

Lactose and Fructo-oligosaccharides Increase Visceral Sensitivity in Mice via Glycation Processes, Increasing Mast Cell Density in Colonic Mucosa

Jasper B.J. Kamphuis, Bruno Guiard, Mathilde Lévêque, Maïwenn Olier, Isabelle Jouanin, Sophie Yvon, Valérie Tondereau, Pauline Rivière, Françoise Guéraud, Sylvie S. Chevolleau, et al.

▶ To cite this version:

Jasper B.J. Kamphuis, Bruno Guiard, Mathilde Lévêque, Maïwenn Olier, Isabelle Jouanin, et al.. Lactose and Fructo-oligosaccharides Increase Visceral Sensitivity in Mice via Glycation Processes, Increasing Mast Cell Density in Colonic Mucosa. Gastroenterology, 2020, 158 (3), pp.652-663.e6. 10.1053/j.gastro.2019.10.037. hal-02558299

HAL Id: hal-02558299 https://hal.inrae.fr/hal-02558299v1

Submitted on 3 Jun 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Lactose and Fructo-oligosaccharides Increase Visceral Sensitivity in Mice via Glycation Processes, Increasing Mast Cell Density in Colonic Mucosa

J.B.J. Kamphuis, B. Guiard, M. Leveque, M. Olier, I. Jouanin, S. Yvon, V. Tondereau, P. Rivière, F. Guéraud, S. Chevolleau, M.-H. Noguer-Meireles, J.-F. Martin, L. Debrauwer, H. Eutamène, V. Theodorou

 PII:
 S0016-5085(19)41528-X

 DOI:
 https://doi.org/10.1053/j.gastro.2019.10.037

 Reference:
 YGAST 62993

To appear in: *Gastroenterology* Accepted Date: 31 October 2019

Please cite this article as: Kamphuis JBJ, Guiard B, Leveque M, Olier M, Jouanin I, Yvon S, Tondereau V, Rivière P, Guéraud F, Chevolleau S, Noguer-Meireles M-H, Martin J-F, Debrauwer L, Eutamène H, Theodorou V, Lactose and Fructo-oligosaccharides Increase Visceral Sensitivity in Mice via Glycation Processes, Increasing Mast Cell Density in Colonic Mucosa, *Gastroenterology* (2019), doi: https://doi.org/10.1053/j.gastro.2019.10.037.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2019 by the AGA Institute



 Lactose and Fructo-oligosaccharides Increase Visceral Sensitivity in Mice via Glycation Processes, Increasing Mast Cell Density in Colonic Mucosa
 J.B.J. Kamphuis^{1,3}, B. Guiard², M. Leveque^{1,3}, M. Olier^{1,3}, I. Jouanin^{1,4}, S. Yvon^{1,3}, V. Tondereau^{1,3}, P. Rivière^{1,3}, F. Guéraud^{1,5}, S. Chevolleau^{1,4}, M.-H. Noguer-Meireles^{1,4}, J.-F. Martin^{1,4}, L. Debrauwer^{1,4}, H. Eutamène^{1,3*}, V. Theodorou^{1,3*}
 Institut National de la Recherche Agronomique (INRA) Toxalim, UMR1331; INRA/INP/UPS, Toulouse, France
 Centre de Recherches sur la Cognition Animale (CRCA), Centre de Biologie Intégrative (CBI), Université de Toulouse, CNRS, UPS, Toulouse, France
 Neurogastroenterology and Nutrition (NGN) team, Toxalim
 AXIOM Platform, MetaToul MetaboHUB, National Infrastructure for Metabolomics and

- 13 Fluxomics, Toulouse, France
- ⁵ Prevention and Promotion of Carcinogenesis by Food (PPCF) team, Toxalim
- 15 *Equally supervised

1

2

3

4

5

6

7

8

9

10

11

12

- 16 Corresponding Author:
- 17 Hélène Eutamène
- 18 UMR1331 INRA Toxalim
- 19 180 Chemin de Tournefeuille
- 20 31027 Toulouse, France
- 21 helene.eutamene@inra.fr
- 22 Conflict of interest statement
- 23 The authors do not have any conflicts of interest to declare.
- 24 Author Contributions
- 25 JBJK, FG, LD, MO, HE, and VT designed the experiments; JBJK, BG, ML, IJ, SY, VTo, PR, SC, M-HN-M
- 26 performed the experiments and analysed data; MO, J-FM analysed data; JBJK, HE, and VT analysed
- 27 data and wrote the manuscript; HE and VT supervised the project.
- 28 Funding
- 29 The research leading to these results has received funding from the People Programme of the EU's
- 30 7th Framework Programme under REA grant agreement no. 607652 (ITN NeuroGut).

1 Abstract

Background and Aims: Irritable bowel syndrome (IBS) is characterized by abdominal pain, bloating,
 and erratic bowel habits. A diet low in fermentable oligo-, di-, mono-saccharides and polyols
 (FODMAPs) can reduce symptoms of IBS, possibly by reducing microbial fermentation products. We
 investigated whether ingestion of FODMAPs can induce IBS-like visceral hypersensitivity mediated by
 fermentation products of intestinal microbes in mice.

7 Methods: C57BI/6 mice were gavaged with lactose, with or without the anti-glycation agent 8 pyridoxamine, or saline (controls) daily for 3 weeks. A separate group of mice were fed a diet 9 containing fructo-oligosaccharides, with or without pyridoxamine in drinking water, or a normal chow diet (controls) for 6 weeks. Feces were collected and analyzed by 16S rRNA gene sequencing 10 11 and bacterial community analyses. Abdominal sensitivity was measured by electromyography and mechanical von Frey filament assays. Colon tissues were collected from some mice and analyzed by 12 13 histology and immunofluorescence, to quantify mast cells and expression of advanced glycosylation 14 end-product specific receptor (AGER).

Results: Mice gavaged with lactose or fed fructo-oligosaccharides had increased abdominal sensitivity compared with controls, associated with increased numbers of mast cells in colon and expression of the receptor for AGER in proximal colon epithelium. These effects were prevented by administration of pyridoxamine. Lactose and/or pyridoxamine did not induce significant alterations in the composition of the fecal microbiota. Mass spectrometric analysis of carbonyl compounds in fecal samples identified signatures associated with mice given lactose or fructo-oligosaccharides vs controls.

22 **Conclusion**: We found that oral administration of lactose or fructo-oligosaccharides to mice 23 increases abdominal sensitivity, associated with increased numbers of mast cells in colon and 24 expression of AGER; these can be prevented with an anti-glycation agent. Lactose and/or 25 pyridoxamine did not produce alterations in fecal microbiota of mice. Our findings indicate that 26 preventing glycation reactions might reduce abdominal pain in patients with IBS with sensitivity to 27 FODMAPs.

28 **KEY WORDS**: functional bowel disorder, mouse model, mastocytes, advanced glycation end products

1 Introduction

2 Irritable Bowel Syndrome (IBS) is a functional gastrointestinal disorder (FGID) characterized by abdominal pain, bloating, erratic bowel habits, and variable changes in the consistency of stools ^{1, 2}. 3 4 It is a heterogeneous disorder, with 4 defined sub-types; Diarrhoea (IBS-D) or Constipation Predominant IBS (IBS-C), Mixed bowel habits (IBS-M), or Unclassified (IBS-U) ³. Because the 5 6 underlying causes for IBS are not well understood, it has proven difficult to design evidence-based therapies with a clear mechanism of effect. However, in recent years a low-FODMAP (Fermentable 7 Oligo-, Di-, Mono-saccharides And Polyols⁴) diet has been successfully used to reduce symptoms of 8 9 IBS ⁵⁻⁸. Dietary FODMAPs have properties that can lead to distension; they are poorly absorbed in the 10 small intestine, osmotically active, and are fermented by the gut microbiota upon reaching the colon ⁹. These dietary components, characterized for IBS patients, can also induce gastrointestinal 11 12 symptoms in healthy subjects. For example, inulin, a fructo-oligosaccharide, can lead to gastrointestinal symptoms such as flatulence and gut cramps¹⁰. 13

14 At first sight, evasion of dietary FODMAPs helps to prevent problematic gut distension, thus alleviating symptom generation in IBS patients¹¹, but it is not necessarily expected to ameliorate 15 underlying reasons for the increased sensitivity to distension. Dietary carbohydrate fermentation has 16 17 been shown to influence seemingly unrelated symptoms as well. A link between perceived food intolerances (mostly to fermentable carbohydrates, or gluten) and IBS symptoms, but also 18 musculoskeletal pain, and fatigue has been described ¹². Lactose and fructose intolerant FGID 19 20 patients report headaches and tiredness in response to a challenge with these carbohydrates at an even higher rate than the more classical symptoms, such as diarrhoea or gastrointestinal cramps ¹³. 21 22 This indicates that dietary carbohydrate fermentation might promote symptoms through other pathways than intestinal distension alone. According to our knowledge, it has not been shown yet 23 that the efficacy of the low-FODMAP diet is higher in IBS-patients presenting visceral 24 25 hypersensitivity, which is not omnipresent in IBS patients^{14, 15}. Apart from gas production and osmotic distension due to FODMAP ingestion, microbial fermentation products have been raised as 26 27 factors involved in symptom generation.

The bacterial metabolic toxin hypothesis, proposed by Campbell et al.¹⁶ poses that harmful bacterial fermentation products, particularly those produced in anaerobic fermentation of unabsorbed carbohydrates by the gut microbiota, are responsible for effects observed from food intolerances such as lactose intolerance. Particularly such metabolites as alcohols, ketones, and aldehydes are held responsible. Indeed, methylglyoxal, a highly reactive dicarbonyl compound, increases visceral sensitivity when administered by enema to female Wistar rats, as well as inducing behaviour indicative of headache ¹⁷. The idea that increased methylglyoxal concentrations can lead to

increased sensitivity is supported by the finding that methylglyoxal drives neuropathic pain in
 diabetic patients, in part through the activation of transient receptor potential ankyrin 1 (TRPA1)^{18,}
 ¹⁹.

4 Moreover, reactive carbonyl compounds like methylglyoxal or glyoxal are major precursors to Advanced Glycation End Products (AGEs)²⁰. Protein conformation modifications by formation of 5 6 dicarbonyl adducts could interfere with the function of host proteins, and AGEs are recognized by 7 the innate immune system as damage-associated molecular patterns (DAMPs), which activates a pro-inflammatory signaling pathway²¹. An increase in such glycating agents during microbial 8 9 processing of FODMAPs in the gut could be expected to enhance the formation of AGEs, and in that way, support a pro-inflammatory state. Interestingly, mast cells can be activated by AGEs through 10 advanced glycosylation end-product specific receptor (AGER) activation²², or even by aldehydes 11 (acetaldehyde) directly ²³. These processes should not be exclusive to FGID patients, so to explain 12 13 the specific symptom generation in these patients, we propose that increased susceptibility due to 14 genetic or environmental factors, variations in intestinal permeability and microbiota, and differences in the handling of carbohydrates are likely responsible. 15

In this study, we have tested the hypothesis that production of carbonyl compounds responsible for increased non-enzymatic glycation reactions produced during fermentation of certain FODMAPs can directly or indirectly induce symptoms of IBS. We used lactose and fructo-oligosaccharides as representatives of FODMAPs in an animal model, to evaluate the effects of chronic increased FODMAP intake on visceral sensitivity and low-grade inflammation through activation of AGER. Finally, we investigated whether mast cells, known to be key players in IBS symptoms and susceptible to AGER activation, were implicated in the effects of this chronic intervention.

23

1 Materials and Methods

2 Animals and sample collection

Lactose experiments: 32 adult male C57Bl/6 mice (Janvier, Le Genest St Isle, France) of 6 weeks old
were housed in polypropylene cages in groups of 8 without mixing experimental groups, mice were
distributed randomly to groups upon arrival, and offered unlimited access to standard rodent food
(Mucedola Global Diet 2018, Harlan, Italy) and water. After a 4 days adjustment period, the lactosetreated group received a daily oral gavage, every morning, of 3mg, 5mg, or 15mg lactose (β-lactose,
Sigma Aldrich, France) in 200 µl saline solution, the control group received only saline, for 3 weeks.

Lactose-pyridoxamine experiment: 80 adult male C57Bl/6 mice (Janvier, Le Genest St Isle, France) of
6 weeks old were housed in polypropylene cages in groups of 8 without mixing experimental groups,
mice were distributed randomly to groups upon arrival, and offered unlimited access to standard
rodent food (Mucedola Global Diet 2018, Harlan, Italy) and water. After a 4 days adjustment period,
the lactose-pyridoxamine treated group received a daily oral gavage, every morning, of 5mg lactose
(β-lactose, Sigma Aldrich, France) and/or 5mg pyridoxamine (pyridoxamine dihydrochlorate, Sigma
Aldrich, France) in 200 µl saline solution, the control group received only saline, for 3 weeks.

Fructo-oligosaccharide experiments: 50 adult male C57Bl/6 mice (Janvier, Le Genest St Isle, France) of 6 weeks old were housed in polypropylene cages in groups of 10 without mixing experimental groups, mice were distributed randomly to groups upon arrival, Animals received a custom modified AIN-93M diet ad libitum, containing 0% or 10% fructo-oligosaccharides (corn-starch partly substituted for fructo-oligosaccharides) (supplementary data table S1), complemented with or without 1mg/mL pyridoxamine in drinking water.

Mice scheduled for immunofluorescence assays were euthanized by cervical dislocation, after which both 1.5 to 2 cm of distal colon and of proximal colon covering regions with and without contents were removed and stored in Carnoy's fixative overnight. Caeca were resected and weighed. Mouse fecal pellets were collected directly from the anus on the last day of the experiment, and collected in 0,5 mL Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C for downstream analyses.

All animal experiments were performed in accordance with EU directive 2010/63/EU and approved
by the local Animal Care and Use Committee of Toulouse Midi-Pyrénées (agreement CEEA-86).

29 Abdominal sensitivity

30 Visceral sensitivity (Electromyography (EMG))

31 Lactose/ Lactose-pyridoxamine experiments: Under xylazine/ketamine anaesthesia (both 1.2 mg,

32 subcutaneously), two nickel–chrome electrodes were implanted into the abdominal external oblique

1 muscle and a third into the abdominal skin and exteriorised on the back of the neck. On the fifth to 2 seventh postoperative day, colorectal distensions were used as noxious stimuli to evaluate visceral 3 hyperalgesia by electromyographic (EMG) recording. Polyethylene perfusion and distension catheters (Fogarty catheter for arterial embolectomy, 4F, Edwards Lifesciences, Nijmegen, The 4 Netherlands) were inserted into the colon. The colorectal distension procedure started 60 min after 5 6 habituation to the tunnel, progressively increasing in 0.02 mL steps, from 0 to 0.08 mL, each step 7 lasting 10 s with 5 min non-distension periods in between. During the distension periods, the striated muscle's EMG activity was recorded and analysed according to Larsson, Arvidsson, Ekman, 8 et al.²⁴. Basal EMG activity was subtracted from the EMG activity registered during the periods of 9 distension. Method adapted from Gecse, Roka, Ferrier, et al.²⁵. Statistical analysis: Two-way ANOVA, 10 multiple comparisons between all groups of the same distension, Tukey's correction for multiple 11 12 comparisons.

13 Mechanical behavioral testing (von Frey)

14 Fructo-oligosaccharide experiment: Animals were placed upon an elevated mesh floor surrounded by a clear plastic enclosure ($10 \times 10 \times 10$ cm). Mechanical sensitivity was assessed using three von 15 Frey filaments with bending force 0.16, 0.6 and 1.4 g (Bioseb Inc., France). In ascending order of 16 17 force, each filament was applied for a duration of 2 seconds to the mid-plantar area of each hind 18 paw five times, with 3 seconds between each application. Rapid retraction, shaking and/or licking of 19 the hind paw were considered to represent nociceptive specific behaviors and only one of these 20 responses needed to be displayed to be considered as a positive withdrawal response. Applications 21 were applied to both hind paws, counted and then expressed as an overall percentage response. The 22 performing researcher was blind to which experimental groups mice belonged. Statistical analysis: Two-way ANOVA, multiple comparisons between all groups for the same filament, Tukey's 23 24 correction for multiple comparisons.

- 25 Microbiota analysis
- 26 Fecal DNA extraction, 16S rRNA gene sequencing, and bacterial community analysis
- 27 For materials and methods, please refer to the supplementary data.
- 28 Immunofluorescence
- 29 Histological sample preparation

30 Collected tissues from animals that did not undergo the visceral sensitivity protocol were rinsed in

- 31 100% ethanol after 1 day in Carnoy's fixative and automatically processed using a Shandon Excelsior
- 32 ES Tissue Processor by the following program: 2x 60min 100% ethanol, 2x 60min butanol, 480min
- 33 butanol, 3x 80min paraffin at 60 °C. Tissue samples were included in paraffin blocks using a Thermo

Scientific HistoStar Embedding Workstation. 5µm tissue sections were made using a Microm HM 340
 E microtome and attached to Superfrost Plus microscope slides (Thermo Scientific, USA).

3 MMCP/AGER: 5µm paraffin embedded sections were deparaffinated by using 3x 5min baths of 4 American Mastertech Clearify followed by 3x 5min 100% ethanol, 3x 5min 95% ethanol, 2x 5min 70% 5 ethanol, 5min demineralised water. Slides were washed 2x in PBS for 5min, followed by a 2-hour 6 blocking step with 10% donkey serum in PBS, and washed 3x 5min under light agitation in PBS. Slides 7 were incubated overnight at 4°C with primary antibodies (Sheep anti-mMCP1 (MS-RM8 (Moredun Group, UK)) diluted 1:400 or Polyclonal Goat-anti-AGER (ab7764 (Abcam, UK)) diluted 1:400)), 8 9 followed by 2x 5min rinsing steps in PBS. Incubation with secondary antibody ((Alexa Fluor 594 10 Donkey-anti-Sheep (A-11016 (Molecular Probes, USA) for MMCP) or (Alexa Fluor 488 Donkey-anti-11 Goat (A-11055 (Molecular Probes, USA) for AGER)) diluted 1:400 in 1% donkey serum PBS was performed for 2 hours, followed by 3x 5min washing steps in PBS, a quick rinse with tap water, 12 13 followed by mounting using ProLong Gold® antifade reagent with DAPI (Thermo Fisher Scientific, 14 USA).

15 Microscopy

16 Samples were imaged using a Nikon Eclipse 90i microscope fitted with a DXM 1200 F Digital Camera.

17 Image sets were taken at 200x magnification.

18 Immunofluorescence analyses and statistics

19 Mast cell count analyses: One ratio mastocyte:crypt per mouse based on analysis of 50-250 crypts, 20 dependent on availability of suitable visual material. Image sets were coded, randomised and 21 analysed blindly. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction 22 for multiple comparisons.

AGER expression analyses: Fluorescence intensity of 3 suitable regions of epithelium was analysed per microscope field, 4 microscope fields per mouse were analysed. Only epithelial cells were selected, without goblet cells, because these presented high unspecific staining throughout. Image sets were coded, randomised and analysed blindly. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

28 Physiological markers of colonic content transit

Transit time: For this experiment, after 2.5 weeks of intervention, 5 mice per group were temporarily housed in individual cages, 0.15 mL paper-filtered saturated carmine red in physiological salt solution was administered by intragastric gavage, and starting from 2 hours after administration, every 15 minutes or when the researcher noted defecation, the cages were checked for red-

coloured droppings, the time of first appearance of a red dropping was used to determine the transit
 time. Statistical analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple
 comparisons.

Fecal water content: Droppings were collected in weighted tubes, weighed to determine wet weight,
and dried in an oven at 80°C for 48 hours. Tubes were weighed again and used to determine the dry
weight. Fecal humidity is the percentage of weight lost between wet and dry weights. Statistical
analysis: One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.
To compare Ctrl v Fructo-oligosaccharides: Student's t-test.

9 Verification of intestinal inflammation

Lipocalin-2 ELISA: Fecal supernatants were prepared by grinding 0.2g feces in 1 mL demineralized water with 5 ceramic beads using a Fast-Prep (MP Biomedicals, Illkirch, France) (3x 15sec 6m/s with 1 min breaks on ice) followed by 20min centrifugation at 8000x, supernatants were collected in 1.5 mL Eppendorf tubes and stored at -20°C until use. ELISAs were performed according to instructions provided by the manufacturer (DuoSet ELISA, Mouse Lipocalin-2/NGAL (DY1857)) (R&D Systems, MN, USA). One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

16 Microscopic score: For materials and methods, please refer to the supplementary data.

17 Results

18 Increase in sensitivity after FODMAP administration

19 Oral gavage with lactose for 3 weeks increased visceral sensitivity compared to control. These 20 increases were statistically significant for the two highest lactose concentrations for both 0.06 mL 21 and 0.08 mL volume of distension ($P \le 0.05$) (Supplemental Figure 1). Based on these results and to 22 reflect a modest lactose consumption, subsequent experiments with lactose were performed using 23 5mg lactose daily. In animals treated with 5mg lactose and/or 5mg pyridoxamine, visceral sensitivity 24 was also increased in the lactose-treated group by ±70 % (P<0.01), ±55% (P<0.0001) versus control, at a distension volume of 0.06 mL and 0.08 mL respectively (Figure 1). Co-administration with 25 26 pyridoxamine effectively reversed the effect of lactose to basal visceral sensitivity response, such as 27 observed in the control group (no significant differences between Lact+Pyr, Pyr and control), 28 Lact+Pyr decreased ±38% (P<0.01), ±36% (P<0.0001) v Lact at a distension volume of 0.06 mL and 29 0.08 mL respectively, and Pyr was reduced $\pm 30\%$ (P<0.05), $\pm 31\%$ (P<0.0001) v Lact at distension 30 volumes of 0.06 mL and 0.08 mL respectively.

Fructo-oligosaccharides significantly increased the abdominal sensitivity in response to mechanical
 stimulation of the hind paw (29% fructo-oligosaccharides v 14% control response rate for 0.6g

1 filament (P<0.05), and 47% v 30% response rate for 1.4g filament (P<0.05)). These increased

2 abdominal sensitivities were reduced by pyridoxamine (21% fructo-oligosaccharides-pyridoxamine v

3 29% fructo-oligosaccharides for 0.6g filament, and 39% fructo-oligosaccharides-pyridoxamine v 47%

4 control response rate for 1.4g filament)(Figure 2), but not significantly so.

5 Mast cell analysis of colonic mucosae

6 Both lactose and fructo-oligosaccharides significantly (P<0.01 v Ctrl and Lact+Pyr, P<0.001 v Pyr,

7 P<0.0001 v Ctrl and fructo-oligosaccharides+Pyr) increased the number of mucosal mast cells in the

8 proximal colon (Figure 3) versus their respective other groups. Lactose did not significantly increase

9 mast cell counts in the distal colon (Supplemental Figure 4), while the fructo-oligosaccharide diet

10 increased mast cells in both proximal and distal colon (Supplemental Figure 5).

11 Expression of AGER in epithelial cells of the proximal colon

AGER immunofluorescence intensities in proximal colon epithelial cells were higher for lactose and fructo-oligosaccharides groups (P<0.0001), compared to all other groups (Figure 4). The FOS+pyridoxamine group showed significantly lower AGER expression than the respective control group (P<0.01).

16 Transit time, fecal water content, and cecal weight

17 The fructo-oligosaccharide-enriched diet increased the fecal water content and cecal weight and 18 reduced the transit time compared to the control diet. Oral gavage of 5mg/d lactose had no 19 significant impact on these parameters. Addition of pyridoxamine did not impact these 20 characteristics in control or FODMAP-treated animals (Figure 5).

21 Microbiota

Lactose and/or pyridoxamine did not induce significant alterations in the composition of the fecal 22 23 microbiota, as demonstrated quantitatively at the OTU level by the prevalence and abundance of 24 the detected OTUs (Figure 6A) and phyla (Figure 6B) respectively. Whichever index of alpha-diversity 25 tested, neither lactose nor pyridoxamine nor a combination of the two significantly altered the 26 number of OTUs, as indicated by the index of richness (chao-1) and evenness (Shannon) (Figure 6C). 27 Multidimensional scaling analysis of unweighted or weighted UniFrac distances revealed no inter-28 sample difference linked to experimental group (Figure 6D). None of the OTUs agglomerated at the 29 genus rank were significantly affected by FODMAP exposure by using DESeq2.

- 1 Intestinal inflammation
- 2 Levels of the sensitive inflammation biomarker Lipocalin-2 were at low, non-inflamed, levels in all
- 3 animals (Figure 7). Similarly, microscopic scoring revealed no inflammation in any of the
- 4 experimental groups (Supplementary Figures 2 & 3).
- 5

Journal Prevention

1 Discussion

2 We have shown that both oral administration of lactose and a high-fructo-oligosaccharides diet can 3 lead to pro-nociceptive effects, as well as an increase in colonic mucosal mast cell counts. 4 Interestingly, these effects were common to the two different FODMAPs at different doses. This 5 indicates that the intake of FODMAPs alone can increase these sensitivities. The dose of lactose 6 (5mg per day) used was chosen to correspond to a modest intake of milk (relative amount of 1 glass of milk for a human of average size), where it has to be noted that mice do not retain lactase activity 7 after weaning and are therefore lactose malabsorbers²⁶. For fructo-oligosaccharides, we chose to 8 9 administer 10% of the total diet based on a previously reported pertinent dose in mice²⁷. Another 10 recent study comparing the effects of different fibers on short-chain fatty acids (SCFA), using doses of 10%, showed that fructo-oligosaccharides had different effects on SCFA production than lignin or 11 12 resistant starch²⁸. Because rodent diets naturally contain a great deal more fructans and glycans 13 than a conventional human diet, to be able to see the effects of an oligosaccharide in mice, we chose this higher dose than would be used in humans. 14

At lower doses of fructo-oligosaccharides (approximately 5% of total dietary intake, half of that used 15 in this work), administered through intragastric gavage, Chen et al. observed an increased 16 17 susceptibility to WAS (water avoidance stress)-induced IBS symptoms, but no difference between 18 fructo-oligosaccharides -treated or control animals in the absence of WAS. This might indicate that WAS, used as a model for IBS, increases the susceptibility to FODMAP induced effects, which could 19 20 explain why IBS patients but not healthy controls report increased discomfort when eating FODMAPs²⁹. Additionally, this indicates that without further challenge mice tolerate this dose of 21 22 FOS, which would be excessive in humans, without symptom generation.

The observed effects of lactose and the fructo-oligosaccharide diet were prevented by coadministration of with pyridoxamine, a recognized anti-glycation agent ³⁰⁻³⁵, indicating the involvement of glycation processes in the generation of the effects observed for both FODMAPs. Accordingly, the expression of AGER on colonic epithelial cells increased in response to the FODMAP experiments, and was, likewise, prevented by pyridoxamine co-administration.

The generation of glycating agents in the colonic lumen was evaluated by LC-MS analysis of carbonyl compounds in colonic contents (Supplementary Figure 4). Indeed, a subset of these compounds in colonic contents was significantly increased for FODMAP treated animals versus their respective controls. Taking into account the high chemical reactivity of certain carbonyl compounds with proteins, we can speculate that the amounts of such compounds produced in the colonic lumen of the FODMAP treated animals are sufficient to generate glycation end products, contributing to the

1 FODMAP-induced effects. Interestingly, despite the prevention of the FODMAP-induced effects by 2 pyridoxamine, no specific differences were detected in the carbonyl compound profiles of the 3 FODMAP versus the FODMAP+pyridoxamine groups, in the colonic content supernatants (Supplemental Figure 3). This can be explained by the strong capacity of the small intestine to absorb 4 pyridoxamine³⁶, leading to systemic, rather than local protection of the gut tissues against glycating 5 reactions, in a similar way as observed in work studying the prevention of glycation reactions by 6 pyridoxamine in animal models of diabetes^{33, 37, 38}. This also implies that the generation of AGEs in 7 vivo by interaction with absorbed carbonyl compounds generated during FODMAP fermentation is at 8 9 the basis of the observed effects, rather than the AGE load of the luminal contents.

10 We observed an increase in AGER expression in the mucosa of animals treated with lactose or 11 fructo-oligosaccharides, which was, again, reversible by co-administration of pyridoxamine, indicating that glycation processes and activation of advanced glycosylation end-product specific 12 13 receptor (AGER) are involved in the induction of visceral and abdominal sensitivity in our animal 14 model. In vitro, AGEs have been shown to interact directly with mast cells, rapidly triggering mast cell exocytosis dose-dependently²². This interaction could be prevented by blocking access to AGER 15 by using an antibody, showing it depends on the interaction between AGEs and AGER ²². The 16 17 observed hypermastocytosis in our animals was prevented by co-administration with pyridoxamine 18 too, indicating it is part of the same pathway that is responsible for the increased sensitivity, and 19 indeed an increased mast cell count in tissues is reported as a possible factor in increased visceral sensitivity of IBS patients ^{39, 40}. In IBS patients on a low FODMAP diet, histamine levels in the urine 20 dropped eight-fold compared to a high-FODMAP intervention⁴¹, supporting the idea that dietary 21 22 FODMAPs can be responsible for an increase in mastocyte proliferation. Mast cell counts and mast cell mediator production are associated ⁴², and mast cell mediator release in turn can lead to mast 23 cell hyperplasia ⁴³. It has been reported that histamine levels are increased in the mucosa of IBS 24 patients ^{40, 44} and it can be directly involved in the sensitization of TRPV1 by its action on HRH1, 25 which can cause symptom generation in IBS patients ⁴⁵. In short, our findings support the idea that 26 27 anaerobic microbial fermentation of FODMAPs can lead to production of glycating agents, which increase the AGE-load locally in the colon, inducing expansion of the mucosal mastocyte population, 28 29 and by mastocyte-nerve cell interactions, increasing visceral sensitivity.

Analysis of intestinal microbiota profiles of lactose experiment groups did not uncover significant differences between the profiles of the 4 groups; control, lactose, lactose-pyridoxamine, and pyridoxamine. It is well-known from literature that fructo-oligosaccharides changes the microbiota composition ^{46, 47}, but as indicated by the reversibility of the effects by pyridoxamine in both experimental groups, and the lack of effect of pyridoxamine on microbiota composition, we assess

1 that the effects of the FODMAPs were mostly due to microbial metabolic changes, rather than a 2 possible dysbiosis. This could alternatively indicate that no significant malabsorption occurs at this 3 modest dose of lactose at 5mg/day, administered by a single gavage, but it's unlikely that we would 4 have observed effects on visceral sensitivity and mast cell numbers if lactose were completely hydrolyzed. Additionally, as mentioned before, we did find differences in carbonyl compound 5 6 profiles between control and FODMAP-treated animals, which would be surprising if lactose were 7 hydrolyzed and absorbed completely in the small intestine. The generation of dicarbonyl compounds 8 every day immediately after exposition to lactose, while only shortly present due to their reactivity, 9 could lead to cumulative effects over time during the experiment because of generation of longer-10 lasting AGEs, and an increased RAGE expression, even if lactose at this dose did not lead to alteration in transit markers as could be expected for lactose malabsorption. Conversely, it has recently been 11 12 reported that a diet high in FODMAPs can induce intestinal inflammation represented by increased mucosal expression of IL-1 β , IL-6, IL-17, TNF- α , and IFN- γ , a visceral hypersensitivity, and an 13 14 increased intestinal permeability in rats, by changing the gut microbiota composition and increasing levels of lipopolysaccharides⁴⁸. We have not observed such changes in the microbiota in our work, 15 nor have we observed intestinal inflammation in either lactose or fructo-oligosaccharides-treated 16 17 groups, according to fecal lipocalin-2 levels (Figure 7) and general histology (Supplemental Figure 2), and while IBS does not normally present with these kind of inflammatory markers ⁴⁹, this interesting 18 work of Zhou et al. indicates that FODMAPs can induce symptoms in even more ways than 19 previously expected. 20

21 We have not observed changes in bowel movement characteristics (fecal water content, output, 22 transit time) in our mice treated with lactose, in contrast to the mice treated with fructo-23 oligosaccharides, which showed both a higher water content and decreased transit time compared 24 to control, though the basic diet between these 2 groups was not the same, and basal characteristics 25 between the 2 control groups were not identical. In our experiments, the application of the FODMAP 26 representatives is dissimilar between lactose and fructo-oligosaccharides. Lactose was administered 27 once daily, diluted in saline, whereas fructo-oligosaccharides were present in the animal feed, as a 28 percentage of the regular diet. This means that lactose represented chronic acute challenges over 3 29 weeks, while fructo-oligosaccharides had a permanent and bulkier presence. It is not surprising then, 30 that fructo-oligosaccharides had significantly more effects on transit time and fecal output than 31 lactose. It is also for this reason that we used the alternative analysis of sensitivity in the fructooligosaccharide-experiment, as fructo-oligosaccharides lead to increased amounts of intestinal 32 content that prevented emptying of the colorectal cavity even after fasting and habituation periods, 33 34 impeding a reliable distension procedure. The measure of effects on transit related markers (cecal

1 weight, fecal water content) in response to exposition to FOS in our experiments indicate that this 2 carbohydrate is not completely fermented and so does not mirror the human situation in a dietary 3 context. However, as mentioned before, administration of FOS at 5% of dietary intake does not induce symptoms in mice without further challenge²⁹, pointing to the different tolerance to these 4 kind of compounds in a rodent model. In contrast to effects related to aldehyde generation, 5 6 pyridoxamine did not reverse fecal water content differences between control and fructo-7 oligosaccharide diets (data not shown), indicating that, like for the lactose-treated groups, 8 differences in transit time were not responsible for the observed symptom generation for this group 9 either, although modification of transit time itself can be seen as a symptom too.

It has been clearly demonstrated that FODMAP ingestion increases water and fermentable material 10 to the proximal colon ⁵⁰, and can lead to distension through increased chyme volume and production 11 of gas ⁵¹. However, it has been found that increased sensitivity rather than increased distension is 12 the cause for complaints in IBS patients related to FODMAP consumption ¹¹ not taking away the fact 13 14 that decreasing the FODMAP intake should reduce this distension. The short-term effects observed 15 upon reducing FODMAP intake in patients are probably thanks to a reduction in distension obtained, whereas the effects obtained in our animal model are on a longer time-scale, in which FODMAPs are 16 17 involved in modulating sensitivity itself. If FODMAP fermentation products increase mast cell numbers, factors that activate mast cell product release such as psychological stress^{52, 53} will have a 18 19 greater effect because there is a larger population to receive these activating signals. It is unlikely 20 that FODMAPs can cause IBS by themselves, but by the effects that we describe, we can conclude 21 that they can cause physiological changes possibly responsible for symptoms of IBS. Recent work by 22 Wilder-Smith et al. in a large patient cohort indicates that Central Nervous System (CNS-) as well as gastro-intestinal (GI-)symptoms are increased in the short-term following lactose and fructose 23 ingestion, likely explained by microbial metabolites ⁵⁴. This illustrates again that FODMAP intake can 24 be linked to other symptoms of FGIDs besides those induced by increased (osmotic) distension. 25

Our study shows that the role of FODMAPs in IBS is multifactorial; apart from the previously reported osmolarity and distension related symptom-generation caused by dietary FODMAPs, reactive carbonyl fermentation products of their microbial processing can cause physiological changes in the colon, specifically reminiscent of IBS. These insights may contribute to the development of functional nutritional strategies focused on the prevention of glycation reactions caused by microbial toxic metabolites. 1 Acknowledgments

- 2 We are grateful to all members of the animal facility staff (EZOP, INRA Toxalim) for housing and
- animal care, and access to facilities. We are grateful to the Get-PlaGe platform (Toulouse) for 16S
- 4 rRNA gene libraries and sequencing, to the Genotoul bioinformatics platform Toulouse Midi-
- 5 Pyrénées and the Sigenae group for providing help and storage resources thanks to the Galaxy
- 6 instance http://sigenae-workbench.toulouse.inra.fr.

Journal Proproof

1 References

- Spiller R. Irritable bowel syndrome: new insights into symptom mechanisms and advances in treatment. F1000Res 2016;5.
- 4 2. Enck P, Aziz Q, Barbara G, et al. Irritable bowel syndrome. Nat Rev Dis Primers 2016;2:16014.
- Drossman DA. Functional Gastrointestinal Disorders: History, Pathophysiology, Clinical
 Features and Rome IV. Gastroenterology 2016.
- Gibson P, Shepherd S. Personal view: food for thought-western lifestyle and susceptibility to
 Crohn's disease. The FODMAP hypothesis. Alimentary pharmacology & therapeutics
 2005;21:1399-1409.
- 105.Halmos EP, Power VA, Shepherd SJ, et al. A diet low in FODMAPs reduces symptoms of11irritable bowel syndrome. Gastroenterology 2014;146:67-75 e5.
- 126.Pedersen N, Andersen NN, Vegh Z, et al. Ehealth: low FODMAP diet vs Lactobacillus13rhamnosus GG in irritable bowel syndrome. World J Gastroenterol 2014;20:16215-26.
- Staudacher HM, Lomer MCE, Farquharson FM, et al. A Diet Low in FODMAPs Reduces
 Symptoms in Patients With Irritable Bowel Syndrome and A Probiotic Restores
 Bifidobacterium Species: A Randomized Controlled Trial. Gastroenterology 2017;153:936 947.
- Staudacher HM, Whelan K. The low FODMAP diet: recent advances in understanding its mechanisms and efficacy in IBS. Gut 2017;66:1517-1527.
- Gibson PR, Shepherd SJ. Evidence-based dietary management of functional gastrointestinal symptoms: The FODMAP approach. J Gastroenterol Hepatol 2010;25:252-8.
- Pedersen A, Sandström B, Van Amelsvoort JM. The effect of ingestion of inulin on blood
 lipids and gastrointestinal symptoms in healthy females. British Journal of Nutrition
 1997;78:215-222.
- Major G, Pritchard S, Murray K, et al. Colon Hypersensitivity to Distension, Rather Than
 Excessive Gas Production, Produces Carbohydrate-Related Symptoms in Individuals With
 Irritable Bowel Syndrome. Gastroenterology 2017;152:124-133 e2.
- Berstad A, Undseth R, Lind R, et al. Functional bowel symptoms, fibromyalgia and fatigue: a
 food-induced triad? Scand J Gastroenterol 2012;47:914-9.
- Wilder-Smith CH, Materna A, Wermelinger C, et al. Fructose and lactose intolerance and
 malabsorption testing: the relationship with symptoms in functional gastrointestinal
 disorders. Aliment Pharmacol Ther 2013;37:1074-83.
- Kuiken SD, Lindeboom R, Tytgat GN, et al. Relationship between symptoms and
 hypersensitivity to rectal distension in patients with irritable bowel syndrome. Aliment
 Pharmacol Ther 2005;22:157-64.
- Wilder-Smith CH, Robert-Yap J. Abnormal endogenous pain modulation and somatic and
 visceral hypersensitivity in female patients with irritable bowel syndrome. World J
 Gastroenterol 2007;13:3699-704.
- Campbell AK, Matthews SB, Vassel N, et al. Bacterial metabolic 'toxins': a new mechanism
 for lactose and food intolerance, and irritable bowel syndrome. Toxicology 2010;278:268-76.
- 41 17. Zhang S, Jiao T, Chen Y, et al. Methylglyoxal induces systemic symptoms of irritable bowel
 42 syndrome. PLoS One 2014;9:e105307.
- 4318.Koivisto A, Pertovaara A. Transient receptor potential ankyrin 1 (TRPA1) ion channel in the44pathophysiology of peripheral diabetic neuropathy. Scand J Pain 2013;4:129-136.
- 45 19. Barragan-Iglesias P, Kuhn J, Vidal-Cantu GC, et al. Activation of the integrated stress
 46 response in nociceptors drives methylglyoxal-induced pain. Pain 2019;160:160-171.
- 4720.Thornalley PJ, Langborg A, Minhas HS. Formation of glyoxal, methylglyoxal and 3-48deoxyglucosone in the glycation of proteins by glucose. Biochem J 1999;344 Pt 1:109-16.
- Thomas MC, Woodward M, Neal B, et al. Relationship between levels of advanced glycation
 end products and their soluble receptor and adverse outcomes in adults with type 2
 diabetes. Diabetes Care 2015;38:1891-7.

1 2	22.	Sick E, Brehin S, Andre P, et al. Advanced glycation end products (AGEs) activate mast cells. Br J Pharmacol 2010:161:442-55.
3	23.	Ferrier L. Berard F. Debrauwer L. et al. Impairment of the intestinal barrier by ethanol
4		involves enteric microflora and mast cell activation in rodents. Am J Pathol 2006;168:1148-
5		
6	24.	Larsson M, Arvidsson S, Ekman C, et al. A model for chronic quantitative studies of colorectal
/		sensitivity using balloon distension in conscious mice-effects of opioid receptor agonists.
8		Neurogastroenterology & Motility 2003;15:371-381.
9	25.	Gecse K, Roka R, Ferrier L, et al. Increased faecal serine protease activity in diarrhoeic IBS
10		patients: a colonic lumenal factor impairing colonic permeability and sensitivity. Gut
11		2008;57:591-9.
12	26.	Detel D, Baticic L, Varljen J. The influence of age on intestinal dipeptidyl peptidase IV (DPP
13		IV/CD26), disaccharidases, and alkaline phosphatase enzyme activity in C57BL/6 mice. Exp
14		Aging Res 2008;34:49-62.
15	27.	Kashyap PC, Marcobal A, Ursell LK, et al. Complex interactions among diet, gastrointestinal
16		transit, and gut microbiota in humanized mice. Gastroenterology 2013;144:967-977.
17	28.	Kaur A, Tuncil YE, Sikaroodi M, et al. Alterations in the amounts of microbial metabolites in
18		different regions of the mouse large intestine using variably fermentable fibres. Bioactive
19		Carbohydrates and Dietary Fibre 2018;13:7-13.
20	29.	Chen B-R, Du L-J, He H-Q, et al. Fructo-oligosaccharide intensifies visceral hypersensitivity
21		and intestinal inflammation in a stress-induced irritable bowel syndrome mouse model.
22		World journal of gastroenterology 2017;23:8321-8333.
23	30.	Berner AK, Brouwers O, Pringle R, et al. Protection against methylglyoxal-derived AGEs by
24		regulation of glyoxalase 1 prevents retinal neuroglial and vasodegenerative pathology.
25		Diabetologia 2012;55:845-54.
26	31.	Cardoso S, Carvalho C, Marinho R, et al. Effects of methylglyoxal and pyridoxamine in rat
27		brain mitochondria bioenergetics and oxidative status. J Bioenerg Biomembr 2014;46:347-
28		55.
29	32.	de Arriba SG, Stuchbury G, Yarin J, et al. Methylglyoxal impairs glucose metabolism and leads
30		to energy depletion in neuronal cellsprotection by carbonyl scavengers. Neurobiol Aging
31		2007;28:1044-50.
32	33.	Illien-Junger S, Grosjean F, Laudier DM, et al. Combined anti-inflammatory and anti-AGE
33		drug treatments have a protective effect on intervertebral discs in mice with diabetes. PLoS
34		One 2013;8:e64302.
35	34.	Voziyan PA, Hudson BG. Pyridoxamine as a multifunctional pharmaceutical: targeting
36		pathogenic glycation and oxidative damage. Cell Mol Life Sci 2005;62:1671-81.
37	35.	Colzani M, De Maddis D, Casali G, et al. Reactivity, Selectivity, and Reaction Mechanisms of
38		Aminoguanidine, Hydralazine, Pyridoxamine, and Carnosine as Sequestering Agents of
39		Reactive Carbonyl Species: A Comparative Study. ChemMedChem 2016;11:1778-89.
40	36.	Hamm MW, Mehansho H, Henderson LM. Transport and metabolism of pyridoxamine and
41		pyridoxamine phosphate in the small intestine of the rat. J Nutr 1979;109:1552-9.
42	37.	Tanimoto M, Gohda T, Kaneko S, et al. Effect of pyridoxamine (K-163), an inhibitor of
43		advanced glycation end products, on type 2 diabetic nephropathy in KK-A(y)/Ta mice.
44		Metabolism 2007;56:160-7.
45	38.	Davies SS, Zhang LS. Reactive Carbonyl Species Scavengers—Novel Therapeutic Approaches
46		tor Chronic Diseases. Current pharmacology reports 2017;3:51-67.
47	39.	Wouters MM, Vicario M, Santos J. The role of mast cells in functional GI disorders. Gut
48		2016;65:155-68.
49	40.	Barbara G, Stanghellini V, De Giorgio R, et al. Activated mast cells in proximity to colonic
50		nerves correlate with abdominal pain in irritable bowel syndrome. Gastroenterology
51		2004;126:693-702.

1 41. McIntosh K, Reed DE, Schneider T, et al. FODMAPs alter symptoms and the metabolome of 2 patients with IBS: a randomised controlled trial. Gut 2017;66:1241-1251. 3 42. Vicario M, Guilarte M, Alonso C, et al. Chronological assessment of mast cell-mediated gut 4 dysfunction and mucosal inflammation in a rat model of chronic psychosocial stress. Brain 5 Behav Immun 2010;24:1166-75. 6 43. Marshall JS. Repeated antigen challenge in rats induces a mucosal mast cell hyperplasia. 7 Gastroenterology 1993;105:391-8. 8 44. Barbara G, Cremon C, Carini G, et al. The immune system in irritable bowel syndrome. J 9 Neurogastroenterol Motil 2011;17:349-59. 10 45. Wouters MM, Balemans D, Van Wanrooy S, et al. Histamine receptor H1-mediated 11 sensitization of TRPV1 mediates visceral hypersensitivity and symptoms in patients with irritable bowel syndrome. Gastroenterology 2016;150:875-887. e9. 12 13 46. Howard MD, Gordon DT, Garleb KA, et al. Dietary fructooligosaccharide, xylooligosaccharide 14 and gum arabic have variable effects on cecal and colonic microbiota and epithelial cell 15 proliferation in mice and rats. J Nutr 1995;125:2604-9. 47. Respondek F, Gerard P, Bossis M, et al. Short-chain fructo-oligosaccharides modulate 16 17 intestinal microbiota and metabolic parameters of humanized gnotobiotic diet induced 18 obesity mice. PLoS One 2013;8:e71026. Zhou SY, Gillilland M, 3rd, Wu X, et al. FODMAP diet modulates visceral nociception by 19 48. 20 lipopolysaccharide-mediated intestinal inflammation and barrier dysfunction. J Clin Invest 21 2018;128:267-280. 22 49. Bennet SM, Polster A, Tornblom H, et al. Global Cytokine Profiles and Association With 23 Clinical Characteristics in Patients With Irritable Bowel Syndrome. Am J Gastroenterol 24 2016;111:1165-76. 25 50. Barrett JS, Gearry RB, Muir JG, et al. Dietary poorly absorbed, short-chain carbohydrates 26 increase delivery of water and fermentable substrates to the proximal colon. Aliment 27 Pharmacol Ther 2010;31:874-82.

- Shepherd SJ, Lomer MC, Gibson PR. Short-chain carbohydrates and functional
 gastrointestinal disorders. Am J Gastroenterol 2013;108:707-17.
- 3052.Gue M, Del Rio-Lacheze C, Eutamene H, et al. Stress-induced visceral hypersensitivity to31rectal distension in rats: role of CRF and mast cells. Neurogastroenterol Motil 1997;9:271-9.
- S3. Van Nassauw L, Adriaensen D, Timmermans JP. The bidirectional communication between
 neurons and mast cells within the gastrointestinal tract. Auton Neurosci 2007;133:91-103.
- Wilder-Smith CH, Olesen SS, Materna A, et al. Fermentable Sugar Ingestion, Gas Production,
 and Gastrointestinal and Central Nervous System Symptoms in Patients With Functional
 Disorders. Gastroenterology 2018;155:1034-1044 e6.
- 37

38

Journal Pre-proof

1 Figure legends

2 Figure 1: Increase in visceral sensitivity after daily gavage with lactose (Lact) and/or pyridoxamine

3 (Pyr) for 3 weeks, in response to increasing volumes of distension. Sensitivity is expressed as a
4 percentage of the maximum average control response. N=12

Figure 2: Increase in sensitivity to mechanical stimulation of the hind paw, after following a diet high
in fructo-oligosaccharides (FOS), with or without pyridoxamine (Pyr) (1mg/mL) in drinking water.
N=10

8 Figure 3: (A) Representative images of mucosal mast cell immunofluorescent staining (mast cells: 9 red; DAPI nuclear counterstain: blue) of proximal colon of animals treated with 5mg lactose and/or 10 pyridoxamine for 3 weeks. (B) Mucosal mast cell (MC) counts of proximal colon in animals treated daily with 5mg lactose (Lact) and/or pyridoxamine (Pyr) for 3 weeks, expressed as MC/crypt. (C) 11 12 Representative images of mucosal mast cell immunofluorescent staining (mast cells: red; DAPI nuclear counterstain: blue) of proximal colon of animals following a diet high in fructo-13 14 oligosaccharides (FOS), with or without pyridoxamine (1mg/mL) in drinking water. (D) Mucosal mast 15 cell (MC) counts of proximal colon of animals following a diet high in fructo-oligosaccharides, with or 16 without pyridoxamine (1mg/mL) in drinking water for 3 weeks, expressed as MC/crypt.

17 Figure 4: (A) Representative images of epithelial AGER immunofluorescent staining (AGER: green; 18 DAPI nuclear counterstain: blue) of proximal colon of animals treated with 5mg lactose (Lact) and/or 19 pyridoxamine (Pyr) for 3 weeks. (B) Intensity of AGER staining in mucosal epithelial cells of proximal 20 colon of lactose experiment. (C) Representative images of epithelial AGER immunofluorescent 21 staining (AGER: green; DAPI nuclear counterstain: blue) of proximal colon of animals following a diet 22 high in fructo-oligosaccharides (FOS), with or without pyridoxamine (1mg/mL) in drinking water. (D) Intensity of AGER staining in mucosal epithelial cells of proximal colon of fructo-oligosaccharide 23 experiment. 24

Figure 5: Fecal water contents of animals treated with FODMAPs; lactose (lact), fructooligosaccharides (FOS) (A) Fecal water content (B) cecal weight and (C) transit time of lactose groups.
(D) Fecal water content (E) cecal weight and (F) transit time of animals following a control, or a
fructo-oligosaccharide-enriched diet.

Figure 6: Effect of lactose (Lact) and/or pyridoxamine (Pyr) on the community distribution and diversity of the fecal microbiota as determined by 16S rRNA gene Illumina Miseq sequencing of animals treated with 5mg lactose and/or pyridoxamine daily. (A) Prevalence per OTU in samples according to groups (B) Relative abundance (%) per phylum according to groups (C) Richness (α-

- 1 diversity) measured by Chao1 and Simpson Indexes (D) Unweighted (left) and Weighted (right)
- 2 Unifrac Multidimensional Scaling (MDS) plots representing structural changes between groups (β-
- 3 diversity). The fraction of diversity captured by the coordinate is given as percentage.
- 4 Figure 7: Lipocalin-2 concentrations of feces, expressed as pg/mg feces, of animals treated with
- 5 lactose (A), or fructo-oligosaccharides (B) and/or pyridoxamine. None of the animals showed
- 6 increased lipocalin levels indicative of inflammation.
- 7

Journal Prevention







Diet











Supplementary data

Materials and Methods - Microbiota analysis

Faecal DNA extraction, 16S rRNA gene sequencing, and bacterial community analysis; Genomic DNA was obtained from frozen faeces using the ZR Faecal DNA MiniprepTM kit (Zymo Research). The microbial 16S rRNA gene was amplified during the first PCR step with adapter fusion primers targeting the V3 to V4 regions (corresponding to a 460-bp region of *Escherichia coli* 16S rRNA gene, GenBank number J01695 with bacterial forward 343F (TACGGRAGGCAGCAG¹) and reverse 784R (TACCAGGGTATCTAATCCT²) primers. Pooled amplicon libraries were sequenced employing an Illumina MiSeq (2 x 250 bp) at the GeT-PlaGe platform in Toulouse (France).

Sequence reads were quality controlled and high quality filtered reads were further processed using FROGS pipeline (Find Rapidly OUT with Galaxy Solution) to obtain OTUs and their respective taxonomic assignment thanks to Galaxy instance (http://sigenae-worbench.toulouse.inra.fr)³: an initial FROGS pre-processing step which allows to select overlapping reads with expected length without N. Swarm clustering method was applied by using a first run for denoising with a distance of 1 and then a second run for clustering with a maximal aggregation distance of 3 on the seeds of the first Swarm. Putative chimerae were removed using Vsearch combined with cross-validation (GitHub repository. DOI:10.5281/zenedo.15524). Cluster abundances were filtered at 0,005%⁴ and/or had to be present at least in 3 samples. 100% of clusters were affiliated to OTU by using the silva132 16S reference database and a taxonomic multi-affiliation procedure (Blast+ with equal multi-hits⁵). Taxonomic assignment at the lowest phylogenetic level and prevalence-based filtering step allowed to obtain of 468 OTUs (after correcting multi-affiliations and some misleading affiliations). Between 15 000 and 22 518 valid sequences per sample were counted.

Richness and diversity indexes of bacterial community, as well as clustering and ordinations, were computed using the Phyloseq package (v 1.19.1) in RStudio software^{6, 7}. Within sample community alpha diversity was assessed using Chao-1 and Shannon indexes. Divergence in community composition between samples was quantitatively assessed by calculating both weighted and unweighted UniFrac distance matrices. Unconstrained ordination was visualized using multidimensional scaling (MDS) and compared using Adonis test (9999 permutations).

In order to evaluate differential abundance in response to experimental treatment and identify important taxa modulated by lactose and prevented by pyridoxamine, OTUs were agglomerated at the genus rank. Univariate differential abundance of taxa was tested using a negative binomial noise model for overdispersion as implemented in the R package DESeq2 (v1.14.1^{8, 9}). A 2x2 factor design combined with a Wald test was applied. Taxa were considered significantly differentially abundant

between groups if their adjusted P-value was below 0.05 and if estimated change was log2FC>|1.5|. Tests were corrected for multiple inferences using the Benjamini-Hochberg method to control the false discovery rate.

Sequences are available on MG-RAST¹⁰, Project name JK_Lactose, temporary project ID: mgp 90034.

Journal Prevention

Dietary information

Composition custom AIN93-M +/- Fructo-oligosaccharide (FOS) diets

Table S1 - Diet composition AIN93M +/- FOS (g/kg)

	AIN-93M	AIN93-M-FOS
	(Energy Density: 3.6 kcal/g)	(Energy Density: 3.4 kcal/g)
Corn-starch	465.692	365.692
Fructo-oligosaccharides	0	100
Casein	140	140
Dextrinized corn-starch	155	155
Sucrose	100	100
Soybean oil	40	40
Powdered cellulose	50	50
Mineral mix (AIN-93M-MX)	35	35
Vitamin mix (AIN-93-VX)	10	10
I-Cystein	1.8	1.8
Choline bitartrate	2.5	2.5
Tert-butylhydroquinone	0.008	0.008

Reference: Reeves, P. G., F. H. Nielsen and G. C. Fahey, Jr. (1993). "AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet." J Nutr **123**(11): 1939-1951.¹¹

Energy densities based on Reeves *et al.*¹¹ for AIN-93M, and calculated for AIN93M-FOS using energy densities of 3.7kcal/g for cornstarch, and 1.75kcal/g for FOS¹².

Diets were prepared and mixed at the UE300 'Unité de Préparation des Aliments Expérimentaux' (UPAE) INRA Jouy-en-Josas



Supplemental figure 1 - Increase in visceral sensitivity after daily gavage with different concentrations of lactose for 3 weeks, in response to increasing volumes of distension. Sensitivity is expressed as a percentage of the maximum average control response. N=8

Jonual

Histological scoring

M&M

Histological processing, ABHE staining; 5µm paraffin embedded sections were deparaffinated by using 3x 5min baths of American Mastertech Clearify followed by 3x 5min 100% ethanol, 3x 5min 95% ethanol, 2x 5min 70% ethanol, 5min demineralised water. Staining was performed by 5 min in Hematoxylin, 10 min in running tap water, 30 min in Alcian Blue solution (pH 3.0) followed by 5 min in running water, 3 min in Eosin, 10 min in 95% ethanol, followed by dehydration (2x 4min 70% ethanol, 2x 5min 95% ethanol, 2x 5min 100% ethanol, 3x 5min American Mastertech Clearify), and finally mounted using Diamount mountant.

Manual Ultra-high resolution Composite Image Overview (MUCIO); datasets of overlapping microscope views covering entire slides were generated by manual microscope photography (single photo resolution: 1280 × 1024pixels) and stitched together using Microsoft Image Composite Editor (MICE), as originally described in Kamphuis, Mercier-Bonin, Eutamène, Theodorou (2017)¹³. Samples were imaged using a Nikon Eclipse 90i microscope fitted with a DXM 1200 F Digital Camera.

Microscopic scoring; composite micrographs were scored according to protocol¹⁴ on a scale from 0-4; 0: no signs of inflammation, 1: very low level of leukocytic infiltration, 2: low level of leukocytic infiltration, 3: high level of leukocytic infiltration, high vascular density, thickening of the colon wall, 4: transmural infiltrations, loss of goblet cells, high vascular density, thickening of the colon wall. Statistical analysis: Scores were averaged per experimental group; One-way ANOVA, multiple comparisons with Tukey's correction for multiple comparisons.

Results



Supplemental figure 2 - Representative images of sections of colon showing no signs of inflammation. (A,B): Controls of lactose, fructo-oligosaccharides (FOS) respectively. (C,D) Lactose-, and FOS-treated, respectively.



Supplemental figure 3 – Microscopic scores of Lactose-pyridoxamine experiments (A) (N=8), and Fructo-oligosaccharides (FOS)-pyridoxamine experiments (B) (N=10). Apart from a very mild presence of leukocytes in 1 or 2 individuals in each group, no signs of inflammation were observed in any group.

No macroscopic (at moment of tissue collection) or microscopic signs of inflammation have been observed in response to FODMAP administration, further excluding the presence of overt active inflammation in these experiments.

ournal prepos

LC/ MS analysis of aldehydes

M&M

Chemicals

Methanol (HPLC grade) and acetonitrile (Optima LC/MS grade) were purchased from Fisher (Illkirch, France), formic acid from Sigma Aldrich (St Quentin Fallavier, France). Ultra-pure water was obtained using a Milli-Q system (Millipore, St Quentin en Yvelines, France).

1-((ammoniooxy)methyl)-2-bromobenzene chloride (BBHA) was purchased from Interchim (Montluçon, France). Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and trifluoroacetic acid (TFA) were obtained from Acros organics (Geel, Belgium). Glyoxal, Methyglyoxal and 3-Deoxyglucosone were purchased from Sigma Aldrich (St Quentin Fallavier, France) and benzaldehyde-d5 (Internal standard) from CDN isotopes (Quebec, Canada). BBHA derivatives were synthesized in house according to previously published methods¹⁵. Briefly, BBHA (50-100 μ mol, 1-2 eq) was added to standard solutions of aldehydes (50 μ mol) in PIPES buffer (0.1 M, pH 6.5, 1 mL), and the mixture was stirred at 6-8°C for one hour. Each BBHA derivative was then purified by SPE.

Sample treatment

Intestinal content samples were prepared by homogenising 0.2g intestinal content in 1 mL demineralised water with 5 ceramic beads using a Fast-Prep (MP Biomedicals, Illkirch, France) (3x 15sec 6m/s with 1 min breaks) followed by 20min centrifugation at 8000x, supernatants were collected in 1.5 mL Eppendorf tubes and stored at -20°C until use.

Intestinal content samples (100 µl) were added to 60 µL of PIPES buffer (0.1 M, pH 6.5) and derivatised with 120 µL BBHA (50 nmol / µL) in presence of 20 µL of internal standard (Benzaldehyde-d5, 1 ng/µL). The samples were stirred at 6-8°C for one hour. SPE was conducted on a Visiprep SPE Vacuum manifold (Supelco, St Quentin Fallavier, France), using Agilent C18 Bond Elut (100mg, 1 mL) cartridges. The sorbent was conditioned with 1 mL of CH₃OH, then 1 mL water. The derivatized samples were vortexed and then deposited on the SPE cartridge. Washing was performed with first 1 mL PIPES (rinse the container and deposit on cartridge) and then 0.05% TFA/CH₃OH (3/2). The cartridge was then dried under vacuum (1min), eluted with 400 µL CH₃OH and collected in glass tubes. Finally, the volume was adjusted to exactly 400 µL with CH₃OH, and the extracts were stored at -20°C until analysis.

Liquid chromatography – mass spectrometry

Sample extracts were analyzed by high-performance liquid chromatography coupled to highresolution mass spectrometry (HPLC-HRMS). The HPLC system consisted in an Ultimate 3000 RS pump fitted with the Ultimate 3000 autosampler (Dionex-Thermo Scientific, Les Ulis, France). The

flow rate was 0.2 mL/min with the following elution gradient program: 0min 0% of B, from 3min to 15min 100% of B, and from 15 to 25min 100% of B. Mobile phases were composed of (A) H₂O/CH₃CN/HCOOH 95/5/0.1 (v:v:v) and (B) CH₃CN/H₂O/HCOOH 95/5/0.1 (v:v:v). 5µL of sample were injected on a Kinetex Core-Shell C18 (150 x 2.1mm, 2.6µm) column (Phenomenex, Le Pecq, France) maintained at 40°C. Detection was achieved on an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific) equipped with an electrospray ionization source used in the positive mode. Ionization parameters were set at +4.5kV for the spray voltage, 35 arbitrary units (au) for the sheath gas flow rate (N_2) , 5 au for the auxiliary gas flow rate (N_2) and 300°C for the capillary temperature. High-resolution mass spectra were acquired at a resolution power of 30,000 from m/z 80 to 800 in centroid mode. Identifications were performed by tandem mass spectrometry experiments (MSⁿ) using the ion trap mass analyzer of the LTQ-Orbitrap mass spectrometer. Solutions of synthesized standard glyoxal-BBHA, methyglyoxal-BBHA and 3-deoxyglucosone-BBHA at different concentration levels were used to characterize the method in terms of linearity of response, repeatability and sensitivity. Statistical analysis: From the lactose-pyridoxamine experiment raw data files, ions were extracted using xcms software¹⁶. Signals corresponding to brominated compounds were filtered based on the HRMS signal of the exact mass of each [M+H]⁺ ion, according to a mass measurement error of \pm 5 ppm, and to the occurrence of two signals of equivalent intensities with $\Delta M = 1.998$ corresponding to the mass difference between the two bromine isotopes. Then isotopic ratio between isotopes was checked. For the fructooligosaccharides-pyridoxamine experiment, the same extraction process was carried out. Supervised multivariate partial least squared discriminant analysis with orthogonal signal filtration (OSC-PLS-DA) and univariate non-parametric Kruskal Wallis tests were carried out on these ions. Discriminant models were validated if PLS Q2 criterion was greater than 0.4 and a permutation test was validated. Significant potential aldehyde ions were selected if PLS variables importance on projection (VIP) was greater than 1 and univariate Kruskal Wallis p-values with false discovery rate correction was lower than 0.05. Heatmap with hierarchical clustering analysis (HCA) using Euclidian distance and Ward method as aggregation criterion was used to present the results of both experiments.





Supplemental figure 3 - Derivatised ions (carbonyl compounds) for which the control group had lower values than the FODMAP treated groups

HPLC/MS analysis of aldehydes in colonic contents

In lactose- and fructo-oligosaccharide-treated animals, a metabolomic analysis by HPLC-MS of colonic contents after BBHA derivatization identified a distinct clustering of global reactive carbonyl compound profiles versus control (Figure S3). Additive administration of pyridoxamine of lactose- and fructo-oligosaccharide -treated animals did not modify internal clustering between these groups.



Supplemental figure 4 - Mucosal mast cell (MC) counts of distal colon in animals treated daily with 5mg lactose (Lact) and/or pyridoxamine (Pyr) for 3 weeks, expressed as MC/crypt.



Supplemental figure 5 - Mucosal mast cell (MC) counts of distal versus proximal colon in animals after following a diet high in fructo-oligosaccharides (FOS), for 3 weeks, expressed as MC/crypt.

References

- 1. Liu Z, Lozupone C, Hamady M, et al. Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic Acids Res 2007;35:e120.
- 2. Andersson AF, Lindberg M, Jakobsson H, et al. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One 2008;3:e2836.
- 3. Escudie F, Auer L, Bernard M, et al. FROGS: Find, Rapidly, OTUs with Galaxy Solution. Bioinformatics 2018;34:1287-1294.
- 4. Bokulich NA, Subramanian S, Faith JJ, et al. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods 2013;10:57-9.
- 5. Camacho C, Coulouris G, Avagyan V, et al. BLAST+: architecture and applications. BMC Bioinformatics 2009;10:421.
- 6. R Development Core Team R. R: A language and environment for statistical computing: R foundation for statistical computing Vienna, Austria, 2011.
- 7. McMurdie PJ, Holmes S. Phyloseq: A Bioconductor Package for Handling and Analysis of High-Throughput Phylogenetic Sequence Data. 2011:235-246.
- 8. McMurdie PJ, Holmes S. Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol 2014;10:e1003531.
- 9. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNAseq data with DESeq2. Genome Biol 2014;15:550.
- 10. Meyer F, Paarmann D, D'Souza M, et al. The metagenomics RAST server a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 2008;9:386.
- 11. Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939-51.
- 12. Hidaka H, Hirayama M. Useful characteristics and commercial applications of fructooligosaccharides. Biochem Soc Trans 1991;19:561-5.
- 13. Kamphuis JBJ, Mercier-Bonin M, Eutamene H, et al. Mucus organisation is shaped by colonic content; a new view. Sci Rep 2017;7:8527.
- 14. Neurath MF, Fuss I, Kelsall BL, et al. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med 1995;182:1281-90.
- 15. Chandra A, Srivastava SK. A synthesis of 4-hydroxy-2-trans-nonenal and 4-(3H) 4-hydroxy-2-trans-nonenal. Lipids 1997;32:779-82.
- 16. Tautenhahn R, Bottcher C, Neumann S. Highly sensitive feature detection for high resolution LC/MS. BMC Bioinformatics 2008;9:504.
- 17. Bachmanov AA, Reed DR, Beauchamp GK, et al. Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behav Genet 2002;32:435-43.

What you need to know

Background and context

A diet low in fermentable oligo-, di-, mono-saccharides and polyols (FODMAPs) can reduce symptoms of irritable bowel syndrome (IBS), possibly by reducing microbial fermentation products. We investigated whether ingestion of FODMAPs can induce IBS-like visceral hypersensitivity mediated by fermentation products of intestinal microbes in mice

New findings

We found that oral administration of lactose or fructo-oligosaccharides to mice increases abdominal sensitivity, which can be prevented with an anti-glycation agent. The lactose or fructo-oligosaccharides did not produce alterations in fecal microbiota composition of mice.

Limitations

This study was performed in mice.

Impact

Agents that prevent glycation reactions might reduce abdominal pain in patients with IBS with sensitivity to FODMAPs.

Lay Summary

Feeding mice lactose or fed fructo-oligosaccharides, which can cause symptoms in patients with irritable bowel syndrome, resulted in an increased abdominal sensitivity in mice. We identified agents that reduced the abdominal pain and changes in the colon that might cause symptoms—these might be developed as treatments for patients.