

Prebiotic role of softwood hemicellulose in healthy mice model

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1
2 Abbreviations:
3 AIEC - Adherent-invasive *Escherichia coli*; AutoH UC - autohydrolysate ultrafiltrated and
4 treated with activated charcoal; DP - degree of polymerization; FOS - fructo-oligosaccharides;
5 GGM - galactoglucomannans; MALDI-TOF MS - Matrix Assisted Laser Desorption Ionization
6 Time of Flight Mass Spectrometry; PP - precipitate; RT- room temperature; SCFA - short
7 chain fatty acids, TSWD - tryptic soy broth without dextrose.

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Abstract (150 words)

We have analyzed the prebiotic effect of softwood hemicelluloses on specific bacterial species *in vitro* and on microbiota diversity and metabolism in healthy mice *in vivo*. The ethanol-precipitated glycans from the softwood hemicellulose autohydrolysate were able to stimulate *in vitro* the growth of *Bifidobacterium adolescentis*, but to a much lesser extent than that of adherent-invasive *E. coli*. *B. adolescentis* was the best producer of SCFA. When mice were fed with the ethanol precipitate fraction, the relative abundance of Bacteroidetes increased while that of Proteobacteria diminished, suggesting change towards a less obesogenic microbiome. Following treatment, lipid analysis showed a decrease in cholesterol, bile acids and free fatty acids, indicating a potential cardioprotection role. Furthermore, analysis of fermentation metabolites revealed an increase in metabolites important for immune health and well-being. Our results highlight the value of hemicelluloses as a potential source of prebiotics supporting both a healthy digestive tract and immune system.

Keywords: softwood hemicelluloses, prebiotics, healthy mice model, microbiota metabolites.

1. Introduction 492 words

The composition and physiology of the gut microbiota is shaped to a large extent by the influx of glycans (polysaccharides) from diet and host mucosal secretions into the intestine. The microbiota confer many metabolic capabilities that the mammalian hosts lack, including the ability to break down otherwise indigestible dietary glycans. Since microbial species differ in their glycan preferences, selective consumption of such nutrients can influence both proliferation and persistence of microorganisms within the gastrointestinal tract.

It has been shown thirty years ago that the non-digestible oligosaccharides could be selectively fermented by Bifidobacteria and at the same time stimulate Bifidobacteria growth [Mitsuoka et al, 1987]. Gibson and Roberfroid put forward the concept of prebiotics: “Non-digestible oligosaccharides in general and fructo-oligosaccharides in particular, are prebiotics [1995]. They have been shown to stimulate the growth of endogenous Bifidobacteria”. In other words, prebiotic is “a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbial, thus conferring benefit(s) upon host health” (International Scientific Association for Probiotics and Prebiotics, 2008). First, a prebiotic oligosaccharide needs to be resistant to gastric acidity, hydrolysis by mammalian enzymes and direct gastrointestinal absorption. Second, it has to be fermentable by the intestinal microbiota. Thirdly it should selectively stimulate the growth and/or activity of intestinal bacteria that are associated with health and well-being. The use of particular dietary glycans, *i.e.* prebiotics, may provide the basis for a non-invasive strategy by which both the composition and the physiology of the microbiota could be modulated, in order to promote host health [Gibson and Roberfroid, 1992; Verbeke et al, 2015].

Currently commercialized glycan prebiotics with a degree of polymerization (DP) below 10 are derived mainly from the oligosaccharides and simple polysaccharides found in plants [Al-Ghazzewi et al, 2012; Yoo et al, 2012]. Thus far, the prebiotic properties of wood hemicellulose have been described in only two publications. Polari et al. [2012] showed that the galactoglucomannans, the predominant hemicellulose in the softwood spruce (*Picea*

1 *abies*), might have prebiotic properties, favoring Bifidobacteria growth, while Rivas et al.
2 [2012] have shown that a fraction obtained from the *Pinus pinaster* (maritime pine) wood
3 hemicellulose and composed of 14 acetylated hexoses was able to stimulate the human
4 bifidobacterial population growth. Thus, oligosaccharides derived from wood hemicelluloses
5 might display prebiotic properties, although their potential impact remains largely unknown.

6 Wood hemicelluloses are an abundant, but still untapped, industrial by-product of the pulp
7 and paper industry; this industry uses more than 300 million tons of wood per year worldwide,
8 of which 20 to 30% is hemicellulose. Exploitation of an industrial by-product avoids the use of
9 arable land and competition with food production resources.

10 Here, we have fractionated softwood hemicelluloses obtained by hot water extraction and
11 analyzed the impact of these hemicellulose fractions on *in vitro* growth of relevant gut
12 bacteria. Furthermore, we have confirmed the prebiotic properties of this fraction *in vivo*, by
13 analyzing its action on both gut microbiota composition and metabolism in healthy mice.

2. Materials and Methods 1633 words

2.1 Preparation of hemicellulose fractions. Hemicellulose was extracted from an industrial mixture of different softwood species containing Sylvester pine (35%), black pine (24%), Alep pine (18%), Douglas fir (16%) and spruce (7%) (provided by the Fibre Excellence Tarascon mill, France) and fractionated. Chemical characterization of fractions was done according to Deloule et al [2016]. Briefly, hemicelluloses were extracted from industrial softwoods chips by hot water extraction (170°C for 1 h). The crude autohydrolysate (AutoH) was fractionated using precipitation with either ethanol: water (PP ethanol) or acetone: methanol (PP acetone), both at a ratio 9:1. In parallel, the ultrafiltration with a molecular weight cut-off of 500 Dalton followed by activated charcoal treatment was carried out on AutoH, yielding AutoH UC.

2.2 Bacteria and growth media. The adherent-invasive *Escherichia coli* (AIEC) LF 82 and *Bifidobacterium adolescentis* CFPL 15196 were isolated from human feces in Lille Pharmacology Department (France). *Akkermansia muciniphila* (ATCC® BAA-835™) was purchased from ATCC. *Lactobacillus salivarius* CIP 103140 was purchased from the Collection of Pasteur Institute (Paris, France). All cultures were carried out in the Don Whitley Scientific A35 workstations (ShIPLEY, UK) under an anaerobic atmosphere consisting of 10% H₂, 5% CO₂ and 85% N₂ (Anaerogaz, Linde). The negative control consisted of tryptic soy broth without dextrose (TSWD) (Becton-Dickinson, Pont de Claix, France). The positive controls consisted of 1% glucose and the dietary fiber P95 containing 1% fructo-oligosaccharides (FOS, BENEEO-Orafti, Oreye, Belgium) with average DP of 4, both in TSWD. TSWD, supplemented with 1% glucose, FOS or hemicelluloses, was filtered (0.22 µm).

2.3 Growth Analysis. The intracellular production of ATP was estimated with VIALIGHT MDA kit (Lonza, Aix-les-Bains, France), using Multimode Reader Tristar LB941 (Berthold Technologies, Zug, Switzerland), while bacterial growth at 37°C was followed by measuring optical density at 595 nm, on the iMark Absorbance microplate reader (Bio-Rad, Marnes-la-Coquette, France).

2.4 SCFA analysis. Concentrated HCl (10 μ L) was added to the bacterial supernatants (100 μ L), then the mixtures were vortexed for 15 s and extracted for 20 min with 500 μ L of diethyl ether. After centrifugation (5 min, 3500 rpm), the upper organic phase was recovered and 50 μ L of 1 M NaOH were added, while 500 μ L of diethyl ether was added to the lower phase for 20 min, to extract again the SCFA. The SCFA-containing samples were combined, cleared by centrifugation and adjusted with concentrated HCl to pH 4. Sample aliquots of 20 μ L were injected into the HPLC-UV apparatus (Ultimate 3000, ThermoFisher Scientific, Waltham, MA, USA).

After mice treatment, the SCFAs were estimated in the fecal water obtained as follows. Stool samples were thawed and resuspended 1:5 in PBS (0.01 mole L^{-1} , pH 7) and homogenized. The fecal slurry was centrifuged at 35,000 g for 15 min at 4°C, and the supernatant (“fecal water”) was collected and sterilized by filtration (0.22 μ m). Chromatographic separation was performed using a Nucleogel Sugar 810H column (300mm \times 7.8mm) containing 10- μ m particles (Macherey-Nagel, Düren, Germany). The column stationary phase was sulfonated polystyrene divinylbenzene spheres in a polymer matrix (PS/DVB) in H^+ form, kept at 40°C. The mobile phase consisted of 10 mM H_2SO_4 in water. The UV detector (Waters 2487) was set at 210 nm wavelength. Data processing was performed using the Chromeleon version 6.80 software (Thermo Scientific). Good peak separation was obtained under the following conditions: a flow rate of 0.6 ml/min, room temperature, 10 mM H_2SO_4 as eluent, and a detector set for UV and refractometer (RI), respectively, at 0.0500 and 64 AUFS. A mix of volatile free acids (Sigma) was used as standard.

2.5 MALDI-TOF-MS. Mass spectroscopy analyses of oligosaccharides were performed using Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry, with apparatus MALDI-TOF-TOF Speed (Bruker Daltonics, Billerica, MA, USA). The matrix used was 2,5-dihydroxybenzoic acid (DHB); N, N-dimethylaniline (DMA) (Sigma-Aldrich, St. Louis, Mo, USA).

2.6 Glycans' variations in culture broth. Dosage of total sugars was done according to [8]. Briefly, 200 μ L of 5% phenol and 1 mL of 97% H₂SO₄ were added to 200 μ L of growth medium sample. After 10 min incubation at RT, the solution was vortexed, and after 30 minutes its OD was measured at 490 nm. The assay was calibrated with glucose standards ranging from 0 to 0.5 mg/mL. The absorbance at 450 nm was obtained with a spectrophotometer (Kontron, La Garde, France) and plotted against the standards. MALDI-TOF spectra were obtained for oligosaccharides in media of *B. adolescentis* grown in the presence of ethanol precipitate fraction. The intensity of carbohydrate-associated peaks was measured.

2.7 Ethical considerations. This research project implies the use of mice for *in vivo* experiments. In line with European laws, this project has been submitted to, and validated by, Grenoble Ethical Committee for Animal Experimentation (Project N012) as well as French Ministry of Higher Education, Research and Innovation (agreement number: APAFIS8502-2016122009136117v1). All along this project mice were carefully handled with respect of ethical rules on Animal Technology Platform of Grenoble University (the agreement n° C3851610006 from the French ministry).

2.8 Mice gavage with ethanol precipitate fraction. C57BL/6N female mice (5 weeks of age) were purchased from Janvier SA (Le Genest-Saint-Isle, France) and were housed (4 mice per cage) with free access to wood and water. Eight control mice were fed for three weeks with control A04 diet (SAFE, Villemoisson-sur-Orge, France), while other 8 mice were fed with A04 supplemented with the ethanol precipitate fraction at 0.3 g/day per mouse (unfortunately, one mouse in the treatment group died from an unknown cause). The freeze-dried ethanol precipitate fraction was dissolved in water to reach 86g/L and filtered through 0.22 μ m membrane. Feeding bottles (100 mL) were changed every 2 - 3 days and replaced with freshly prepared solution. At the end of experimental period the mice were euthanized with isoflurane, and serum was withdrawn by cardiac puncture. A ventral midline incision was made to excise cecum and colon. Cecal contents were collected and immediately frozen in

liquid nitrogen. Samples of cecal content were prepared through homogenization with PBS (cecal water), similarly as for fecal water, and frozen.

2.9 Cecal microbial diversity. The 16S rDNA sequencing (including DNA extraction and PCR) was subcontracted to Vaiomer (Vaiomer SA, Labège, France). PCR amplification was performed using 16S universal primers targeting the V3-V4 region of the bacterial 16S ribosomal gene (Vaiomer universal 16S primers). The joint pair length was set to encompass 467 base pairs amplicon thanks to 2 x 300 paired-end MiSeq kit V3. The detection of the sequencing fragments was performed using MiSeq Illumina® technology. Sequences were analyzed and normalized with the pipeline FROGS (Find Rapidly OTU with Galaxy Solution) [Escudié et al, 2018]. Reads were clustered into Operational Taxonomic Units (OTUs) using the Swarm clustering method. Phylogenetic affiliations were manually checked (using RDP seqmatch (https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) and/or BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LIN_K_LOC=blasthome)). The alpha diversity, which represents the richness and diversity within a group, was evaluated using the number of Observed OTU and the Chao1 index (community richness), using the Shannon index (community diversity) and the Simpson and InvSimpson indexes. Beta diversity, which reports diversity between groups was assessed by computing weighted UniFrac distances between each sample pairs.

To normalize data, 18763 reads were randomly selected for each sample. 289 OTUs were assigned at different taxonomic levels (from phylum to species). *P* values of ≤ 0.05 from statistical analyses (t-tests) were considered significant.

2.10 Tryptophan catabolites. Kynurenine and tryptophan concentrations were determined in 40µL of mice serum by HPLC coupled to mass spectrometry according to [Boulet et al, 2017]. Briefly, 10 µL of internal standard mixture (13C-labeled tryptophan and kynurenine) was added to samples. Protein precipitation was made by addition of 700 µL of MeOH, 79 g/L zinc sulfate in water (80/20, v/v) containing 0.05% trifluoroacetic acid. After mixing, samples were left at 4°C during 15 min and centrifuged at 10,000 g for 15 min at 4°C. 700 µL of clear

supernatant were transferred into a new tube and dried under a stream of nitrogen. The dried residues were reconstituted with 35 μ L of water containing 0.08% ascorbic acid. LC-MS/MS was performed using a Kinetex C18 column (100 mm x 2.1 mm; 5 μ M particle size) with a 2.1 mm C18 guard column, both obtained from Phenomenex (Torrance, USA), piloted by a Dionex Ultimate 3000 liquid chromatography instrument (ThermoFisher, Waltham, USA) connected to a Sciex API 4000 tandem mass spectrometer (SCIEX, Ontario, Canada). Mann-Whitney *U* test gave values $p < 0.05$ (*), $p < 0.01$ (**).

2.11 Lipid analysis. Total lipids were extracted from 10 μ L serum in the presence of 1 nmol tridecanoic acid and 1 nmol dihenecosanoyl phosphatidylcholine (extraction control), with 600 μ L chloroform/methanol, 2:1 (v/v), followed by biphasic separation through the addition of 150 μ L 0.2% KCl (w/v). Organic phase was dried with 1 nmol pentadecanoic acid and derivatized online to fatty acid methyl ester (FAME) with trimethylsulfonium hydroxide (TMSH, Macherey-Nagel). For cholesterol, the organic phase was dried with 1 nmol pentadecanoic acid and derivatized to cholesterol-TMS with N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + TMCS, 99:1 (Sigma). Resulting FAME and cholesterol-TMS was analyzed by GC-MS (5977A-7890B, Agilent). FAME was then quantified using Mass Hunter Quantification software (Agilent). All statistical analyses were conducted using GraphPad Prism software. *P* values of ≤ 0.05 from statistical analyses (Ttests) were considered significant.

2.12 Other metabolites. Lipopolysaccharide-Binding Protein was estimated in mice sera, using Mouse LBP PicoKine ELISA kit (Boster Biological Technology, Pleasanton, CA, USA). Secretory IgA, myeloperoxidase, folic acid and bile acids were estimated in cecal water (cecal samples diluted in PBS at 1:5 ratio w/v) by using Mouse sIgA ELISA Kit (ElabSciences, Houston, TX, USA), Mouse MPO ELISA Kit (HycultBiotech, Uden, Netherlands), Mouse Folic Acid ELISA Kit (Cusabio Technology, Houston TX, USA) and Mouse total Bile Acids assay Kit (Crystal Chem, Zaandam, Netherlands), respectively, according to manufacturer's instructions. Statistical differences were considered significant when $*p < 0,005$ or $**p < 0,001$ using unpaired t-test, when comparing control versus PP ethanol fed mice ($n = 7-8$ / group).

3. Results 2779 words

3.1 Wood hemicellulose-derived fractions extraction and characterization

Hemicellulose (20-30% of wood) is usually a rejected by-product of paper production from wood. It consists of polymers of arabinose, glucose, xylose, galactose and mannose with DP = 50-150, that undergo hydrolysis during hot water extraction. The extraction of crude autohydrolysate yielded three different fractions containing the same major component, galactoglucomannan (GGM, the main hemicellulose glycan of softwoods), but differed in purity and molecular weight (Fig. 1, and [8]). The three fractions derived from crude autohydrolysate were acetone precipitate, ethanol precipitate and autohydrolysate-treated by ultrafiltration (cut-off 500) and activated carbon. The PP ethanol contained glycans with the highest DP (Fig. 1B). Naturally occurring acetylation of GGM is also visible, as each peak on Fig. 1B exhibits a second and a third, slightly delayed, replicates. Acetylation has been shown to impact the solubility of polysaccharides in water through structural reorganization, and chemical acetylation of wood has been used to improve its durability and resistance to fungi, bacteria, and termites [Kishani et al, 2018; Pawar et al, 2013 and references therein]. In our hands, the acetyl groups represented 7.9% of the mass in crude autohydrolysate, 8.9% in ethanol precipitate and 8.3% in PP acetone (measured through hydrolysis to acetic acid and estimated by HPLC). The ratio of one O-acetyl per three monomers was persistent over a wide range of DP. The acetylated GGMs exhibited peaks of higher intensity than the non-acetylated ones for ethanol precipitate, with DP 12 to 22, and with DP 6 to 16 for acetone precipitate.

3.2 Effect of hemicelluloses on bacterial growth *in vitro*

In order to determine the effect of the hemicellulose fractions and glucose or FOS (as control) on different gut bacteria, we first used a growth control experiment. To follow bacterial growth and metabolism, we followed intracellular ATP production measurements rather than relay on OD₅₉₅ measurements. Indeed, the ATP assay detects bacteria that are dying and is not influenced by size, shape and sedimentation ability of involved bacteria. In our choice of

bacterial species, we were guided by their gut predominance. The AIEC LF82 (Gram negative) is abnormally predominant in the ileac mucosa of Crohn's disease [Carvalho et al, 2012]. It has been recently shown recently that this AIEC is able to adhere, invade and survive within prostate epithelial cells, inducing strong inflammatory response, and could conceivably play a role in prostate infection/inflammation [14]. This bacterium was used to pinpoint the hemicellulose fractions to be eliminated, in case they would boost AIEC growth. *Bifidobacterium adolescentis*, together with the *B. catenulatum* group, dominate in healthy gut microbiota [Conte et al, 2016]. This Gram-positive bacterium is present in 79% of the adults [Kim et al, 2008] and was found to inhibit harmful fecal enzymes, hamper proliferation of human colon cancer cells *in vitro* and exhibit an antiviral activity [Lee et al, 2013; Derrien et al, 2017]. *Akkermansia muciniphila* is a Gram-negative mucin-degrading bacterium that resides in the gastrointestinal tracts of humans and animals. Reduced levels of *A. muciniphila* have been observed in patients with inflammatory bowel diseases and metabolic disorders, which suggests it may have anti-inflammatory properties [Neville and O'Toole, 2010]. *Lactobacillus salivarius* is a Gram-positive bacterium, that is able to influence composition of gut microbiota through production of bacteriocins, some with an anti-Listeria activity [Messaoudi et al, 2013]. *L. salivarius* is also able to stimulate the human immune system, mainly through increased production of interleukins [Barker, 1981].

Medium containing glucose induced a rapid ATP production by all examined bacteria (Fig. 2), but it was also the fastest to become depleted medium, with low or no ATP produced on glucose after about 100 h of growth. In contrast, oligomers in all other fractions continued to sustain low ATP production for quite a long time. Importantly, hemicelluloses were rather poorly fermented by *E. coli* LF82. These media gave growth values close to that of FOS or even to that of TSWD. *A. muciniphila* was not able to achieve high ATP production on glucose, in contrast to the other three bacteria. When the effect of the autoH UC, fraction obtained from autoH but devoid > 500 kDa compounds and soluble lignins, was compared with that exerted by autoH, some increase of ATP production (meaning bacterial growth) was observed for all bacteria, probably due to the removal of deleterious impurities. Interestingly,

1 growth of *B. adolescentis* was significantly prompted by 1% and even more by 3% PP ethanol
2 fraction, this fraction inducing a second ATP production peak at between 50 and 170 hours of
3 growth (Fig. 2). It is conceivable that these bacteria produce, after induction, glycan-
4 hydrolyzing enzymes, thus providing the low-molecular weight glycans to sustain bacterial
5 growth. For the remaining three bacteria, a second ATP production peak (albeit significantly
6 smaller) was also observed around 150 h of growth. When 2% of AutoH, FOS and glucose
7 were used, no major changes were observed (not shown).

8 As bacterial growth was also monitored with OD₅₉₀ readings, we could extract the
9 maximum OD₅₉₀ values just before bacteria reached the stationary phase was reached.
10 Maximum OD₅₉₀ values not only show the effect of hemicellulose fractions on both the level
11 and rate of bacterial growth (Fig. 3A). These measurements showed again that the highest
12 growth was obtained on glucose. However, while the *E. coli* grew exponentially on glucose for
13 only 9 h, *A. muciniphila* grew efficiently on glucose for 64 hours. The latter also grew longer on
14 FOS and TSWD, without, however, reaching a high OD. Surprisingly, all bacteria could weakly
15 propagate on TSWD (no sugar). Of note, amino acids can serve as energy sources for some
16 anaerobic bacteria [19]. *A. muciniphila* had the longest growth on these different fractions,
17 followed by *B. adolescentis*, *L. salivarius* and *E. coli* LF 82, with the highest OD obtained by *B.*
18 *adolescentis*. These observations suggest that AIEC LF 82 grew well on the simple glycans
19 present in control media but was not very efficient at hydrolyzing the longer oligomers that
20 could sustain lengthier growth periods. On the other hand, the longer growth periods
21 demonstrated by the three other bacteria suggest that they might extend their growth by
22 hydrolyzing higher-DPs glycans and utilizing the resulting shorter sugars.

23 The degradation of carbohydrate oligomers was measured in *B. adolescentis* culture broth
24 using the MALDI-TOF MS and total sugar dosage (Fig. 3B). *B. adolescentis* easily fermented
25 the low-DP glycans and acetylated glycans, which were consumed quite rapidly but also
26 reappeared at longer culture times – see results for 4HOAc, 5H and 5HOAc. Furthermore, the
27 longer oligomers (DP 9 and above) diminished clearly at 144h. Taken together these results
28 indicate quite probable the induction of *B. adolescentis* glycolases, able to digest high-DP

glycans in the PP ethanol fraction. Note that the MALDI-TOF MS technique was applied to ascertain the degree of acetylation on spruce-derived AcGGM, and to characterize the acetylated hemicelluloses in hardwoods (Jacobs et al, 2002; Teleman et al, 2003; Lundquist et al, 2003).

In conclusion, *B. adolescentis* achieved the highest growth on all media, with an ability to efficiently ferment hemicelluloses, in particular during later growth periods. The health-detrimental *E. coli* LF82 was unable to reach significantly better growth on hemicelluloses than on low-molecular-weight oligosaccharides present in control media, displaying a rather low ability to ferment softwood hemicelluloses.

3.3 Effect of hemicellulose fractions on SCFA production *in vitro*

The important end products of fermentation of dietary fibers by the anaerobic intestinal microbiota are short-chain fatty acids (SCFAs). The combined concentrations of SCFAs produced in the colon reach ~150 mM, making SCFAs the most abundant anions in the colon. SCFAs are absorbed in the colon and either utilized in colonocytes or transported via the portal vein to reach the blood circulation and other organs [Kim et al, 2014]. We determined the amount of SCFA induced during growth of *E. coli*, *B. adolescentis* and *A. muciniphila* on different hemicellulose-derived fractions (Fig. 2S). It appears that the acetate was the main SCFA produced by all bacteria, with *B. adolescentis* producing more SCFA than *E. coli* LF82 or *A. muciniphila*. In addition, *B. adolescentis* produced significant amount of propionic acid, which was formed at a negligible level by the two other bacteria (Figs. 2S). Interestingly, at the beginning of LF82 growth, formic acid was produced, with, however, a steady decline in all media. The analyzed bacteria did not seem to produce butyric acid, known for its anti-cancer effects and immunity modulation [Fung et al, 2012; Meijer et al, 2010]. It is relevant that among 313 bacterial isolates from human fecal samples, only 74 were observed to produce butyrate [Barcenilla et al, 2000]. As acetic and propionic acids are fuel for colonocytes [Floch, 2010], we wanted to better characterize the SCFA-generating effect of hemicellulose fractions, by following the changes in the ratio of propionic/acetic acid (Fig. 4). This comparison

confirmed quite low production of SCFAs by *E. coli* LF82 and *A. muciniphila*. The ratio observed with *B. adolescentis*, using the hemicellulose fractions underlines the positive effect of autohydrolysates on propionic acid production, with a peak appearing at about 30 h of growth. It seems that autohydrolysates containing glycans with lower DP are able to sustain propionate production by *B. adolescentis* at earlier stages (up to ~47 h, see the first peak of bacteria growth on Fig. 2S). In contrast, the PP ethanol fraction with higher DP glycans rather efficiently maintained the longer growth phase of *B. adolescentis* (after 60 h, see Fig. 2S), which correlated with an efficient propionate production.

3.4 Effect of PP ethanol fraction on mice microbiota

Mice were fed with PP ethanol fraction and the different phyla from their caecum were identified through analysis of 16S rRNA genes as previously [Mangin et al, 2018]. Microbiota of 15 mice were analyzed: 8 for the control group and 7 for the PP ethanol fraction gavage group. 18763 sequences were analyzed per sample. The 289 OTUs belonged to six phylogenetic phyla: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Verrucomicrobia and Candidatus Saccharibacteria. The phyla Bacteroidetes, Firmicutes and Proteobacteria accounted for more than 99.5 % of the diversity. Only 32 OTUs were known (less than 2% divergence with a sequence of a cultivated species in databases), for example *Acetatifactor muris*, *Akkermansia muciniphila*, *Bacteroides acidifaciens*, *Bifidobacterium animalis*, *Clostridium disporicum*, *Escherichia coli*, *Lactobacillus gasseri*, *Pseudomonas aeruginosa* and *Streptococcus salivarius*. The phylum Bacteroidetes was dominant (based on the number of sequences), but the phylum Firmicutes was the most diversified (based on the number of OTUs) (Figs. 5 and 3SA). Analysis of the beta diversity enlightened the microbiota modification due to the prebiotic fraction: weighted UniFrac distances allowed to separate samples from treated and untreated mice (Figs. 3SB). There were two main consequences on the microbiota when feeding mice with the PP ethanol fraction. Firstly, the number of OTUs was less important in the caecum of treated mice, 246.4 for the PP ethanol fraction \pm 8.5

versus 285.5 ± 18.6 for the control microbiota (mean \pm SEM), $p < 0.01$. This decrease was statistically significant for the phyla Firmicutes, Proteobacteria, Actinobacteria and Verrucomicrobia (Fig. 5A). The alpha diversity measure was in agreement with this finding, with all indexes smallest for the PP ethanol group (Observed, Chao1, Shannon, Simpson and InvSimpson). Secondly, the amount of Bacteroidetes sequences increased from 58 to 65 % ($p < 0.05$) and the Proteobacteria sequences decreased from 11 to 7 % ($p < 0.01$) (Fig. 5B). The observed decrease for the Firmicutes phylum was not statistically significant. Of note, *B. adolescentis* belongs to phylum Actinobacteria, while *E. coli* LF82 belongs to Proteobacteria, confirming our *in vitro* results. Glycans from the PP ethanol fraction impacted the cecal microbiota of mice: the proportion of the Bacteroidetes phylum was increased, the proportion of Proteobacteria decreased and the number of species diminished except for the Bacteroidetes phylum. It has been observed that gut dysbiosis leading to several human diseases is characterized by an increased Firmicutes/Bacteroidetes (F/B) ratio. Importantly, the F/B ratio is a marker of an obesogenic microbiome, a high F/B ratio is more likely to support obesity, while a low F/B ratio is associated with weight loss [Ussar et al, 2016]. Our *in vivo* experiments led to a decrease in F/B ratio from 0.527 in control mice to 0.433 in mice fed with PP ethanol glycans, indicating a potential beneficial effect of this fraction on mice microbiome, reshaping it toward less obesogenic. This could be tested in a further study more focused on the physiological impacts of these fibers.

3.5 Effect of hemicellulose fractions on SCFA production *in vivo*

SCFAs, that play a key role in the maintenance of colonic homeostasis and seem to be the most important microbial end products of the colon fermentation involved in the regulation of the immune cell function [Flint et al, 2015; Corrêa-Oliveira et al, 2016]. Administration of a hemicellulose-derived fraction had a significant impact by increasing the production of acetate (50 vs $39 \mu\text{mol. g}^{-1}$) and propionate (11 vs $7 \mu\text{mol. g}^{-1}$), with little effect on the levels of formate or butyrate in the cecum (Fig. 6A). The bacterial species belonging to the *Bacteroidetes* phylum produced predominantly acetate and propionate, whereas butyrate is primarily

1 produced by the *Firmicutes* phylum [Vital et al, 2014] SCFA production (Fig. 6A) correlates
2 well with changes in bacterial populations (Fig. 5).

3
4 **3.6 Tryptophan metabolites.** Since gut microbial dysbioses are linked to aberrant
5 immune responses, we subsequently carried out a detailed analysis of some immune system-
6 relevant products and fermentation metabolites. A major group of immunomodulatory gut
7 bacteria metabolites derives from catabolism of the aromatic amino acids. A principal route for
8 tryptophan catabolism is the kynurenine pathway. It plays an important role in several
9 fundamental biological processes, because tryptophan metabolites are ligands of aryl
10 hydrocarbon receptor (AhR), which regulates immune responses and inflammation through its
11 effects on cytokine production [Haase et al, 2018]. It might also directly influence gut health,
12 as 5-hydroxytryptamine (5-HT, serotonin) produced by the gut epithelial cells contributes to
13 the development of microvilli in the intestine [Nakamura et al, 2008]. The hemicellulose diet
14 induced in mice an increase in tryptophan level and a decrease in kynurenine level (Fig. 6B).
15 As a result, the ratio of Trp to Kyn increased significantly, from 100 to 170. In addition, the
16 level of 5-HT rose, suggesting that the neurotransmitter serotonin pathway might be
17 privileged. Indeed, it has been recently shown, that gut microbes promote colonic serotonin
18 production through action of SCFA [Reigstad et al, 2015].

19
20 **3.7 Lipid analysis.** Lipid analysis included an estimation of the changes in the level of
21 serum free fatty acids (FFA) and cholesterol in treated mice. Upon feeding mice with the PP
22 ethanol fraction, the total level of FFA significantly diminished with reduction noted for palmitic
23 acid (C16:0), stearic acid (C18:0) and linolenic acid (C18:2) (Fig. 7A). In parallel, a reduction
24 of cholesterol was also observed (Fig. 7B). Free fatty acids, in addition to be utilized as an
25 important source of energy, are involved in a variety of both beneficial and detrimental effects.
26 Under conditions of overload, FFAs become toxic, inducing reactive oxygen species
27 production, endoplasmic reticulum stress, apoptosis and inflammation [Legrand-Poels et al,
28 2014]. Elevated cholesterol is known to be one of the main risk factors for cardiovascular

1 diseases, with reduction of cholesterol being cardioprotective [London et al, 2014]. Thus,
2 reduction of FFA and cholesterol is obviously health-beneficial.

3 Furthermore, the analysis of the toxic effect on Microbiota bile acids was carried out (Fig.
4 7C). Bile acids are synthesized by the liver from cholesterol, transformed into secondary bile
5 acids by the intestinal microbiota and reabsorbed back into the blood. Nearly 100% of the bile
6 acid pool in the large intestine consists of bacterially modified bile acids, implicated in a variety
7 of signaling pathways relevant to metabolic and hepatic diseases [Klaassen and Cui, 2015].
8 Almost two-fold reduction observed in bile acid of treated mice is significantly health-
9 beneficial, as bile acids convert the intestinal microbiota to higher Firmicutes/Bacteroidetes
10 ratios that are observed after a high-fat diet [Klaassen and Cui, 2015], during obesity and gut
11 dysbiosis [Mangin et al, 2018].

12 In addition, we monitored the changes in the level of the lipopolysaccharide-binding protein
13 (LBP) that is associated with obesity and which through lipopolysaccharide binding may
14 promote immune responses [Moreno-Navarrete et al, 2012; Kheirandish-Gozal et al, 2014], of
15 the secretory IgA (sIgA) that promote intestinal homeostasis [Rogier et al, 2014], of the
16 myeloperoxidase (MPO) that has been detected in a wide range of inflammatory conditions
17 [Nauseef, 2014]. We also measured changes in the levels of folic acid, an essential vitamin,
18 exclusively supplied by commensal microbes in the gut (by Bifidobacteria among others
19 [Sugahara et al, 2015]) or by dietary supplementation. The changes observed for these
20 metabolites were not significant upon feeding mice with the PP ethanol fraction (Fig. 4S).
21 Thus, the levels of some metabolites were not changed, which is understandable, as only
22 healthy mice were treated with the PP ethanol fraction.

23

4. Discussion

Hemicellulose (20-30% of wood) is a rejected by-product of paper production. Hardwoods hemicelluloses (from dicot angiosperm trees that reproduce by flowering, such as teak, mahogany, maple, walnut, oak, cherry, apple) contain 15-30% of xylan but only 2-5% of glucomannan. In contrast, the softwoods hemicelluloses (from gymnosperm trees such as evergreen conifers, like pine or spruce) contain only 5-10% xylan but 14-20% glucomannan [Timmel, 1964; Álvarez et al, 2016]. Not much is known about the eventual biological role of glucomannans, apart from the fact that galactomannan does not support growth of some human *Lactobacilli* [McLaughlin et al, 2015]. When the fermentability of GGM was studied in broiler chickens, it was observed to protect against *S. typhimurium* colonization [Rajani et al, 2016]. Very recently, when the effect of acetylated galactoglucomannan (AcGGM) from Norwegian lignocellulosic feedstock has been investigated, it appeared to promote beneficial bacteria, suggesting a prebiotic activity *vis-a-vis* human gut health [La Rosa et al, 2019].

In our hands, the majority of hemicellulose-derived fractions contained glycans with a degree of polymerization comprised between 3 and 8, while the ethanol-precipitated fraction contained GGM with DP comprised between 4 and 13. In order to measure growth of chosen gut bacteria, we monitored ATP production, which follows the growth and metabolism of living bacteria better than OD measurements. We observed that the ethanol precipitate was able to modify growth behavior of analyzed bacteria, inducing robust growth of *B. adolescentis*. Moreover, this hemicellulose fraction induced quite high second peak of bacteria growth at later culture time, suggesting the transformation of initial GGMs to lower-DP glycans, readily used by bacteria. Analysis of glycan kinetics upon feeding *B. adolescentis* with the ethanol precipitate fraction showed that at lengthier culture times the concentration of longer glycans decreased, with a concomitant appearance of shorter glycans. It is relevant that *B. adolescentis* produces several secreted glycan-degrading enzymes [van den Broek et al, 2008]. *E. coli* LF82 was able to quickly and efficiently use furnished glycans, which confirms high fitness of adherent-invasive *E. coli* LF82 [Martinez-Medina and Garcia, 2014]. *E. coli*

1 LF82 is a pathogenic strain, and our hemicelluloses fractions did not induce a strong growth of
2 this bacterium. The highest growth on both controls and hemicellulose-derived fractions was
3 achieved by *B. adolescentis*, but the longest periods of growth on these fractions were
4 observed for *A. muciniphila*, showing a slow increase of viable bacteria (and suggesting rather
5 long doubling time).

6 Commensal microbes attune the innate and adaptive immune responses [Donia and
7 Fischbach, 2015; Lee and Hase, 2014, among others], through the production of short-chain
8 fatty acids, the important end products of fermentation of dietary fibers by the anaerobic
9 intestinal microbiota. SCFAs act as a link between the microbiota and the immune system, by
10 modulating different aspects of intestinal epithelial cells and leukocytes development, survival
11 and function through activation of G protein coupled receptors and by modulation of the
12 activity of enzymes and transcription factors [Corrêa-Oliveira et al, 2016]. In addition, the
13 SCFAs exert multiple beneficial effects on mammalian homeostasis of healthy mucosa. The
14 most abundant SCFAs produced by colonic bacteria are acetic acid (C2), propionic acid (C3),
15 and butyric acid (C4). Butyric acid is an energy source for the colonic epithelial cells
16 [Tsujikawa et al, 2003], the other SCFA are absorbed in the host circulation, joining various
17 metabolic pathways [Cummings JH and Macfarlane, 1991; Wolever et al, 1993; Henningsson
18 et al, 2002]. The low level of SCFAs in the human gut has been associated with the
19 development of different diseases ranging from metabolic and inflammatory diseases to
20 cancers [den Besten et al, 2013; Bultman, 2016; Stilling et al, 2016]. Furthermore, the
21 presence of SCFA is proposed to confer colonization resistance against Enterobacteriaceae
22 (containing disease-causing bacteria) through “metabolic exclusion” [Lawley and Walker,
23 2013].

24 *B. adolescentis* is known as the best producer of acetate among different Bifidobacteria
25 [Marx et al, 2013], and the production of acetate by *B. adolescentis* may result in cross-
26 feeding to *Faecalibacterium prausnitzii*, leading to production of health-promoting butyrate by
27 the latter [Moens et al, 2016]. Upon supplementing medium with hemicellulose-derived

fractions, *B. adolescentis* was able to produce significant amounts of acetate but also of propionic acid, in particular induced by autohydrolysate and PP ethanol fraction. Propionic acid is generally associated with hypocholesterolemic effects and might be an anti-obesity, satiety-enhancing and anti-cancer factor [Illman et al, 1988; Arora et al, 2011; Havenaar, 2011]. Furthermore, it is associated with reduced stress behavior and protection of blood-brain barrier (BBB) from oxidative stress, which suggests that gut-derived microbial metabolites might interact with the BBB [Burokas et al, 2011; Hoyles et al, 2018]. Interestingly, mice fed with Western diet (high sugar, high fat) showed a decrease in propionate production and were more susceptible to colonization with AIEC strains [Agus et al, 2016]. We did not detect *in vitro* production of butyrate. It is known that Bifidobacteria do not produce butyrate, as no butyrate production pathway is present in their genomes [Klijn et al, 2005]. It has also been noted that the mucin-degradation activity of *A. muciniphila* led to the production of propionate and acetate only [Derrien et al, 2004].

During *in vivo* experiments, no significant effect on body mass of mice was observed with the hemicellulose diet. The analysis of 16S rRNA genes in cecal samples of control and ethanol PP-fed mice showed a significant increase in the proportion of Bacteroidetes and a diminished proportion of Proteobacteria. Some Proteobacteria, in particular AIEC strains such as LF 82, have been associated with the pathogenesis of inflammatory bowel conditions [Mazzarella et al, 2017]. Bacteroidetes and Firmicutes are prevalent phyla (more than 90%) in normal human gut microbiota and also in the cecum of our mice. The Bacteroidetes phylum generally makes up half or more of the gut microbiome, with other phyla, such as Actinobacteria and Verrucomicrobia, comprising 10% or less of the microbiome [Jandhyala et al, 2015; Lloyd-Price et al, 2017]. Similar proportions were found in our study (see control in Fig. 5). Bacteroidetes genomes encode a high number of inducible carbohydrate-active enzymes that enable the use of dietary and host mucosal glycans [El Kaoutari et al, 2013], explaining the rise of Bacteroidetes upon feeding mice with hemicelluloses. Similarly, in the study on the effect of fiber diet in humans, levels of Bacteroidetes correlated positively with fiber intake (Simões et al, 2013). A high Firmicutes/Bacteroidetes ratio has been implicated in

1 predisposition to obesity [Ley et al, 2006; Turnbaugh et al, 2009; Compare et al, 2016].
2 Therefore, an increase in Bacteroidetes observed upon feeding with glycans contained in PP
3 ethanol fraction suggests potentially an anti-obesity effect of this fraction.

4 Our *in vivo* data showed the potential of hemicellulose-derived fractions to modulate the
5 intestinal microbiota. By feeding healthy mice with particular glycans, we observed the diverse
6 and varied effects resulting in gut microbiota modification, boosting of the immune system,
7 lipids alterations and bile sequestration. Data obtained on murine models have shown that
8 tryptophan catabolites are able to enhance regulatory T cells and protect mice from chronic
9 hyperinflammatory responses [Mezrich et al, 2010]. The increase of the Trp/Kyn ratio is a sign
10 of good health [Keszthelyi et al, 2009]. Moreover, the increase in serotonin (5-HT) might be a
11 sign a well-being since serotonin is well known for mood control in the brain (Liu and Deneris,
12 2011). Accordingly, the administration of prebiotics (FOS and GOS) reduced depression and
13 anxiety in mice [80]. Finally, the increased 5-HT might also be a sign of good bowel health,
14 serotonin being involved in the peristaltic reflex pathway [Burokas et al, 2017] and the
15 development of microvilli in the intestine [Nakamura et al, 2008].

16 The total level of fatty acids was significantly diminished in mice fed with PP ethanol
17 fraction, with a reduction noted for palmitic, stearic and linolenic acids. It has been described
18 that the Palmitic acid has been shown to induce anxiety-like behavior in mice [Moon et al,
19 2014] and it has been associated with an increased risk of coronary heart disease and tumor
20 development [Fattore and Fanelli, 2013], while stearic acid apparently induces
21 proinflammatory cytokine production [Miao et al, 2015]. Linoleic acid (C18:2) is an essential
22 fatty acid that te human body cannot synthesized. When released from membrane
23 phospholipids, it can be enzymatically oxidized to a variety of derivatives involved in cell
24 signaling. It is a precursor of arachidonic acid that can be converted to prostaglandins and
25 leukotrienes, which, when produced in excess, are known to contribute to a number of chronic
26 diseases, such as inflammation and cancer [Fritsche, 2014]. These data should, however, be
27 treated with caution as it seems that the response of fatty acid metabolism to the
28 administration of Bifidobacteria is strain-dependent [Wall et al, 2012], and we do not know

whether these changes in lipidome result from an increase in *B. adolescentis* due to the mice being fed with the PP ethanol fraction. In parallel, a reduction in cholesterol level was observed. Elevated blood cholesterol is a major risk factor for coronary heart disease, a risk factor for breast cancer and a significant public health problem. Despite use of cholesterol-lowering statins, quite often a residual vascular risk remains [Sampson et al, 2012]. Also, the reduction of bile acids was observed for treated mice. It is known that dietary or genetic obesity inducing alteration of gut microbiota increases the level of deoxycholic acid (DCA), a gut bacterial metabolite known to cause DNA damage, which may lead to liver cancer [Yoshimoto et al, 2013]. Decreasing bile acid concentration in the colon in cirrhosis leads to reduction in bile acid 7 α -dehydroxylating gut bacteria, which produce DCA [Ridlon et al, 2013]. Until now, the manipulation of the bile acid pool was done with drugs able to sequester bile acids. The bile sequestrants have found clinical application for dyslipidemia leading to regression of atherosclerotic plaque [Insull, 2006; The Lipid Research Clinics, 1984] and, more recently, application to type 2 diabetes [Ooi and Loke, 2014]. Remarkably, the action of some anti-diabetic drugs is accompanied by increase in *Lactobacilli* and *Bifidobacteria* [Gu et al, 2017]. In our hands, upon feeding healthy mice with glycans in PP ethanol fraction, the amount of bile acids diminished significantly, suggesting a decreased toxicity to microbiota. In conclusion, the observed modulation of healthy mice metabolites might conceivably lead to a better health through prevention of some diseases. These effects can be obtained through utilization of the usually rejected softwood fraction and achieved without deleterious side effects.

In a recent review spruce galactoglucomannan (GGM) was described as a potential wood-based food ingredient and the examples were given of plant-based polysaccharides that are already authorized for food purposes (Pitkänen et al., 2018). However, these examples are limited to food thickeners and stabilizers and large safety analyses would be needed to introduce GGMs as a mainstream food.

A change in the intestinal microbiota through diet modification can occur within a single day [Turnbaugh et al, 2009; Wu et al, 2011; Faith et al, 2013]. However, over the long-term,

microbial populations are generally stable; each individual's microbiota is most similar to their family members, with two unrelated individuals sharing ~30% of strains in their microbiota [Wu et al, 2011; Faith et al, 2013]. Thus, enrichment with *B. adolescentis* alone would not lead to long-term persistence of this bacteria within gut microbiota. In addition, it is thought that any strain that increases its numbers relative to that of other bacteria occupying the same ecological niche will quickly be eliminated by phage predation (nothing is known about phages activity against *B. adolescentis*). However, treatment with *B. adolescentis* applied together with glycans from the PP ethanol fraction may provide a long-term selective advantage, decreasing resilience of an unhealthy state. The addition of particular glycans might induce specific pathways utilization [Lammerts van Bueren et al, 2017]. Such synbiotic intervention consisting of the simultaneous use of probiotics and prebiotics might open novel prophylactic and therapeutic strategies to human health, such as the treatment of obesity or chronic inflammatory and immunity-related diseases.

Conclusion

Softwood hemicellulose is a rejected by-product of paper production from wood but our work suggest that high added value could be obtained for this by-product. Indeed, we showed that hemicellulose oligosaccharides obtained by autohydrolysis stimulate growth of beneficial gut bacteria species. Furthermore, in mice fed with these oligosaccharides we observed a reshaping toward a less obesogenic microbiome, and production of metabolites interesting for health. These results suggest that hemicellulose from softwood could be a source of important prebiotics for humans. Work is under investigation to further characterize the active oligosaccharide fractions and to optimize the production process.

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