans) that are considered as prebiotics since they change the gut microbiota (mainly in favor of the genus *Bifidobacterium*). Similarly, arabinoxylo-oligosaccharides (AXOS) and FOS that are, respectively, hydrolysis products of arabinoxylans and fructans, can improve adiposity and metabolic disorders associated with obesity in rodents [19–21] and human subjects.[22,23] In the present study, we tested if the administration of AXOS or FOS can counteract the effects of gluten in a murine model of diet-induced obesity.

2. Experimental Section

2.1. Animals and Treatments

Forty-five mice (9-week old C57BL/6J male) were purchased from2 Janvier Labs (Saint Berthevin, France). Mice were housed in individually ventilated cages by groups of three mice per cage and kept with 12-h daylight cycle and free access to food and water. The acclimatization period lasted ten days with a standard diet (Research Diet Inc., New Brunswick, NJ, USA). The experiment was approved by and performed following the guidelines of the local ethics committee of Université catholique de Louvain. Housing conditions were as specified by the Belgian Law of May 29, 2013 regarding the protection of laboratory animals (Agreement number LA 1230314).

Randomization of mice into five groups ($n = 9$) was done based on body composition assessed by NMR (LF50 minispec, Bruker, Germany) to minimize baseline differences. The experimental groups consisted in mice fed: 1) Control diet (according D12450K, Ssniff, Soest, Germany) with 10% kcal from fat; 2) high fat/high sugar diet (from here on called Western diet [WD]) (according D12451, Ssniff) with 45% kcal from fat and 17% kcal from sucrose; 3) WD + 5% gluten at the expense of 5% of casein (Western diet with gluten [WD + G]; Ssniff); 4) WD + G + 5% wheat bran derived AXOS (w/v, Cargill, Belgium) in the drinking water; and 5) WD + G + 5% FOS (w/v, Orafti, Beneo, Belgium) in the drinking water.

The 7th week a 24 h feces collection test was performed. The 8th week and after 6 h of fasting, mice were anesthetized using isoflurane gas (Abbot, Ottignies, Belgium). Blood from cava vein was harvested in EDTA tubes. Plasma was immediately collected after centrifugation (12 000 × *g* for 3 min). One aliquot of plasma was kept in ice to assess intestinal permeability and one aliquot was stored at −80 °C for biochemical analysis. Mice were necropsied after cervical dislocation. Liver, brown and white adipose tissues (epididymal, visceral, and subcutaneous), muscles (gastrocnemius, tibialis, and soleous), cecal content, and intestinal tissues were dissected and immersed in liquid nitrogen before storage at −80 °C.

2.2. Diet and Dosage Regimen

The composition of the diets is shown in Table S1, Supporting Information. The WD was supplemented with 5% gluten from wheat (G5004, Sigma-Aldrich, MO, USA). This amount of gluten can be found in human food as, for instance, regular wheat flour has a content of 10–12% of gluten.[13] Prebiotics were administered in the drinking water to minimize the stress of the animals at a concentration of 5% w/v. This dose is equivalent to the content of cellulose in the diets and is within the range used in previous studies.[19–21,24] Taking into account the volume of water ingested, each mouse received an approximate dose of 0.2 g per day. Recent recommendations propose a dietary fiber intake of 50 g per day.[25] Consequently, the dose of prebiotics in experimental conditions is higher than the desirable dose for humans (≈0.70 g kg^{-1} body weight in humans vs ≈8 g kg^{-1} body weight in animals).

The gluten immunogenic peptides in the diets and the drinking water were quantified with 5% of AXOS or FOS as described below. The WD with 5% of gluten contained 3.8% of gluten immunogenic peptides. There was no gluten in the control diet and the Western diet, and the drinking water supplemented with the prebiotic compounds.

2.3. Intestinal Permeability

FITC-dextran 4 kDa (Sigma-Aldrich, MO, USA) was administrated by oral gavage (600 mg kg^{-1}) 1 h before necropsy. Plasma was diluted in an equal volume of PBS (pH 7.4), and the fluorescence was measured at an excitation wavelength of 485 nm and emission of 535 nm (SpectraMax M2, Molecular Devices). Standard curves were obtained by diluting FITC-dextran in non-treated plasma with PBS.[26]

2.4. Biochemical Analysis

Lipids were extracted from muscle gastrocnemius and liver and quantified.[21] Plasma insulin, triglycerides, cholesterol, and non-esterified fatty acids (NEFA) were measured. All the procedures are explained in Supporting Information.

2.5. Quantification of Gluten Immunogenic Peptides

In the diets, gluten was quantified using a commercial kit for foodstuff (GlutenTox ELISA Sandwich, Biomedal. Seville, Spain LOD: 0.600 μg g^{-1}).[27] Gluten peptides were also quantified in the cecal content using another commercial kit (iVYLISA GIPS,

Biomedal. LOD: 0.156 μg g^{-1}).[28] The method is based on the G12 monoclonal antibody that recognizes the following sequences of gluten immunogenic peptides: QPQLPY, QPQQPY, QPQQPF, QPQLPF, QPQLPL, QPELPY.

2.6. Analysis of the Gut Microbiota Composition

Genomic DNA was extracted from cecal content using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), including a bead-beating step. The gut microbiota was analyzed by Illumina sequencing of the 16S rRNA gene. Illumina sequencing was performed using a previously described approach.[29] Briefly, the V5–V6 region of the 16S rRNA gene was amplified by PCR with modified primers. The amplicons were purified, quantified, and sequenced using an Illumina Miseq to produce 2 × 300-bp sequencing products at the University of Minnesota Genomics Center. Subsequent bioinformatics and biostatistics analyses were performed as previously described.[30] The full protocol is described in Supporting Information.

2.7. Gene Expression Analyses

Total RNA was isolated from different sections of the intestine, adipose tissue, skeletal muscle and liver using the TriPure reagent kit (Roche Diagnostics, Basel, Germany). Complementary DNA was prepared by reverse transcription of 1 μg of total RNA using the kit GoScript Reverse Transcriptase (Promega, Madison, WI, USA). RT-qPCR was performed with the StepOne System (Applied Biosystems, Walttham, MA, USA). Samples were run in duplicate and the data were analyzed using the $2^{-\Delta\Delta CT}$ method. Negative controls of the RT reaction and RT-qPCR were included. Targeted genes were normalized with the expression of the ribosomal protein L19 (*Rpl19*) as the reference gene. Primers sequences are given in Table S2, Supporting Information.

2.8. Histological Analysis

The visceral adipose tissue was stained with hematoxylin/eosin to quantify the size of the adipocytes. The scanned images were analyzed on ImagenJ software as previously described.[31] The cross-sectional area of each adipocyte was automatically recognized and calculated by the software. Artifacts were manually discarded. At least 700 adipocytes were quantified per measurement and two measurements were done for each mouse.

In the ileum, the crypt depth and villus length were measured after hematoxylin/eosin staining. The sections were digitized (Leica SCN400, Leica Microsystems, Germany), and the images were captured using the Leica Image Viewer Software (Version 4.0.4). Crypt depth and villus length measurements were made on sections (at least 10 measurements per animal).

2.9. PEP and DPP-4 Activities

In the jejunal mucosa, PEP and DPP-4 activities were quantified by measuring the release of para-nitroanilide (PNA) from the respective substrates Z-Gly-Pro-PNA and Gly-Pro-PNA.[32,33] 20–50 mg of the samples were suspended in Tris-base buffer (pH 7.0 for PEP activity and pH 8.3 for DPP-4 activity), and homogenized with a TissueLyser for 2 min. Samples were centrifuged (3000 × *g*, 20 min) and 20 μL of the supernatant was incubated with the corresponding substrate. The enzymatic activity was measured in a kinetic of 30 min at 380 nm (SpectraMax M2, Molecular Devices). Both enzymatic activities were quantified with a standard curve of free PNA and the values normalized by the amount of protein quantified with the Bradford method.

2.10. Protein Extraction and Immunoblotting

Proteins were extracted and separated from muscle gastrocnemius and liver as previously described with slight modifications.[34] Membranes were incubated with the following primary antibodies: phospho-Akt (Ser473), phospho-ribosomal protein S6 (rpS6, Ser235/236) (Cell Signaling Technology, Leiden, The Netherlands), phospho-forkhead box O (FOXO)3a (Thr32) (Millipore, Burlington, MA, USA). The μ-actin (Abcam, Cambridge, UK) and α-tubulin (Sigma) were used as a loading control. All the antibodies were used in a 1:1000 dilution in Tris-buffered saline with Tween-20 containing 1% of bovine serum albumin. The detailed protocol is shown in Supporting Information.

2.11. Statistical Analysis

The number of mice allocated per group was based on previous experiments of the research group allowing the detection of the primary outcome (the WD-induced increase in body weight) with a minimal number of animals.[21] Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison tests using GraphPad software (version 5, San Diego, CA, USA). For the body weight evolution, a two-way ANOVA followed by Bonferroni post-test was performed. For gut microbiota analyses, significantly affected operational taxonomic units were identified using one-way ANOVA followed by Tukey post-tests in R. The *p*-value of the one-way ANOVA test was adjusted (*q*-value) to control for the false discovery rate (FDR) for multiple tests according to the Benjamini and Hochberg procedure.[35] Associations between the abundance of bacteria and the content of gluten peptides were based on Spearman correlation. The results were considered statistically significant at $p < 0.05$. For all analyses, any exclusion decision was supported by the Grubbs test for outlier detection. Plots were generated using GraphPad Prism version 5.

2.12. Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. The sequences used for analysis can be found in the SRA database under the accession ID PRJNA549149.

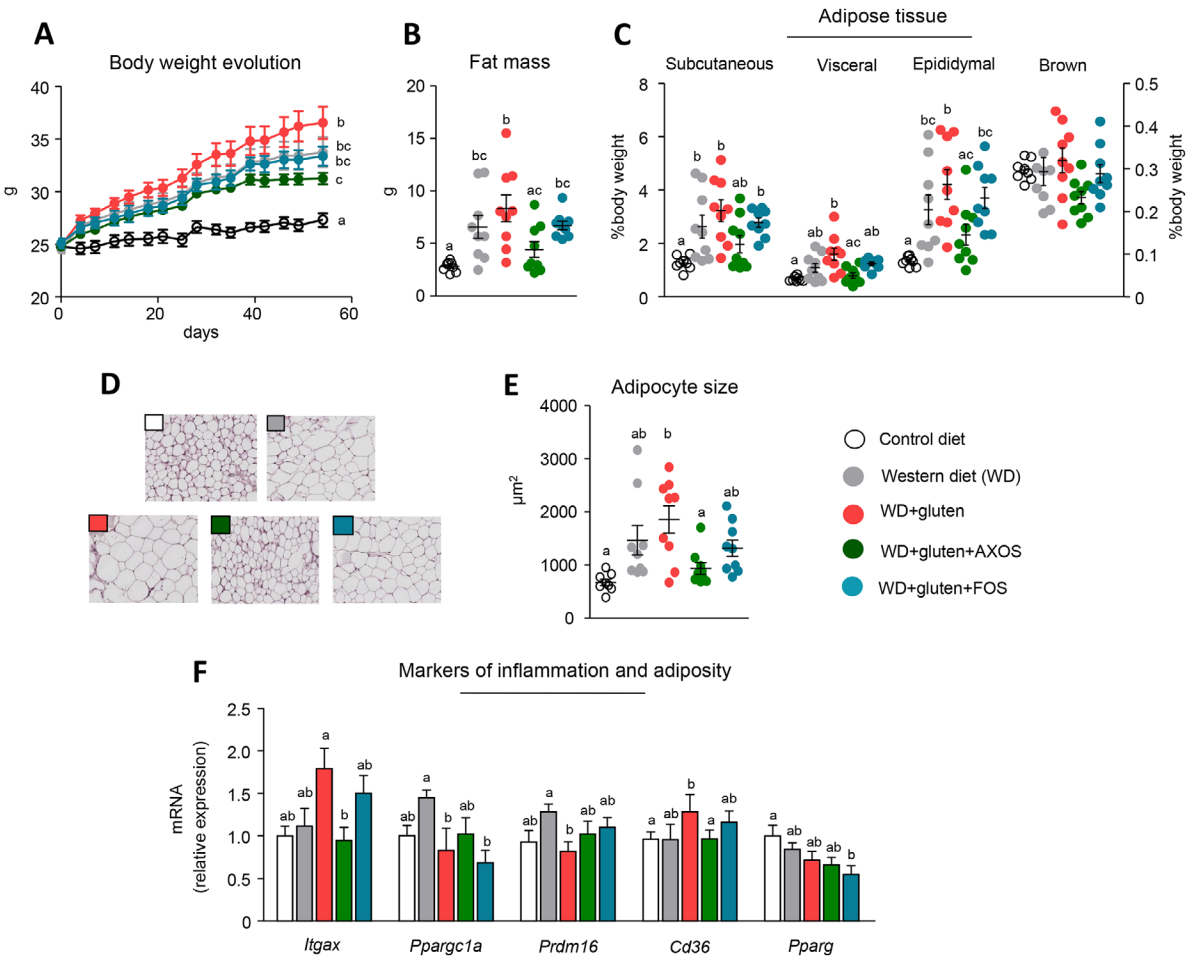

Figure 1. A) Body weight evolution, B) weight of the fat mass, C) percentage of the white pads (Yscale on the left) and brown adipose tissue pads (Yscale on the right) with respect to the total body weight, D) histology of the visceral adipose tissue, E) size of the adipocytes, and F) expression in the subcutaneous adipose tissue of markers of expression of markers of inflammation and adiposity. Data are presented as the mean ± SEM (for A–E: $n = 8–9$; for F: $n = 6–9$. Mice were fed a control diet, Western diet (WD), WD with gluten, and WD with gluten and arabinoxilo-oligosaccharides (AXOS) or fructo-oligosaccharides (FOS) in the drinking water. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison tests, with the exception of body weight evolution that was analyzed with a two-way ANOVA followed by Bonferroni post-test. Significant differences between groups are expressed by using different superscript letters. *Itgax* codes for CD11c.

3. Results

3.1. AXOS Counteracts Body Weight Gain and Fat Mass Expansion Induced in Mice Fed a WD Containing Gluten

The WD significantly increased the body weight due to an expansion of the adipose tissue, particularly in the subcutaneous and epididymal fat pads (**Figure 1**A–C). Mice fed a WD containing gluten exhibited the highest body weight gain and adiposity, and the greater adipocyte size. However, the difference did not reach the statistical cut-off when compared to the WD (Figure 1A). Prebiotic administration prevented obesity and fat mass expansion due to WD containing gluten, although only the administration of AXOS reached significance. AXOS lessened adiposity in the visceral and epididymal fat pads and decreased the adipocyte size compared to the WD plus gluten group (Figure 1A–E). All these changes occurred without differences in the water or food intake (Table S3, Supporting Information).

While gluten tended to increase *Itgax* (coding for CD11c), AXOS significantly reduced its expression in the subcutaneous adipose tissue (Figure 1F). When compared to the WD, the addition of gluten significantly dropped the markers of browning (*Ppargc1a* and *Prdm16*) and tended to promote *Cd36* mRNA level. CD36 is involved in fatty acid uptake and storage, and in contrast to the effect of gluten, its expression is reduced by AXOS (Figure 1F).

3.2. AXOS Counteracts the Detrimental Effect of Gluten on Ectopic Lipid Deposition

Neither gluten nor the prebiotics caused differences in the systemic levels of cholesterol, NEFA, or triglycerides (Table S3, Supporting Information). When gluten was added in the WD, fasting hyperglycemia was significantly increased compared to the control diet (**Figure 2**A). Despite the high variability in the response

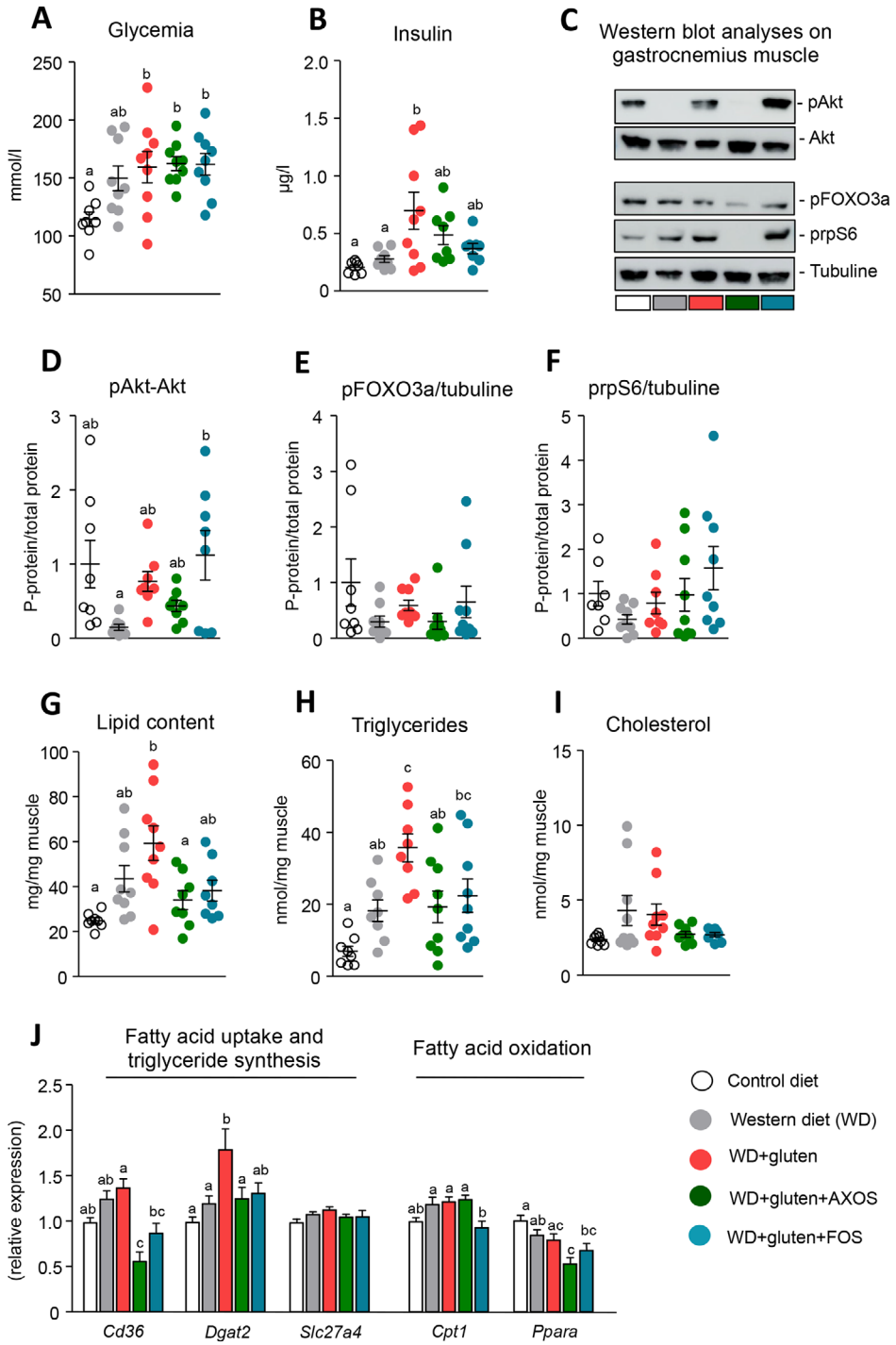

Figure 2. In the systemic circulation, values of A) glycemia and B) insulinemia. In the muscle gastrocnemius, C) representative blots of the phosphorylation of Akt, ribosomal protein S6 (rpS6) and forkhead box O (FOXO)3a, D–F) show their corresponding quantifications, G) total lipid content, H) triglycerides and I) cholesterol, and J) markers of the metabolism of lipids. Data are presented as the mean ± SEM (for A, D–J: $n = 8–9$; for B: $n = 7–9$). Mice were fed a control diet, Western diet (WD), WD with gluten, and WD with gluten and arabinoxilo-oligosaccharides (AXOS) or fructo-oligosaccharides (FOS) in the drinking water. Statistical analyses were performed by one-way ANOVA followed by post hoc Tukey's multiple comparison tests. Significant differences between groups are expressed by using different superscript letter. *Slc24a4* codes for Fatp4.

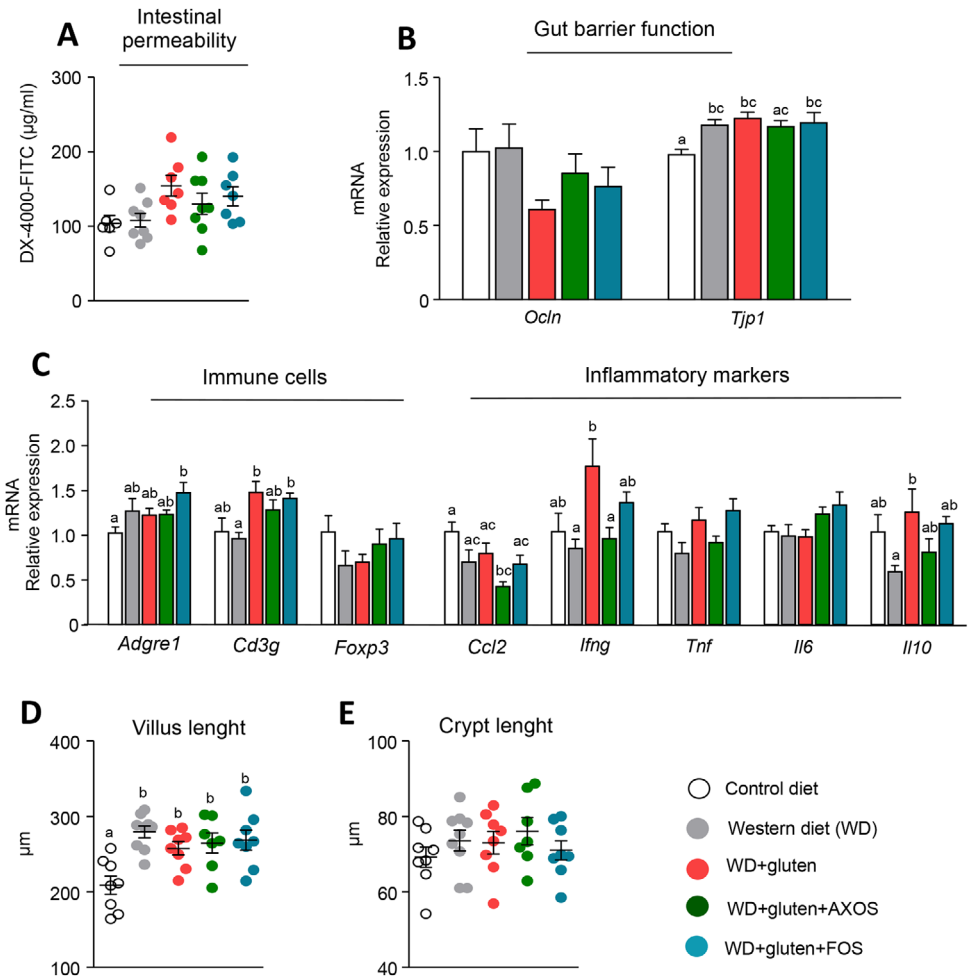

Figure 3. Gut barrier function assess A) by FITC translocation in the systemic circulation, and B) expression of tight functions markers in the ileum, C) expression of immune cells and cytokines, D) villus length, and E) crypt length in the ileum. Data are presented as the mean ± SEM (for A: n = 6–8; for B-E: n = 7–9). Mice were fed a control diet, Western diet (WD), WD with gluten, and WD with gluten and arabinoxilo-oligosaccharides (AXOS) or fructo-oligosaccharides (FOS) in the drinking water. Statistical analyses were performed by one-way ANOVA followed by post hoc Tukey's multiple comparison tests. Significant differences between groups are expressed by using different superscript letter. *Tjp1* codes for Zo1 (tight junction protein 1), *Adgre1* codes for F4/80 (adhesion G protein-coupled receptor E1).

of the gluten group, the values of insulinemia were also significantly higher in the WD with gluten when compared to the control and WD diets (Figure 2B). Of note, this effect in the gluten group was not related to a "cage effect."

Prebiotics did not significantly modify these parameters. Akt phosphorylation and two of its direct targets—the FOXO3a transcription factor and the protein rpS6—were analyzed by Western blot to evaluate the insulin signaling pathway activation in the gastrocnemius muscle. The administration of gluten and FOS led to an increase in pAkt/Akt in the skeletal muscle as compared to the WD alone (Figure 2C,D). No differences in the FOXO3a or rpS6 activation were found (Figure 2E,F). Gluten addition in the WD led to fat accumulation (increase in triglycerides but not in cholesterol compared to the WD group) (Figure 2H,I). This effect was prevented by AXOS supplementation (Figure 2G,H). AXOS lowers fatty acid uptake and esterification as illustrated by the reduction in the expression of *Cd36* and *Dgat2* (Figure 2J).

In the liver, AXOS also significantly decreased both triglycerides and cholesterol levels, without affecting hepatic insulin signaling and inflammation (Figure S1, Supporting Information).

3.3. Gluten and Prebiotics Do Not Affect Intestinal Permeability and Have Minor Effects on Ileal Inflammation

When added in the WD, gluten did not modify the translocation of FITC-Dextran (gut permeability) and did not induce any significant change in the ileal expression of occludin and tight junction proteins (**Figure 3**A,B). In the ileum, we also measured the expression of immune cells and inflammatory markers. No significant changes of *Tnf* or *Il6* due to gluten or prebiotic occurred. Compared to the WD group, gluten increased the expression of *Cd3g* (a marker of T cells) as well as the expression of the cytokines *Il-10* and *Ifng*, the latter effect being counteracted by

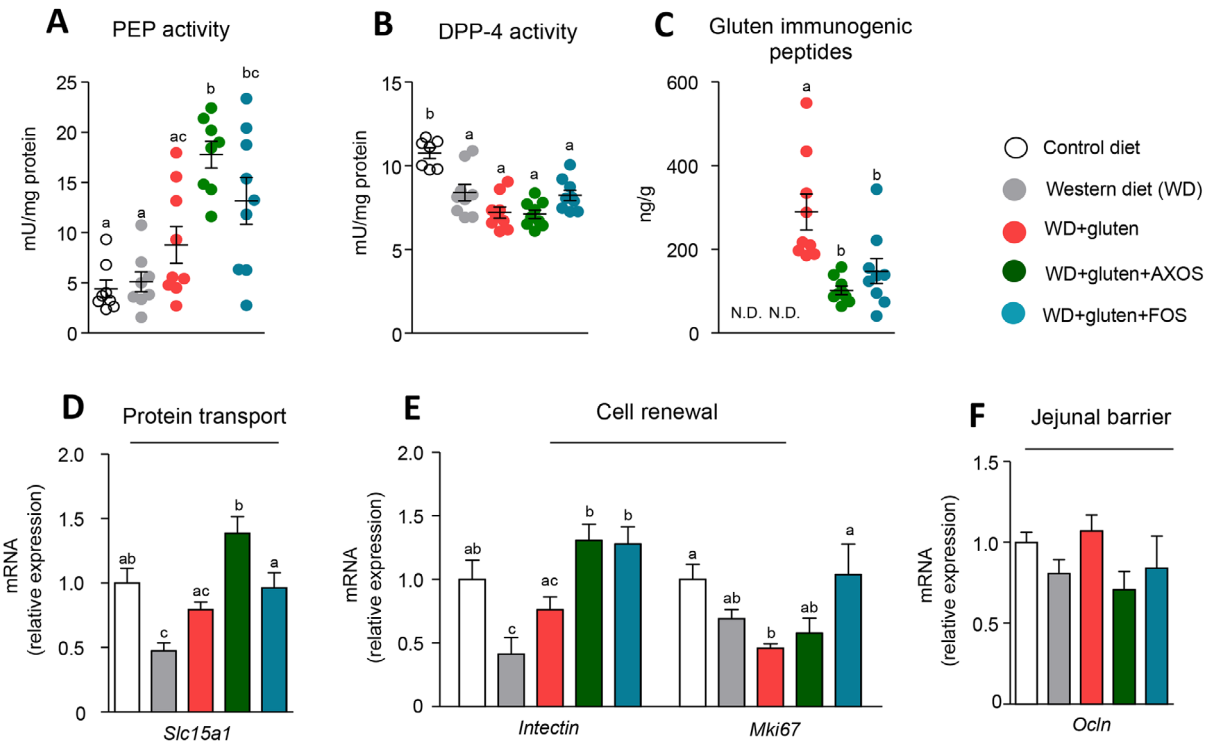

Figure 4. A) Prolyl endopeptidase (PEP) and B) dipeptidyl peptidase 4 (DPP-4) activities in the jejunal mucosa, C) content of gluten immunogenic peptides in the cecal content, expression of markers of D) protein transport, E) cell renewal, and F) tight functions markers in the jejunum. Data are presented as the mean ± SEM (for A–F: $n = 7$–9). Mice were fed a control diet, Western diet (WD), WD with gluten, and WD with gluten and arabinoxylo-oligosaccharides (AXOS) or fructo-oligosaccharides (FOS) in the drinking water. Statistical analyses were performed by one-way ANOVA followed by post hoc Tukey's multiple comparison tests. Significant differences between groups are expressed by using different superscript letter. *Slc15a1* codes for PepT1. Not detectable, N.D.

AXOS (Figure 3C). An increase in villus length without changes in crypt length occurred upon WD feeding, with no impact of gluten or prebiotics (Figure 3D,E).

Although minor, the effects of gluten on markers of the gut barrier and inflammation seem to be specific for the ileum since no differences were found in the colon (Figure S2, Supporting Information).

3.4. Prebiotics AXOS, and to a Lesser Extent FOS, Reduce the Concentration of Gluten Immunogenic Peptides in the Cecal Content

We next looked if the protective effects of AXOS preventing lipid deposition could be related to changes in gluten digestion. To test this hypothesis, we measured the activity of two of the enzymes involved in gluten cleavage. In the jejunal mucosa, AXOS significantly increased the PEP activity; FOS also caused an increased, but it did not reach the statistical significance due to the high variability within the group (**Figure 4**A). No differences in the DPP-4 activity were attributed to gluten or prebiotics (Figure 4B). A higher PEP activity could translate into changes in the concentration of gluten peptides. Indeed, we observed that both prebiotics caused a significant reduction in gluten immunogenic peptides in the cecal content (Figure 4C). It has been suggested that prebiotics and gluten could modify intestinal motility and/or gastric emptying that could modify nutrient excretion.[36] However, the changes of fecal gluten content are not likely explained by the differences in the intestinal motility since we did not observe any change in the 24 h fecal mass excretion (Table S3, Supporting Information). In the AXOS group, a more efficient digestion of gluten was associated with a higher absorption in the jejunum of small peptides as concluded from the increase in the expression of *Slc15a1* (coding for the oligopeptide transporter PepT1, which mediates the uptake of di- and tripeptides from the lumen into the enterocytes) (Figure 4D). The PEP activity is considered a differentiation marker of the intestinal mucosa,[37] and in addition, we quantified the expression of *Intectin* and *Mki67*, two additional markers of cell renewal.[38,39] Both prebiotics, AXOS and FOS, increased the expression of *Intectin*, with FOS also impacting *Mki67* (Figure 4E). These effects of FOS and AXOS on the jejunum occurred without any change in the gut permeability assessed by dextran FITC (Figure 3A), and without affecting the expression of occludin in the jejunum (Figure 4F) or inflammatory markers (Figure S3, Supporting Information).

3.5. Prebiotics Influence the Gut Microbiota Composition, Gluten Having a Very Limited Impact

Changes in the gut microbiota could contribute to the effects observed above. AXOS and FOS are largely and fully fermented in the ceco-colon, which is translated by an increase in cecal tissue and cecal content, as shown in many studies in mice,[20,21,40,41]

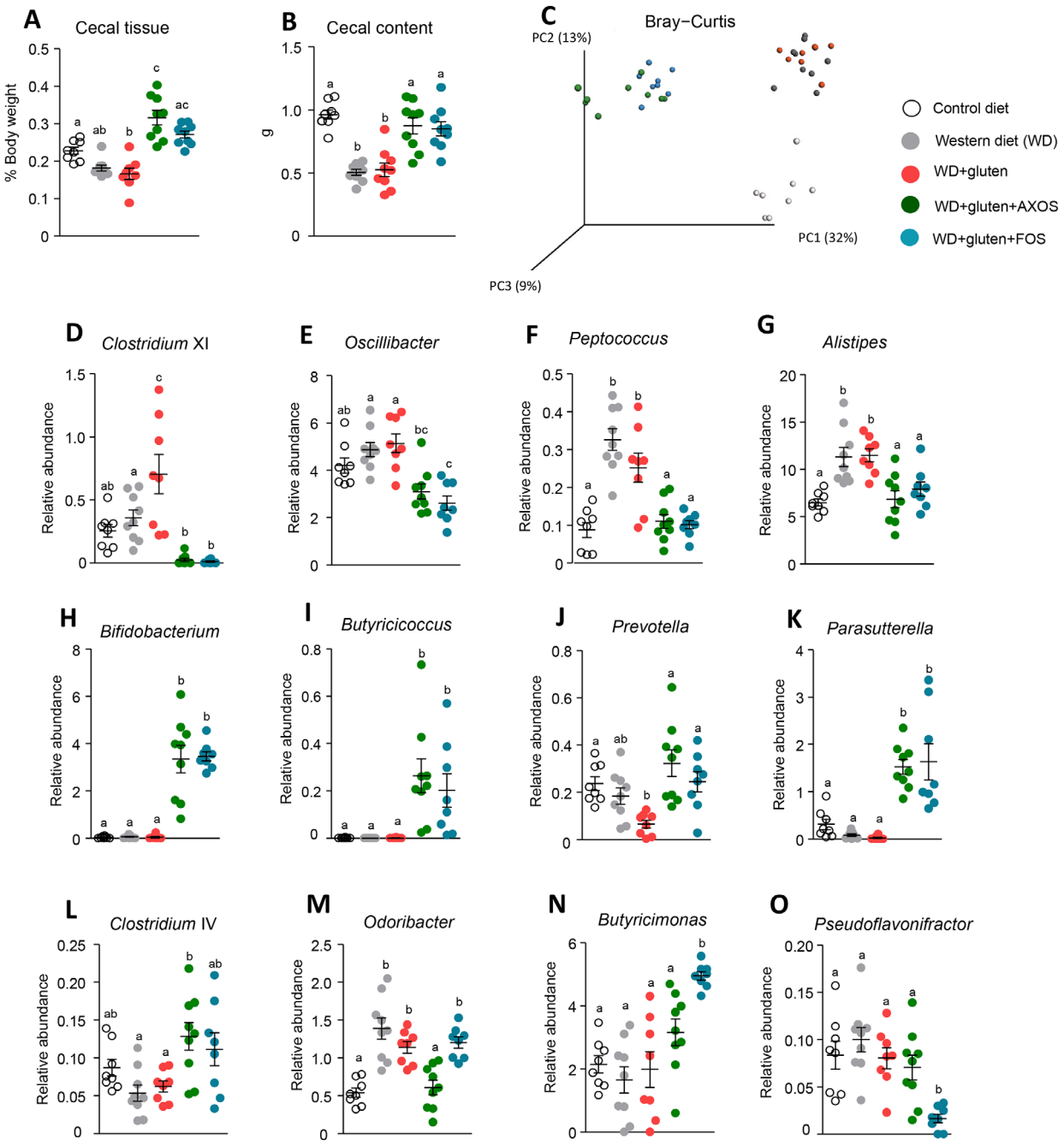

Figure 5. A) Weight of the cecal tissue, normalized by total body weight, and B) weight of the cecal content, C) principal coordinate analysis (PCoA) plot of β-diversity based on Bray–Curtis distance, and D–O) genera modified by gluten, or any of the prebiotics tested assess by 16S rDNA sequencing (Illumina Miseq). Data are presented as the mean ± SEM (for A–F: n = 8–9). Mice were fed a control diet, Western diet (WD), WD with gluten, and WD with gluten and arabinoxilo-oligosaccharides (AXOS) or fructo-oligosaccharides (FOS) in the drinking water. The relative abundance is expressed as percentage of the total read number. Data were analyzed by one-way ANOVA followed by post hoc Tukey's multiple comparison tests. The p-value was corrected for multiple tests. Significant differences between groups are expressed by using different superscript letter.

which was confirmed here (**Figure 5**A,B). In the cecal content, we investigated the composition of the gut microbiota using 16S rDNA sequencing. Principal coordinate analyses of β-diversity based on Bray–Curtis distance revealed three clear clusters: one for the mice fed a control diet, one for the WD and WD with gluten groups and one for the prebiotics groups (Figure 5C). Other indexes of β-diversity showed a similar clustering (Figure S4, Supporting Information). Six indexes of α-diversity are also shown in Figure S4, Supporting Information. Of the latter, AXOS increased the richness compared to the WD independently of gluten treatment, while FOS is associated with a reduction in evenness.

A total of 162 taxa were identified, of which 73 were significantly different between the five groups when the FDR was applied for *p*-value correction (Table S4, Supporting Information). Looking at the taxonomic distribution, 19 of these taxa corresponded to the genus level. Specifically, one genus (*Clostridium XI*) was modified by the addition of gluten to WD, whereas ten genera were modified by the addition of AXOS or FOS in the WD diet (Figure 5D–O). The seven remaining taxa comprise differences between other groups (Figure S5, Supporting Information). As commented above, the addition of gluten to the WD diet caused minor changes in the gut microbiota composition as it only increased the relative abundance of the genus *Clostridium* XI (Figure 5D). As expected from the multivariate analyses, the influence of both prebiotics was broader, including shared changes, like the reduction in *Oscillibacter*, *Peptococcus* and *Alistipes* (Figure 5E–G); or the increase in *Bifidobacterium*, *Butyricicoccus*, *Prevotella*, and *Parasutterella* (Figure 5H–K). These four genera together with *Clostridium* IV showed a negative correlation with the concentration of gluten immunogenic peptides in the cecal content; whereas the correlation was positive between gluten peptides and *Peptococcus* and *Alistipes* (Figure S6, Supporting Information).

Additional changes were specific to AXOS, such as the increase in *Clostridium* IV (Figure 5L) and the reduction in *Odoribacter* (Figure 5M), or in the case of FOS, the increase in *Butyricimonas* (Figure 5N) and the decrease in the relative abundance of *Pseudoflavonifractor* (Figure 5O).

4. Discussion

Gluten has a high content of proline that confers partial resistance to digestion and leads to the generation of peptides with biological activity. The activities of gluten peptides are not limited to the induction of an autoimmune response, but may also affect gluten-tolerant individuals.[42,43] Here, we replaced the 5% of the casein of the WD by gluten, in a way that we obtained two isocaloric obesogenic diets. With this approach we assessed, first, the contribution of dietary gluten on obesity development and metabolic dysfunction and, second, the beneficial effect of two prebiotics—AXOS and FOS—that can be extracted from gluten-containing cereals.

As reported previously in mice fed diets with very high-fat levels (60% kcal as energy from fat),[11–13] we showed here that the addition of gluten to the WD (45% kcal as energy from fat) promotes adiposity by decreasing markers of browning and thermogenesis in the subcutaneous fat. The adipocyte size tended to increase due to the addition of gluten in the WD diet; this contrasts with the findings of other authors who reported a trend toward a decrease.[13] In agreement with our study, Freire et al. detected greater body weight and less energy expenditure when gluten was added in a high-fat diet.[12] This response was also observed in a control diet enriched with gluten demonstrating that gluten can disrupt fatty acid metabolism independently of the level of fat intake.[12] Accordingly, an intervention study in humans showed that a low-gluten diet resulted in weight loss.[17] The observed reduction in body weight was explained by an enhanced thermogenesis due to increase in the hormone peptide YY and b-aminoisobutyric acid.[17] If changes in these two mediators can also explain the lower thermogenesis observed here will need further investigation.

In addition to the effect on the adipose tissue, we show that dietary gluten also leads to fat accumulation in the skeletal muscle. This effect is explained by a higher expression of *Dgat2*, which encodes acyl-CoA:diacylglycerol acyltransferase that catalyzes triglycerides synthesis. Accordingly, the overexpression of *Dgat2* in mice induces lipid accumulation in the muscle and is accompanied by impaired insulin signaling.[44] In humans, the amount of intermuscular adipose tissue is positively associated with insulin resistance and increased risk of type 2 diabetes.[44,45] Despite the considerable variability in the response, we find out that gluten increased the levels of insulin in the systemic circulation. However, given that mice have different insulinemia in the basal state, we cannot evaluate if gluten drove to insulin resistance in the skeletal muscle. We observed that the addition of 5% of gluten into a high fat/high sugar diet (Western diet) for 8 weeks induced an increase in lipid content in the muscle, but not in the liver. This contrasts with a previous study, where the inclusion of gliadin (the water-insoluble component of gluten) into a high-fat diet (60% kcal from fat) induced hepatic lipid accumulation.[13] Probably, the differences in the experimental procedures (e.g., different diet composition, lasting of the treatment) explain the discrepancies across studies.

Our work innovates in evaluating the protective effect of oligosaccharides obtained from arabinoxylans and fructans when administered with gluten. Both prebiotics have previously been shown to improve obesity and related metabolic disorders in nutritional and genetic models of obesity.[46] Here we show that AXOS, and FOS to a lower extent can oppose to the detrimental effect of gluten on metabolism. AXOS prevents body weight gain, and in the adipose tissue, it reduces the hypertrophy of adipocytes and the expression of *Itgax* (coding for CD11c) which is the primary subpopulation of macrophages increased in adipose tissue in obesity.[47] AXOS also reduces triglyceride accumulation in skeletal muscle and liver, a protective effect that can be linked to a lower lipid uptake as illustrate the expression of *Cd36* in both tissues.

The mechanism by which gluten impairs the host metabolism might involve its translocation from the gastrointestinal tract. Accordingly, radiolabelled gluten was detected in the circulation and peripheral organs, like the adipose tissue where it influences the adipocyte metabolism,[12] or the pancreas where gluten induces the release of insulin.[48,49] AXOS—and to a lesser extent of FOS—promotes gluten digestion that is translated into a reduction of gluten immunogenic peptides in the cecal content. The methodology used here quantifies gluten immunogenic peptides of relevance in the adaptive immunity (e.g., 33-mer epitopes)[50]; however, gluten can induce an innate response.[51] Then, it remains to be investigated if AXOS and/or FOS can also be effective in reducing the content of other gluten sequences triggers of this response. In this sense, it has been already shown that microbes also can degrade wheat proteins reducing innate immunity.[52]

Gluten digestion requires diverse proteolytic enzymes. Since proline confers the resistance to protein digestion, the first class of enzymes studied to cleave gluten were those that act on proline bonds. More recently, the importance of other enzymes, such as elastases and carboxypeptidase A1 has been demonstrated.[53,54] To assess gluten digestion, we look at two canonical enzymes

involved in the proteolysis of gluten; these are the PEP and DPP-4 activities. PEP and DPP-4 enzymes hydrolyze post-proline peptides bond with the difference that the PEP cleaves internal bonds and DPP-4 cuts two residues in the N-terminus. In the AXOS group, we observed a boost in the PEP activity in the jejunal mucosa with consequences for the absorption of small peptides as showed the expression of the transporter PepT1. The PEP activity is proposed as a differentiation marker of the intestinal mucosa.[37] As we confirm here, prebiotics induce a higher intestinal cell renewal,[19] this effect might explain the higher PEP activity that has a secondary impact on gluten digestion. However, we cannot rule out that the administration of food components recalcitrant to mammalian enzymes per se (gluten and prebiotics) might have induced the higher production of PEP activity as an adaptive response to ease their digestion.

In addition to the host, enzymatic activities coded by the microbiome have also been proposed as means to detoxify gluten. In particular, *Bifidobacterium* can break gluten-derived peptides and attenuated their pro-inflammatory response.[55] Interestingly, both prebiotics promoted the bloom in *Bifidobacterium*, and also in *Butyricicoccus*, *Prevotella*, and *Parasutterella*, all of them negatively correlated with the concentration of gluten immunogenic peptides. In agreement, in human studies the genera *Bifidobacterium* and *Prevotella* have been linked to gluten metabolism.[53] Besides compositional changes, non-digestible carbohydrates (AXOS and FOS) can shift the microbial metabolism (from proteolytic to saccharolytic) that might potentially have influenced gluten digestion.[17] However, this point remains hypothetical, as we exclusively performed taxonomical analyses. Aside from commensals, opportunistic pathogenic bacteria, like *Pseudomonas*, can metabolize gluten.[56] *P. aeruginosa* with gluten-degrading elastase activity, but not the mutant blocked for the elastase, synergizes with gluten to induce more severe inflammation and villus blunting.[52] According to our data, *Pseudomonas*, which is increased in celiac disease patients, was not susceptible to modification by any of our treatments.

Other members of the gut microbiota were also profoundly influenced by prebiotics. Both prebiotics prevented the increases of the obesity-related bacteria *Alistipes* induced by the WD with and without gluten.[57,58] In other cases, the reduction of genera linked to obesity was specific to one prebiotic; for instance, AXOS decreased the genus *Odoribacter*, whereas FOS did in *Pseudoflavonifractor*.[59] In contrast to the prebiotics, gluten caused minor changes in the microbiota as only increased *Clostridium* XI, which contains species that are potential pathobionts.[13] Accordingly, the increases in *Cd3g* and *Infg* in the small intestine of this group could have been triggered by this genus or by gluten-peptides,[60,61] or both factors. We cannot exclude the contribution of certain components of wheat (e.g., α-amylase trypsin inhibitors) that are difficult to remove during gluten purification and that have proven innate activation properties.[62,63] It is particularly important when interpreting the effect of gluten on immune activation we have reported.

In conclusion, we show that gluten worsens the metabolic dysfunction of Western diet-fed mice. We also discovered that prebiotics that can be extracted from gluten-containing cereals play an interesting role to control the metabolic dysfunction induced by gluten. We propose that the modulation of the gut microbiota may contribute to changes in intestinal enzymes associated with gluten cleavage that could, in turn, contribute to the improvement of host health. Overall, our finding constitutes new evidence to back up the interest of dietary fiber and whole grains interacting with the gut microbiota to promote human health, not only in the context of metabolic disorders, as suggested by our data, but also, probably, on gluten intolerance and sensitivity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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M.O., J.R., A.M.N., and N.M.D. conceived and designed the experiments. M.O., J.R., and A.M.N. performed the animal experiments. M.O., J.R., S.A.P., and A.M.N. acquired and analyzed the data. M.O. and N.M.D. wrote the paper. J.R., A.M.N., P.D.C., and L.B.B. revised the article and provided important intellectual content. N.M.D. planned and supervised all experiments and manuscript preparation. All authors read the manuscript and approved the version to be published. The authors thank V. Allaeys, I. Blave, R. Selleslagh, and B. Es Saadi (Louvain Drug Research Institute, Université catholique de Louvain, Belgium) for their skillful technical assistance. This work was supported by EU grant 613979 (MyNewGut). M.O. is a beneficiary of a "MOVE-IN Louvain" Incoming Post-doctoral Fellowship co-funded by the Marie Curie Actions of the European Commission. P.D.C. is senior research associate of the FRS-FNRS (Fonds de la Recherche Scientifique) and is supported by the FRFS-WELBIO (WELBIO-CR-2017-02) and the Funds Baillet Latour (Grant for Medical Research 2015). N.M.D. is a recipient of grants from Wallonia (FOOD4GUT project; FiberTAG project from European Joint Programming Initiative "A Healthy Diet for a Healthy Life") and from Belgium National Scientific Research Fund (FRS-FNRS).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

arabinoxylo-oligosaccharides, fructo-oligosaccharides, gluten, prebiotics, Western diet

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