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Heteropolysaccharides from *S. cerevisiae* show anti-adhesive properties against *E. coli* associated with Crohn's disease

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Abstract

The *Saccharomyces cerevisiae* CNCM I-3856 was previously reported to strongly inhibit adherent-invasive *Escherichia coli* (AIEC) adhesion to intestinal epithelial cells *in vitro* and to favor AIEC elimination from the gut in a murine model of Crohn's disease *in vivo*. In order to identify which cell wall components of yeast are responsible for AIEC elimination, constituent polysaccharides of yeast were isolated and their anti-adhesive ability against AIEC adhesion *in vitro* were screened. A fraction containing mannan, β-glucan and α-glucan extracted from yeast cell-walls was shown to inhibit 95% of AIEC adhesion *in vitro* and was thus identified as the strongest anti-adhesive yeast cell wall component. Furthermore, this mannan-glucan-containing fraction was shown to accelerate AIEC decolonization from gut *in vivo*. This fraction could be proposed as a treatment to eliminate AIEC bacteria in patients with Crohn's disease, a microbial trigger of intestinal inflammation.

Highlights

- Mannan extracted from yeast inhibits *E. coli* adhesiveness to intestinal cells *in vitro*.

- A soluble fraction containing β-glucan/α-glucan/mannan (GGM) was extracted from *S. cerevisiae* cell wall.

- This soluble GGM fraction strongly inhibits *E. coli* adhesiveness to intestinal cells *in vitro*.

- This soluble GGM fraction favors elimination of *E. coli* from gut *in vivo.*

Keywords: mannan-glucan heteropolysaccharides; Adherent-Invasive *Escherichia coli*; *Saccharomyces cerevisiae*; anti-adhesive strategy; NMR analysis

Abbreviations: AIEC, adherent-invasive *Escherichia coli*; CD, Crohn's disease; CEACAM6, carcinoembryonic antigen-related cell adhesion molecule 6; YCW, yeast cell wall; Man, mannose; Glc, glucose; PPM, phospho-peptido-mannan; GGM, β-glucan/α-glucan/mannan

1. Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) cause a dysfunction of the immune response to gut microbiota occurs. Among those, Crohn's disease (CD) leads to a reduction of global microbial diversity and an increase in mucosal bacteria in patients (Gevers et al., 2014; Manichanh et al., 2006; Sommer et al., 2017). Into this context, ileal mucosa-associated microbiota at time of surgery was shown to be predictor of post-operative endoscopic recurrence in CD (Sokol et al., 2019). Among this mucosa-associated community, Enterobacteriaceae, and particularly the *Escherichia coli* species, are over-represented at the ileal mucosa of CD patients (Martin et al., 2004) (Baumgart et al., 2007) (Lopez-Siles et al., 2014). Abnormal prevalence of *E. coli* was observed worldwide in CD patients (Agus, Massier, Darfeuille-Michaud, Billard, & Barnich, 2014). These CD-associated *E. coli* possess adherent and invasive properties *in vitro* on intestinal epithelial cells, the defining characteristics of this pathobiont of *E. coli* that confers its name, "Adherent-Invasive *E. coli*" (AIEC) (Boudeau, Glasser, Masseret, Joly, & Darfeuille-Michaud, 1999; Darfeuille-Michaud et al., 1998; Eaves-Pyles et al., 2008). Besides the adherent and invasive phenotypes, AIEC invade and replicate inside macrophages, which induces elevated secretions of pro-inflammatory cytokines TNF-α, and promotes granuloma *in vitro* (Bringer, Glasser, Tung, Meresse, & Darfeuille-Michaud, 2006; De la Fuente et al., 2014; Glasser et al., 2001; Meconi et al., 2007). Furthermore, AIEC bacteria induce or aggravate intestinal inflammation in various models of mice (Carvalho et al., 2008; Carvalho et al., 2009; Eaves-Pyles et al., 2008; Small, Reid-Yu, McPhee, & Coombes, 2013). Therefore, it appears essential to develop therapies targeting AIEC in CD patients in order to eliminate a triggering agent of intestinal inflammation.

Anti-adhesive strategy could be of interest to the eradication of AIEC from the gut by focusing on the initial step of virulence: attachment to ileal mucosa. Bacterial adhesin FimH, located at the tip of the type 1 pili, recognizes mannose (Man) residues on host cells (Abraham, Sun, Dale, & Beachey, 1988). AIEC possess genetic polymorphisms in *fimH* that allow them to bind strongly to mannose residues on intestinal epithelial cells (Dreux et al., 2013). The glycoprotein carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) was shown to be overexpressed at the ileal mucosa of CD patients (Barnich et al., 2007). AIEC reference strain LF82 preferentially persists in the gut of transgenic mice expressing the

human CEACAM6 protein (CEABAC10 mouse model) (Carvalho et al., 2009), demonstrating that oligomannosylated glycans exposed by CEACAM6 provides to AIEC increased opportunities to bind to the intestinal mucosa.

From 1980's, type 1 pili-mediated interactions between enterobacteria and host cells were characterized using yeast agglutination assays (Eshdat, Speth, & Jann, 1981). Because of the numerous mannose residues exposed on the yeast cell surface, yeasts can bind type 1 pili-expressing bacteria and thus, can act as a competitive inhibitor of the ligand system, preventing bacterial attachment to the host cells. *Saccharomyces cerevisiae* CNCMI-3856 was previously selected for its high ability to agglutinate AIEC bacteria and to prevent bacterial attachment to intestinal epithelial cells *in vitro* (Sivignon, de Vallee, et al., 2015). In CEABAC10 mice, oral administration of *S. cerevisiae* CNCM I-3856 accelerated AIEC elimination from the gut and decreased the subsequent symptoms of colitis. Interestingly, crude extracts enriched in yeast cell wall still possess beneficial effects against AIEC infection in mice (Sivignon, de Vallee, et al., 2015).

In this study, the nature of glycoconjugates from *S. cerevisiae* that exert the anti-adhesive ability against AIEC was investigated in order to identify the active part of the yeast and to develop a rationally defined probiotic-based medicinal product targeting AIEC bacteria. Yeast mannan exerts good inhibition properties against AIEC. In comparison, a glycoconjugate fraction composed of mannan, β6-glucan, and glycogen together was shown to exhibit much superior anti-adhesive effect *in vitro* and *in vivo*. This work presents new alternatives in personalized antimicrobial therapies in CD, and more generally, to various pathologies involving type 1 pili-expressing enterobacteria.

2. Material and methods

2.1 Materials

All yeast products used in this study were provided by the Lesaffre Group (Marcq-en-Baroeul, France). CNCM I-3856, LV04 and LV08 are active live *Saccharomyces cerevisiae* yeasts commercially available in dry form. CNCM I-3856 is a patented probiotic yeast strain registered in the French National Collection of

Cultures of Microorganisms (CNCM) under the number I-3856. LV04 is a specific yeast strain used in the bioethanol production process. LV08 is a specific yeast strain used in the beer high fermentation (Ale). YCW CNCM I-3856 and YCW CNCM I-5268 are yeast cell wall fractions obtained from primary culture and then the autolysis of yeast cells from respective strain CNCM I-3856 and CNCM I-5268 (Reed & Nagodawithana, 1991). After cell lysis is completed, the soluble intracellular fraction (the yeast extract) is separated from the insoluble yeast cell wall fraction by centrifugation. Obtained yeast cell walls are then spray dried.

2.2 Purification of mannan fraction

50 g of yeast product (either whole yeast or yeast cell wall) was suspended in 300 mL of 0.02 M citrate buffer (pH 7), autoclaved at 121°C for 90 min. After autoclaving, the yeast extracts were cooled down, followed by centrifugation at 4300 g, at 4 °C for 30 min. The supernatant was collected, and the pellet was suspended in another 300 mL of 0.02 M citrate buffer, autoclaved at 121°C for 90 min followed by centrifugation again. Two supernatants were pooled together, called crude mannan. The pellet was kept for the extraction of glucan, which was described in section 2.4.

The conventional method to purify mannan uses Fehling solution through copper-mannan complex precipitation (Kocourek & Ballou, 1969). Fehling solution was prepared freshly by mixing equal volume of two prepared solutions; one solution contains 35 g of copper(II) sulfate pentahydrate, 5 mL of 2N sulfuric acid, addition of water until a final volume of 500 mL, and the other solution contains 87.5 g of potassium sodium tartrate, 38.5 g of sodium hydroxide in 500 mL of water. Fehling solution was added to the pooled supernatant (supernatant: Fehling solution, 1:1 v/v), and stirred overnight at 4°C. The mannan-copper complex was formed, and the precipitate was collected by centrifugation 4300 g at 4°C for 30 min. 100 mL of 3N HCl was added to the precipitate, and stirred at 4°C until the precipitate was dissolved to dissociate the mannan from copper. Another 300 mL of ethanol was added to precipitate mannan, and stirred overnight at 4°C. The white precipitate corresponding to mannan was collected by centrifugation at 4300 g at 4 °C for 30 min. The mannan precipitate was re-dissolved in 50 mL of water and put into

dialysis membrane (MWCO 3500) against 1L of water, then stirred overnight at 4 °C. The dialyzed mannan was collected and lyophilized.

2.3 Acetolysis of mannan

Dried mannan was aliquoted into 50 mL beaker, and 10 mL of 100% acetic acid, 10 mL of acetic anhydride, 1 mL concentrated sulfuric acid were subsequently added into beaker under hood and stirred at room temperature for 24 h. Then, solution was transferred into glass tube, followed by centrifugation at 4500 g at 4 °C for 10 min. The supernatant was collected and added slowly into 40 mL of frozen ice in 250 mL beaker, on ice. Sodium bicarbonate powder was added slowly into solution over ice until pH at 6-7. The solution was transferred into separatory funnel (250 mL), and 100 mL of chloroform was added into separatory funnel, followed by agitation 5-10 times. The funnel was kept stationary after agitation until two distinct layers were formed. The peracetylated oligomannosides-containing chloroform-rich bottom layer was collected under fume hood. Another 100 mL of chloroform was added into separatory funnel for further agitation and extraction. Two bottom layers were pooled together and transferred into separatory funnel again. 20 mL of saturated sodium bicarbonate solution (in water) was added into funnel, then stirred and the bottom layer was collected. The chloroform was removed by rotary evaporator. The dried peracetylated oligomannosides was re-dissolved in 50 mL of methanol and 50 mL of 0.2 M sodium hydroxide and stirred at 4 °C for 90 min for deacetylation. The solution was concentrated until 10 mL by rotary evaporator. The concentrated solution was loaded into AG®Dowex 50W-X8 column (bed volume 50 mL, 25-50 mesh ion exchange resin, Bio-Rad, No.1421421) to remove excess sodium salts. All the eluates were collected, followed by concentration until 5 mL by rotary evaporator. For separation of oligomannosides, Bio-Gel P6 gel filtration column (80 cm long, 2 cm diameter, Bio-Rad, No.1504130) was prepared. The oligosaccharides fraction was loaded into column, and water was used as eluent. All the eluates were collected and separated to 3 fractions, called oligo F1, oligo F2, oligo F3.

2.4 Purification of soluble glucan fraction containing β6-glucan-glycogen-mannan

The methods to purify soluble glucans fractions were adapted from the methodologies developed by Manners *et al*. and Sugawara *et al*. to study β-glucans (Manners, Masson, & Patterson, 1973; Sugawara, Takahashi, Osumi, & Ohno, 2004). The pellet from yeast product treated with hot citrate buffer, as described previously in section 2.2, was solubilized in 1 L of 1N NaOH, stirred at room temperature overnight to hydrolyze the proteins, lipids and residual mannan. The pellet was collected by centrifugation at 8400 g at 4 °C for 30 min. The pellet was washed by 1 L of water followed by centrifugation at 8400 g at 4 °C for 30 min, extracted by 800 mL of 0.5 N acetic acid and stirred at 90 °C for 3 h under chemical hood. After 3 h, solution was cooled down and centrifuged at 8400 g at 4°C, for 20 min. The pellet was extracted again by 800 mL of 0.5 N acetic acid and stirred at 90 °C for 3 h and centrifuged at 8400 g at 4°C, for 20 min. The two supernatants were pooled into the same flask. The extraction of the pellet was repeated at least 5 times. The pellet was used for phosphorylation as described in section 2.5. All the supernatants were pooled together, neutralized by NaOH and concentrated by rotary evaporator down to 500 mL. This soluble fraction was put into dialysis membrane (MWCO 3500) and dialyzed against 5-10 L of water at 4 °C, overnight. This dialyzed fraction was collected and concentrated down to 5 mL by rotary evaporator.

This soluble glucan fraction was further purified by hydrophilic-lipophilic-balanced cartridge (HBL, Waters Oasis®). The soluble polysaccharides were not bound onto this reverse-phase cartridge and all the impurities containing lipids and peptides/proteins were retained on the HLB cartridge. The cartridge was equilibrated by 5mL of acetonitrile and 5 mL of 0.5 N acetic acid in a stepwise manner. This glucan fraction was loaded into HLB cartridge and eluted by 5 mL of 0.5 N acetic acid. The collected flow through was dialyzed in dialysis tube (MWCO 3500) against 2 L of water at 4 °C, overnight and lyophilized.

2.5 Phosphorylation of insoluble β3-glucan

The pellet from hot acetic acid extraction was dialyzed into dialysis membrane (MWCO 3500) against 1 L of water at 4 °C, overnight and lyophilized. This fraction contains β3-glucan, which was identified by linkage analysis and NMR analysis. To solubilize this fraction, phosphorylating β3-glucan was used

(Williams et al., 1991). 0.4g urea solubilized in 5 mL of dimethyl sulfoxide (DMSO) were added into the beaker containing 100 mg β3-glucan then stirred until it dissolved. 1 mL of 85% H_3PO_4 was added and stirred at 100°C for 6h. After cooling down, 100 mL of water was added and the product was filtered through 1.2 μm membrane. The filtered solution contains phosphorylated β3-glucan that was dialyzed against water, followed by lyophilization.

2.6 NMR Analysis

All NMR data were acquired on Avance II Bruker spectrometer equipped with BBO or TBI 5mm probe resonating at 400 MHz for ^{1}H, 100.6 MHz for ^{13}C and 162 MHz for ^{31}P. Mannan and β6-glucan-glycogen-mannan polysaccharides were dissolved in 500 μL D_2O (99.96% ^{2}H, Eurisotop®) at 10 mg/mL. β3 glucan was dissolved in d6-DMSO (99.96% ^{2}H, Eurisotop®) with or without D_2O, at 10 mg/mL. The solubilized polysaccharides were then transferred into 5 mm Shigemi tubes (Allision Park, USA). NMR experiments were performed at 293 K for mannan, 341 K for β6-glucan-glycogen-mannan, 353 K for β3 glucan. The signals corresponding to methyl group of acetone (δ^1 H 2.225 and δ^{13} C 31.55 ppm.) or signals corresponding to methyl group of DMSO at δ^1 H 2.52 and δ^{13} C 40.98 ppm were used as to calibrate the spectra. The ^{1}H/^{1}H-COSY, ^{1}H/^{1}H-TOCSY, ^{1}H/^{13}C-HSQC experiments were performed by using the Bruker standard sequences and optimized for each experiment.

2.7 Bacterial strain and intestinal epithelial cell line

The ampicillin/erythromycin-resistant AIEC strain LF82 was isolated from an ileal biopsy specimen of a CD patient. Bacterial strain was conserved at -80 °C in glycerol stocks. Bacteria were seeded from the LF82 glycerol stock to Luria Bertani (LB, Conda, Madrid, Spain) broth and grown overnight at 37°C without shaking. The growth was spectrophotometrically evaluated at 600nm.

The human intestinal epithelial T84 cell line was obtained from the American Type Culture Collection (ATCC® CCL248™) and stored in liquid nitrogen. T84 cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient mixture F-12 (DMEM/F12, Gibco) supplemented with 2 mM L-Glutamine (Gibco), 0.1M

HEPES buffer (Dominique Dutscher, Brumath, France), 10% fetal bovine serum (FBS, Dominique Dutscher, Brumath, France), and antibiotics-antimycotics to a final concentration of 100 U/mL penicillin G, 100 μg/mL streptomycin and 0.25μg/mL of amphotericin B (GE Healthcare Österreich, Austria). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

2.8 Adhesion assays on AIEC LF82-infected intestinal epithelial cells in the presence of yeast glycoconjugates

T84 cells were seeded at 1.5×10^5 cells per well in 48-well plates and incubated for 48 h at 37°C. Before infection, monolayers were washed 3 times with phosphate buffer saline (PBS, Gibco) and culture medium was replaced by cell medium without antibiotics/antimycotics. LF82 bacterial suspension was prepared at a concentration of 1.2×10^7 CFU/mL in cell culture medium. Bacteria were incubated for 1 h at room temperature with increasing concentrations of yeast glycoconjugates on an orbital agitator (ratio1:1; final bacterial concentration 6×10^6 CFU/ml). Intestinal epithelial cells were infected with bacteria/yeast product mixtures for 3 h at 37°C in a humidified 5% CO_2 atmosphere at a multiplicity of infection of 10 bacteria per cell. T84 cells were washed 3 times with phosphate buffer (PBS) and lysed with 1% Triton X-100 (Sigma) in deionized water. After appropriate dilutions in physiological water, adhering bacteria were plated on Luria-Bertani agar medium and were incubated overnight at 37°C to determine the number of colony-forming units (CFU). The average adhesion rates (from at least 3-4 independent experiments) were expressed as a percentage of residual adhesion, which is the ratio of the bacterial adhesion level in the presence of yeast products and the adhesion level in absence of treatment (considered as 100 %). The error bars correspond to the standard error at mean or SEM.

2.9 Animals

Mice were housed in specific-pathogen-free conditions (21–22°C, 12:12-h light-dark cycle), with access to food and water *ad libitum*, in the animal facility of the University Clermont Auvergne (unité de stabulation et d'expérimentation animale, Clermont-Ferrand, France). SPF FVB/N wildtype female mice

purchased from Charles Rivers Laboratories (France) and FVB/N CEABAC10 transgenic male mice (heterozygous, (Chan & Stanners, 2004)) were coupled to obtain 50% WT mice and 50% CEABAC10 mice. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the University of Clermont Auvergne (Clermont-Ferrand, France). The animal protocol was approved by the Committee for Research and Ethical Issues of the Department of Auvergne (CEMEA Auvergne; Permit Number: CEMEAA, 20160111171036870).

2.10 Mice infection protocol and AIEC LF82 gut colonization assessment

Eight-week-old CEABAC10 heterozygous male mice (body weight, ~24–26 g) were orally pretreated with 5 mg per day (in 0.2 mL of PBS, phosphate buffer saline) of the soluble glucan fraction extracted from YCW CNCM I-5268 or with PBS for non-treated mice, from day-7 to the day of the bacterial challenge (day 0). After infection, soluble glucan was administered twice a day until day 4 post-infection. Mice received 0.5 % of dextran sulfate sodium (DSS, MP Biomedicals, Illkirch, France) in drinking water from day-3 to day 4 and they were treated *per os* with 5 mg of streptomycin (Sigma-Aldrich) at day-1 in order to disrupt normal resident bacterial intestinal microbiota and induce a low-grade inflammation. An overnight bacterial culture of AIEC LF82 strain was harvested by centrifugation at 5 500 g for 10 min. The bacterial pellet was resuspended in PBS to reach a concentration of 2.5×10^{10} bacteria/mL. Mice were challenged with 5×10^9 LF82 (in 0.2 mL) at day 0 immediately after an oral administration of 0.1 mL of sodium bicarbonate at 0.2M (Sigma-Aldrich). Body weight was monitored throughout the experiments. At days 1, 2, 3 and 4 post-infection, fresh fecal pellets (≈100 mg) were collected from individual mice and resuspended in PBS. After appropriate serial dilutions, bacteria were enumerated by plating on Luria-Bertani agar medium containing 100 µg/ml ampicillin (Sigma-Aldrich) and 20 µg/ml erythromycin (Sigma-Aldrich) to isolate AIEC LF82 and incubated at 37°C overnight. The severity of colitis was assessed by the disease activity index (DAI) score, which ranges from 0 (healthy) to 12 (high activity of colitis) (Sivignon, Yan, et al., 2015). At day 4 post-infection, they were anesthetized with isoflurane and killed by cervical

dislocation. Colon specimens were collected to quantify LF82-associated to the tissue by plating onto antibiotic-selective culture medium for LF82.

2.11 Fecal lipocalin-2 assay

Fecal samples from days 2 and 3 post-infection were homogenized and centrifuged at 10,000 g for 10 min. Lipocalin-2 levels were assessed in the supernatant with the Mouse Lipocalin-2/NGAL DuoSet Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Results are expressed as ng/g of feces.

2.12 Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA) software package for PC. Data are expressed as means ± standard error of the mean (SEM) or as medians. Data were compared by Student's t-test analysis (Mann-Whitney test if not parametric), or ANOVA followed by a post-Bonferroni (Kruskal–Wallis if not parametric). A p value of less than 0.05 was considered to be statistically significant. * $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3. Results

3.1 Purification of glycoconjugates from Saccharomyces cerevisiae yeasts

The yeast cell wall is a multilayered structure that provides physical protection and osmotic support to the cell. The outer layer consists of heavily glycosylated mannoproteins collectively referred as phospho-peptido-mannans (PPM) or mannan, glycogen-like α1,4-glucan, and cell wall proteins that are covalently linked or associated with the inner layer made of β1,3-glucan-chitin network and β1,6-glucans (Klis, Mol, Hellingwerf, & Brul, 2002) (**Figure 1A**). Initially, five yeast samples were chosen as starting material in order to compare their structures and activities, including three strains of intact dry yeasts (CNCM I-3856, LV04, LV08), and two yeast cell wall (YCW) enriched fractions generated by autolysis of yeasts (YCW CNCM I-3856 and YCW CNCM I-5268). Herein an experimental procedure aimed at purifying individual

glycoconjugate fractions from the chosen whole yeasts and yeast cell walls, including PPM, β3-glucan, β6-glucan, and α4-glucan, was optimized in order to subsequently assay their capacity to inhibit bacterial adhesion (**Figure 1B**). In brief, crude PPM fraction was solubilized from whole yeast or YCW in citrate buffer at 120°C, and enriched by Fehling method through the formation of insoluble copper-mannose complex (Kocourek & Ballou, 1969). Then, remaining insoluble fraction was treated with successive chemical extractions in order to purify different types of glycoconjugates (Aklujkar, Sankh, & Arvindekar, 2008; Manners, Masson, & Patterson, 1973; Sugawara et al., 2004). Herein, hot diluted acetic acid was finally utilized to separate insoluble β3-glucan from soluble β6-glucan and α4-glucan fractions.
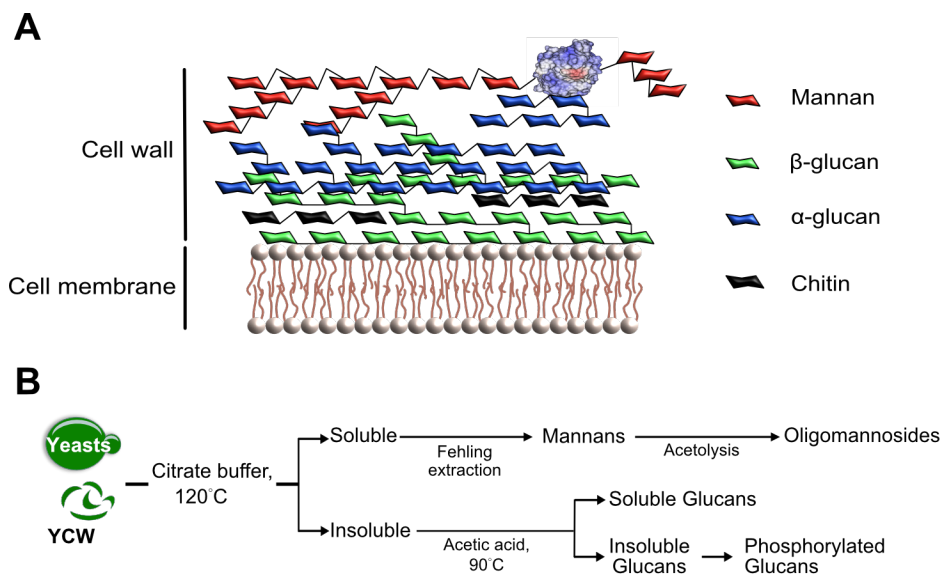

Figure 1. (A) Schematic representation of major glycoconjugates on the cell wall of *S. cerevisiae*. (B) Flowchart of polysaccharide extractions from intact yeasts or yeast cell walls (YCW) isolated from *S. cerevisiae* by autolysis. The overall fractionation pipeline was adapted from existing methodologies developped to purify mannans and β-glucans (Kocourek & Ballou, 1969; Manners, Masson, & Patterson, 1973; Sugawara et al., 2004).

3.2 PPM from CNCM I-3856 yeast strain exhibits good anti-adhesive property in vitro

The outmost layer PPM consists of polymannosylated *N*-glycans covalently attached to proteins. In *S. cerevisiae*, the polymannosides are composed of a long stretch of (α-1,6)-linked D-mannopyranose units substituted in C2 positions by short side chains of (α-1,2)-linked mannose units that may be further

capped by terminal Man(α-1,3) residues. So called acid-labile mannan domain is further attached to the (α-1,2)-oligomannosides through phospho-di-ester bond (Nelson, Shibata, Podzorski, & Herron, 1991). PPM fractions from different *S. cerevisiae* strains were extracted to identify their structures and examine their inhibitory activity effects on the AIEC LF82 adhesion to T84 intestinal epithelial cells.

The structures of PPM from the five samples were characterized by a combination of NMR experiments including 1D-^{1}H-NMR, ^{1}H-^{1}H COSY-NMR and ^{1}H-^{13}C HSQC-NMR analyses, based on the known structural features and NMR parameters of *S. cerevisiae* polymannosylated glycans (Dulary et al., 2018; Vinogradov, Petersen, & Bock, 1998). Here, the analysis was focused on the main structural features of the polymannosylated moiety (mannan) such as the nature, the density and the terminal capping of mannosylated side chains. The anomeric region (5.5-4.8 ppm) of 1D-^{1}H-NMR spectra shows five broad signals annotated as I, II, III, IV, V corresponding to five different epitopes, which are internal -2)Man(α-1,2) residues (I); terminal Man(α-1,3) residues (II); -2,6)Man(α-1,6) branched residues (III); terminal Man(α-1,2) and -3)Man(α-1,2) residues (IV); unbranched -6)Man(α-1,6) residues (V), respectively (**Figure 2A, 2B**). The identity of these signals was confirmed according to their ^{1}H-^{13}C NMR parameters as established by ^{1}H-^{13}C HSQC experiments (**Supplementary Figure 1, Supplementary table 1)** and to the partial spin systems of the associated monosaccharides as established by ^{1}H-^{1}H COSY experiments (data not shown). Altogether, all five PPMs expressed complete sets of I-V NMR signals, except LV08 that was totally devoid of terminal Man(α-1,3) residues, as shown by the absence of signal II. However, the relative quantification of individual signals showed that all five PPMs dispatched similar but slightly different mannose patterns (**Supplementary Figure 1B**). Among those, PPM extracted from whole yeast CNCM I-3856 and from YCW CNCM I-3856 showed very similar structures according to NMR data, which indicates that prior sub-fractionation of whole yeast does not modify the nature of extracted mannans. It is noteworthy that mannans from both CNCM I-3856 samples and from LV04 strain are characterized by a higher branching level than the two other strains, as shown by the very low intensity of signal V. Intriguingly, as already mentioned, whereas the LV08 mannan does not present α3 mannose capping, LV04 mannan contains more α3 mannose capping than the four other PPM samples, accordingly to the relatively very high intensity of signal II (**Figure 2A, Supplementary Fig 1B**). Altogether, the five PPMs

could be categorized in four different groups according to the combination of structural features of their mannan moieties, as depicted in **Figure 2C**.
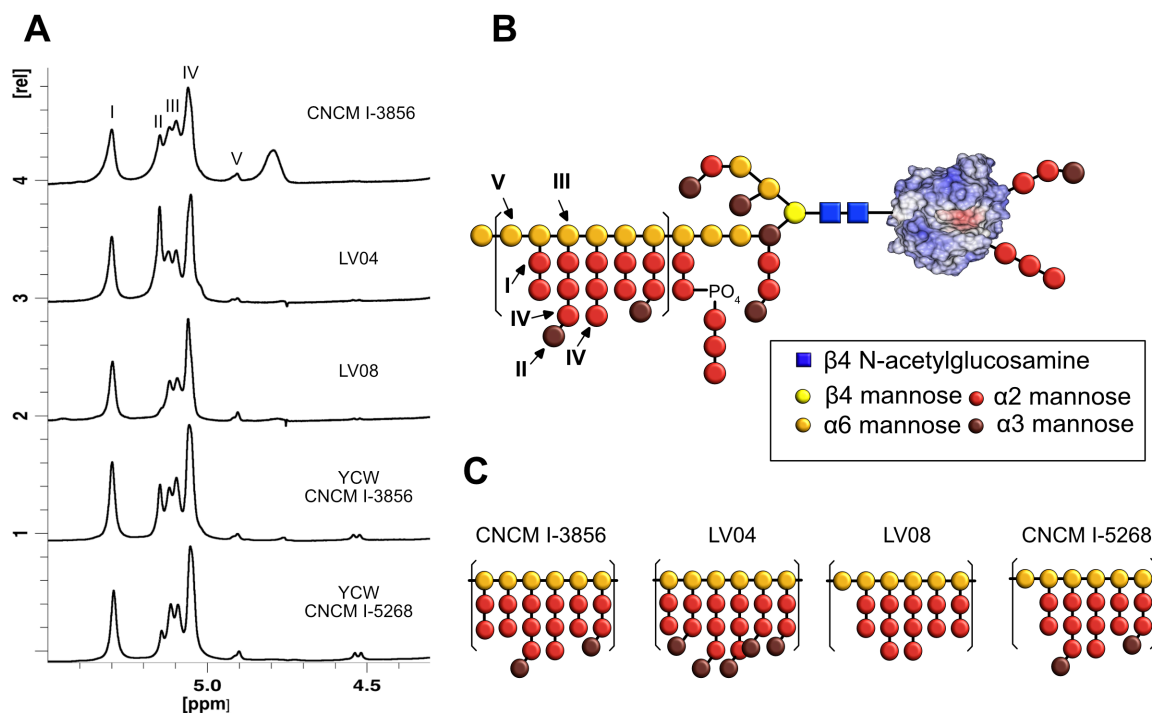

Figure 2. Structural characterizations of mannan fractions extracted from CNCM I-3856, LV04, LV08 yeasts and yeast cell walls from CNCM I-3856 and CNCM I-5268. (A) 1D-^{1}H-NMR analyses of mannan (acquisition at 400 MHz, D_2O, 300K). (B) Schematic representation of PPM. (C) Schematic representation of mannan moieties from four different strains.

The capacity of five PPMs extracted from yeasts and yeast cell walls (**Figure 3A**) to inhibit bacterial adhesion was assessed *in vitro*. Different concentrations of PPM were pre-incubated with AIEC LF82 bacterial strain. The glycoconjugates/bacteria mixtures were then added onto T84 cells and cell-associated LF82 bacteria were quantified in order to determine the residual adhesion percentage related to the yeast treatment. Among these, PPM from CNCM I-3856 yeast exerted the highest anti-adhesive effect for LF82 adherent to T84 cells in a dose-dependent manner (**Figure 3B**). Indeed, at 0.25 mg/mL, PPM of CNCM I-3856 inhibited 50.06% of bacterial adhesion, whereas a 4-fold higher concentration was needed for PPM from LV04 and LV08 strains to reach the same efficiency. Intriguingly, inhibition efficiency of CNCM I-3856 is decreasing when PPM were extracted from YCW. At the maximal concentration of 1

mg/mL, PPM from YCW CNCM I-3856 led to an AIEC LF82 residual adhesion level of 54.64% compare to 22.87% for PPM from whole CNCM I-3856 (**Figure 3B and 3C**). PPM from YCW CNCM I-3856 was less efficient to decrease AIEC LF82 attachment to cells that the one extracted from the whole yeast. Maximal inhibitory effect of PPM fraction from YCW CNCM I-5268 was slightly better than PPM from YCW CNCM I-3856 with 46.23% of residual adhesion at concentration of 1 mg/mL (**Figure 3C**). Altogether, combined structural and *in vitro* analyses showed that all PPMs exhibited anti-adhesive activities toward AIEC LF82, although with different efficiencies. The PPM activity level could not be unambiguously correlated with a specific trait such as terminal capping, which suggests that inhibitory efficiency may be dependent on other factors such as epitope presentation or density that cannot be evaluated by the used methodologies.
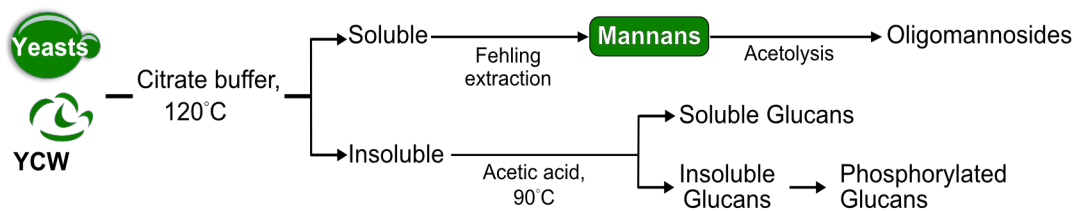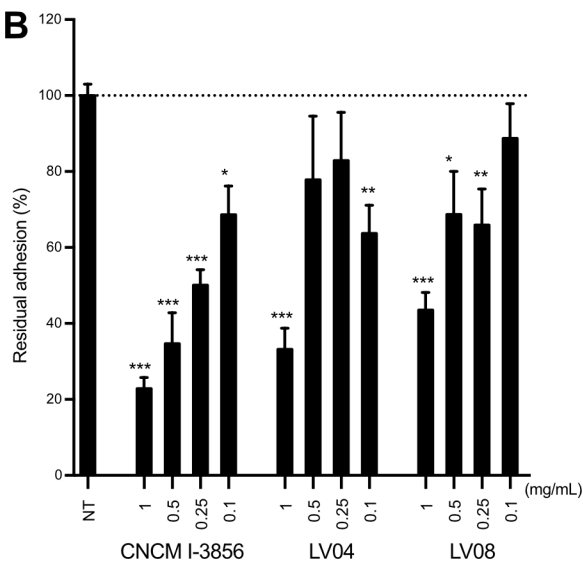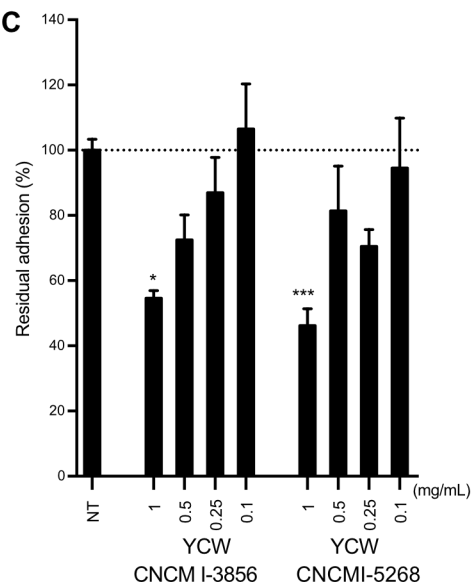

Figure 3. Mannan fractions extracted from CNCM I-3856, LV04, LV08 yeasts and CNCM I-3856, CNCM I-5268 yeast cell walls reduce AIEC adhesion to T84 cells. The fraction containing phosphopeptidomannan (PPM), which was utilized for inhibition assay, was highlighted in (A). Adhesion assays: infection of T84 cells with AIEC LF82 bacteria at a multiplicity of infection of 10 bacteria/cell for 3 hours. Bacteria were

pre-incubated 1 hour with different concentrations (in mg/mL) of PPM from CNCM I-3856, LV04, LV08 yeasts in (B), and from CNCM I-3856 and CNCM I-5268 YCWs (C), before adhesion assay. Results are expressed as percentages of adherent bacteria, LF82 infection in absence of treatment was considered as 100% (mean±SEM). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ in comparison with the non-treated condition. One-way ANOVA with Dunnett's Multiple Comparison Test.

3.3 The production of oligomannosides from PPM does not potentiate anti-adhesive activity

In order to determine the region of mannan that plays the role of inhibition and possibly enhance the anti-adhesive potency of the purified fractions, mannans were chemically degraded in order to generate defined sets of oligomannosylated compounds. PPM was subjected to mild acetolysis that preferentially cleaves the Manα1-6Man linkage over the more stable Manα1-2Man and Manα1-3Man linkages (Kocourek & Ballou, 1969; Stewart & Ballou, 1968), and then, the generated oligosaccharides were separated by gel filtration. Three major fractions F1-F3 were collected (**Figure 4A**). MALDI-MS profiles (**Supplementary Figure 2**) of these three fractions showed that fraction 1, which is the earliest eluting fraction, contains abundant phosphorylated oligosaccharides (Man)$_n$-phosphate, n=3-10 with a prevalence for low molecular mass oligosaccharides Man$_3$-PO$_3$ and Man$_4$-PO$_3$. Early elution of phosphorylated oligosaccharides, irrespectively of their low mass range, is due to the negative charges repulsion between phosphate groups and remnant acidic groups on the polyacrylamide medium, as previously observed (Garenaux et al., 2008). F2 contains a mixture of smaller phosphorylated oligomannoses Man$_3$-PO$_3$ and Man$_4$-PO$_3$ as well as two groups of neutral oligosaccharides with higher degree of polymerization: one group of oligomannoses Hex$_2$ to Hex$_9$ and one group of oligosaccharides corresponding to HexNAc$_2$Hex$_4$ to HexNAc$_2$Hex$_6$. The monosaccharide composition strongly suggests that it is composed of the trimannosylated core *N*-glycans (GlcNAc$_2$Man$_3$) further extended by a variable number of mannose residues. Finally, F3 contains smaller oligomannoses Hex$_2$ to Hex$_8$ dominated by Man$_2$, Man$_3$ and Man$_4$.

These three fractions (F1, F2 and F3) of oligosaccharides significantly decreased LF82 adhesion to T84 cells at the concentration of 1 mg/mL, but this effect did not match the inhibition efficiency of the intact PPM at the same concentration (**Figure 4B**). Altogether, the complex and branched structure of the mannan from CNCM I-3856 is the best inhibitor against LF82 bacterial adhesion.
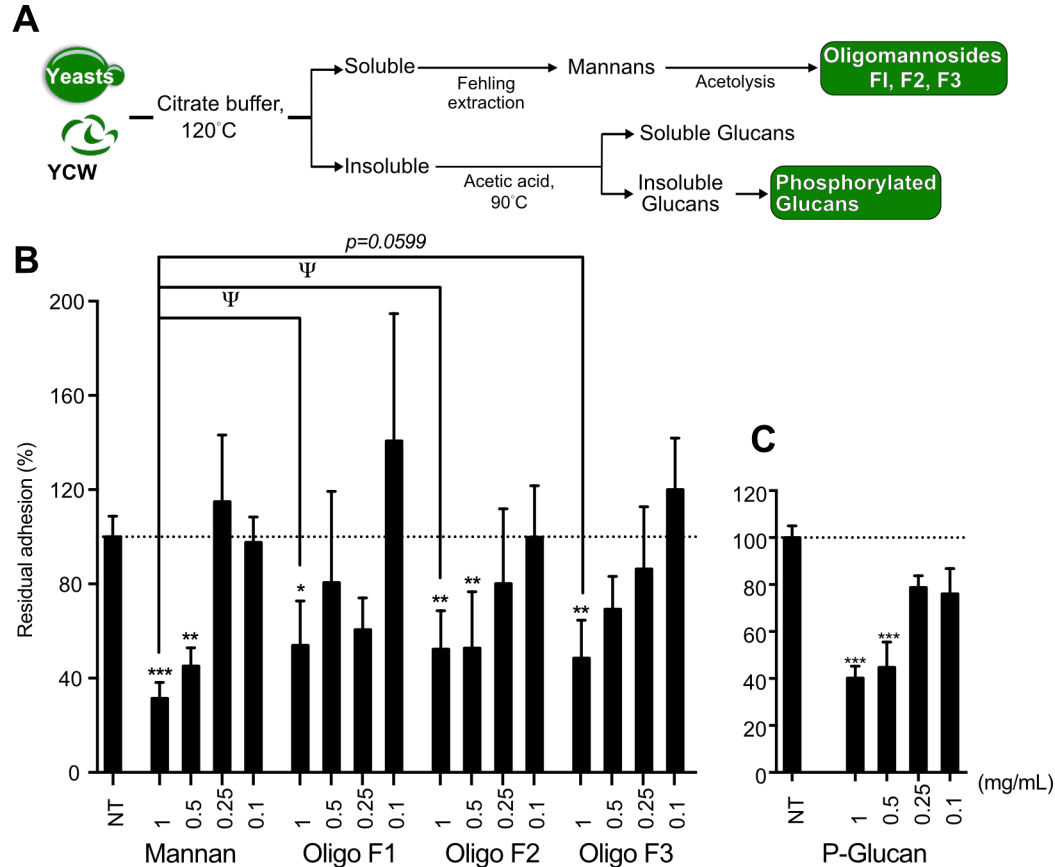

Figure 4 Activity of mannan-derived oligomannosides and phosphorylated β3-glucan from CNCM I-3856 yeast on AIEC LF82 bacteria adhering to T84 cells. Two different fractions utilized for inhibition assay were highlighted in (A). Oligo F1, F2, F3 are different fractions of oligomannosides, which were generated from mannans by acetolysis and gel filtration. P-Glucans are soluble phosphorylated β3-glucan that are otherwise insoluble. Adhesion assays: infection of T84 cells with AIEC LF82 bacteria at a multiplicity of infection of 10 bacteria/cell for 3 hours. Bacteria were pre-incubated 1 hour with different concentrations (in mg/mL) of oligomannosides and phosphorylated β3-glucan before adhesion assay. (B) Residual adhesion after incubation with mannan and the three major fractions (F1, F2 and F3) of acetolyzed mannan. (C) Residual adhesion after incubation with phosphorylated β3-glucan. Results are expressed as percentages of adherent bacteria, LF82 infection in absence of treatment was considered as 100%

(mean±SEM). *p < 0.05; **p < 0.01; ***p < 0.001 in comparison with the non-treated condition. Ψ p<0.05 between 1 mg/mL conditions. One-way ANOVA with Dunnett's Multiple Comparison Test.

3.4 Phosphorylated β3-glucan from CNCM I-3856 has an intermediate anti-adhesive effect in vitro

β3-glucans were purified from the three intact yeast strains (CNCM I-3856, LV04 and LV08) and from YCW CNCM I-5268. According to the purification protocol, GC-MS composition analysis confirmed that these fractions were composed by more than 95% of glucose (Glc) and less than 5% of mannose. Overall, structural analysis showed that all four fractions were essentially similar. In particular, GC-MS analysis of partially methylated acetylated alditols (PMAA) showed a majority of 3-linked Glc residues and 3,6-linked Glc residues, in accordance with the presence of a β3-glucan polymer further substituted in C6 position (**Supplementary Figure 3**). In addition, minor signals associated to 6-linked Glc suggests the presence of either 6-linked side chains or β6-glucan.

Considering the structural similarity of all purified β3-glucans, only β3-glucan isolated from CNCM I-3856 was used to conduct *in vitro* assay to examine the inhibiting activity toward AIEC attachment on intestinal cells. However, because of its insolubility, β3-glucan could not be used straightly for *in vitro* assays. Therefore, β3-glucan from CNCM I-3856 was phosphorylated in order to solubilize it prior to assay (Du, Zhang, Wang, & Xiao, 2012; Shi, Shi, & Li, 2014; Williams et al., 1991). Presence of phosphorylated glucan was first confirmed by observing a strong signal at 0 ppm on 1D ^{31}P-NMR (**Supplementary Figure 4A**). ^{1}H-^{31}P-HSQC NMR analysis established that the phosphate groups were in majority associated to the C6 positions of 3-linked as well as C2 and C4 position of 6-linked of β-Glc residues (**Supplementary Figure 4B**). Then, linkage analyses by GC-MS of phosphorylated β3-glucan (**Supplementary Figure 4C**) from CNCM I-3856 showed an increase in the proportions of terminal Glc and 6-linked Glc counterbalanced by a decrease in the proportion of 3-linked Glc, compared to original β3-glucan (**Supplementary Figure 3**). ^{1}H-^{13}C-HSQC NMR analysis of this fraction also confirmed that the phosphorylated β3-glucan was made of equivalent proportions of 3-linked and 6-linked glucans (**Supplementary Figure 4D, Supplementary table 2**). These results indicated that phosphorylated β3-

glucan fraction is constituted by shorter chains of β3-glucan compared with the original β3-glucan. It may be caused by the high temperature phosphoric acid that partially depolymerized the β3-glucan polysaccharides. Of interest, even the small proportion of mannose in phosphorylated β3-glucan fraction (5%) compared to PPM fraction from CNCM I-3856 (92%) (known by monosaccharide analysis, data not shown), this fraction was able to inhibit 60% of bacterial adhesion at 1 mg/mL (**Figure 4C**).

3.5 Soluble glucan fraction exert a better anti-adhesive effect when extracted from yeast cell wall rather than intact yeast

According to the purification scheme, citrate-insoluble fraction of whole yeasts (CNCM I-3856, LV04, LV08) and of YCW (CNCM I-3856 and CNCM I-5268) treated by hot diluted acetic acid solution generates a soluble glucan fraction (**Figure 1B**). This soluble glucan fraction was supposedly constituted of a mixture of β6-glucan and α4-glucan (Arvindekar & Patil, 2002; Bacon, Farmer, Jones, & Taylor, 1969; Manners, Masson, & Patterson, 1973; Manners, Masson, Patterson, Bjorndal, & Lindberg, 1973). In agreement to this hypothesis, 1D-^{1}H-NMR spectra of the glucan soluble fractions isolated from the five yeast samples showed intense signals at 4.3 ppm and at 5.37 ppm tentatively attributed to internal β6-linked glucose residues and to α4-linked glucose residues, respectively (**Figure 5A**). Their attribution was further confirmed by ^{1}H-^{13}C HSQC NMR experiments. These two monosaccharides were predominant in soluble glucan fractions isolated from CNCM I-3856, LV04 and LV08 as they represented from 82% (CNCM I-3856) to 96% (LV08) of the total signal intensities of anomeric protons. Unexpectedly, NMR analysis of soluble glucan fractions isolated from yeast cell walls (YCW CNCM I-3856 and YCW CNCM I-5268) showed an additional set of intense ^{1}H signals previously identified as α-Man residues signals I to V associated to mannan (**Figure 2**), which established the presence of significant quantities of mannan in these two samples (**Figure 5A**). The nature of these signals was further confirmed by ^{1}H-^{13}C HSQC NMR analyses (**Figure 5B, Supplementary table 3**). This set of signals could also be observed in the soluble fractions extracted from CNCM I-3856, LV04 and LV08, but with much lower intensity. The proportions of β-glucan, α-glucan and mannan (expressed as β-glucan/α-glucan/mannan ratios, GGM) in the soluble glucan fractions isolated from each sample were estimated by area integration of individual signals detected by

1D-^{1}H-NMR analyses (**Supplementary Figure 5**). The soluble glucan fraction isolated from CNCM I-3856, LV04 and LV08 were characterized by very high proportions of α-glucan (from 56 to 88%) and low proportions of mannan (from 2 to 18%), as depicted on **Figure 5C**. On the contrary, soluble glucan fractions from YCW CNCM I-3856 and YCW CNCM I-5268 contain a high content of both β-glucan and mannan but low content of α-glucan with GGM index around 40:15:45 (Supplementary Figure 5). The high mannose content of soluble glucan fraction extracted from yeast cell wall was confirmed by GC-MS monosaccharide and linkage analyses (**Supplementary Figure 6**). Thus, these results demonstrated that when the soluble glucan fraction is purified from whole yeasts (CNCM I-3856, LV04 and LV08), it is almost mainly constituted by α-glucan whereas the soluble glucan fraction is highly enriched in β-glucan and mannan when it is purified from cell walls.
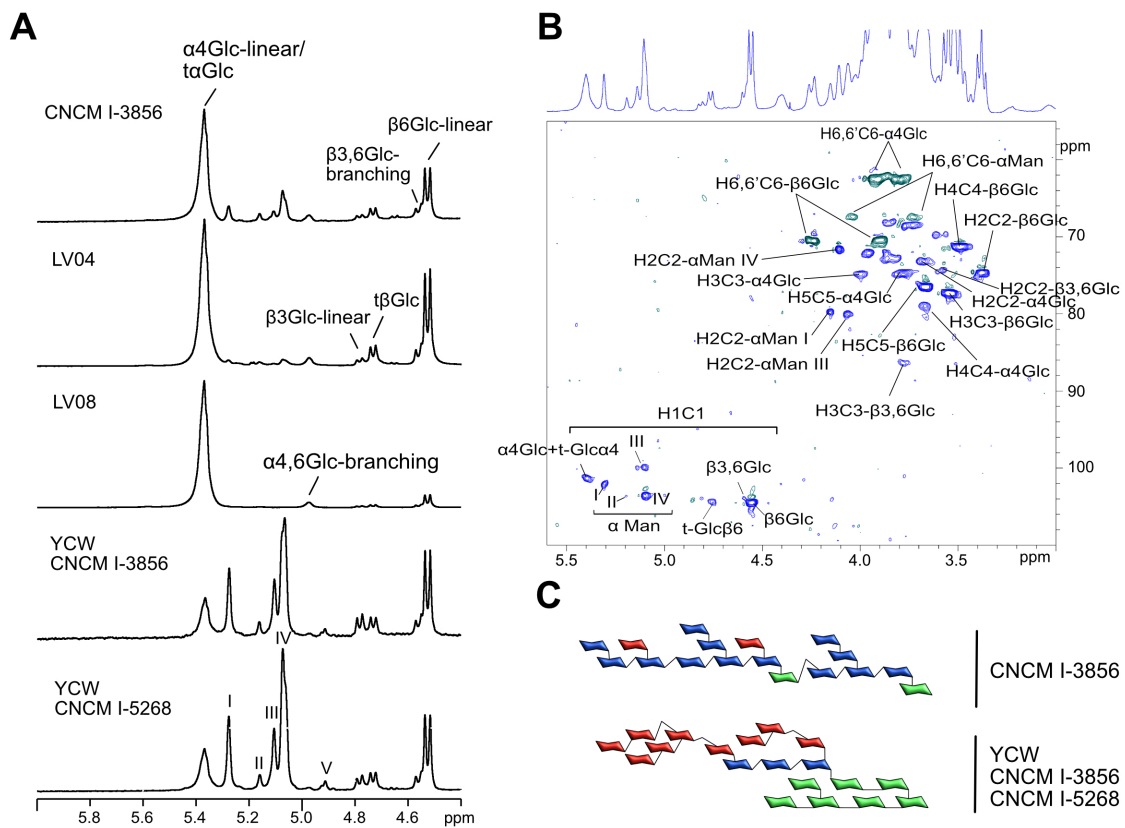

Figure 5. Structural characterizations of soluble glucan fractions extracted from CNCM I-3856, LV04, LV08 yeasts and CNCM I-3856, CNCM I-5268 yeast cell walls. (A) 1D-^{1}H-NMR analyses of glucans (acquisition at 400 MHz, D$_2$O, 341K., Signals annotated as peaks I, II, III, IV, V are the signals corresponding to mannan as shown in Figure3. (B) ^{1}H-^{13}C HSQC spectrum (C) Schematic

representation and proportions of heteropolymers consisting in mannan (red), α-glucan (blue) and β-glucan (green).

Considering the clear structural differences observed between soluble glucan fractions depending on the starting material (**Figure 6A**), their *in vitro* cellular activities toward bacterial adhesion were compared. In intestinal epithelial cells T84, adhesion inhibition property of the soluble glucan fraction extracted from whole yeast CNCM I-3856 was shown to be moderate with a residual adhesion level of 42.3% at the concentration of 1 mg/mL (**Figure 6B**). At the same concentration, soluble glucan fraction from YCW CNCM I-3856 exhibits a much higher anti-adhesive effect since it decreases LF82 adhesion at 16.7% of residual adhesion (**Figure 6C**). Interestingly, soluble glucan fraction from YCW CNCM I-5268 exhibited the highest inhibitory effect, as 94.3% of the bacterial adhesion to T84 cells was prevented at the concentration of 1 mg/mL.

The high proportion of mannose in these fractions may be instrumental to the increased anti-adhesive activity. However, the fact that the inhibitory effect is far superior to that of the mannan fraction demonstrates that richness in mannan in not sufficient to trigger the activity. The presence of the three partners β-glucan/α-glucan/mannan in a precise ratio may be required to potentiate the anti-adhesive efficacy.
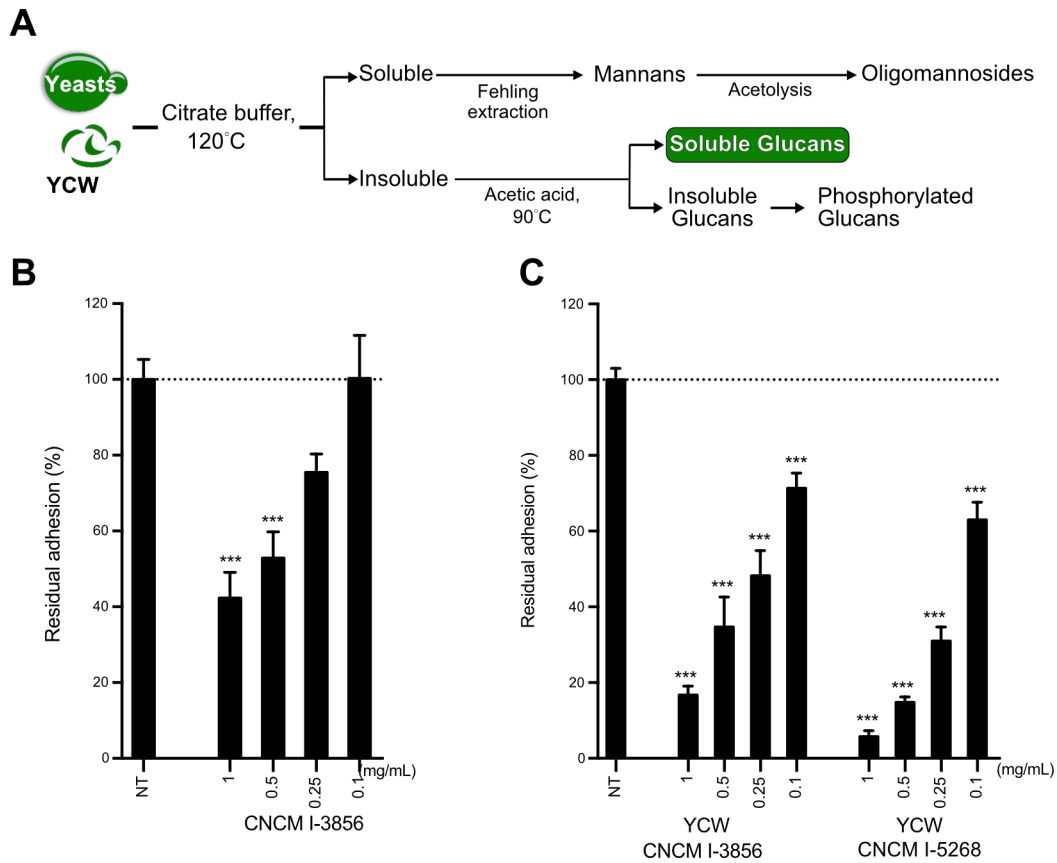
Figure 6. Soluble glucan fractions extracted from CNCM I-3856 and YCW from CNCM I-3856, CNCM I-5268 strongly reduce AIEC adhesion to T84 cells. Soluble glucans were extracted according to (A). Adhesion assays: infection of T84 cells with AIEC LF82 bacteria at a multiplicity of infection of 10 bacteria/cell for 3 hours. Bacteria were pre-incubated 1 hour with different concentrations (in mg/mL) of soluble glucan from CNCM I-3856 yeasts (A), or from CNCM I-3856 and CNCM I-5268 YCWs (B) before adhesion assay. Results are expressed as percentages of adherent bacteria, LF82 infection in absence of treatment was considered as 100% (mean±SEM). ***p < 0.001 in comparison with the non-treated condition. One-way ANOVA with Dunnett's Multiple Comparison Test.

3.6 Soluble glucan fraction isolated from yeast cell wall favors the elimination of AIEC LF82 bacteria from the gut of mice

Considering the high capacity of soluble glucan fraction isolated from yeast cell wall to inhibit the bacterial adhesion to T84 cells *in vitro*, the *in vivo* activity of the most effective soluble glucan fraction isolated from YCW CNCM I-5268 was assessed in the CEABAC10 mouse model mimicking CD susceptibility

infected with the AIEC LF82 bacterial strain. Bacterial infection induced a decrease of the body weight of mice for 48 hours. Yeast treatment succeeded in reversing the body weight curve from 2 days post-infection (dpi), and body weights were almost restored at the initial level at dpi 4 (**Figure 7A**). AIEC LF82 bacterial loads in the feces of mice were significantly decreased from dpi 2 to dpi 4 by the soluble β-glucan fraction treatment (**Figure 7B**). This result was confirmed by the analysis of the area under curve, which reflects the global colonization of the gut during the infection period for each mouse ($2.34×10^8$ cfu versus $2.95×10^7$ cfu for non-treated mice and treated mice respectively, **Figure 7C**). Yeast treatment also reduced the number of LF82 bacteria associated to the colonic mucosa. At dpi 4, 62.5% of mice still presented AIEC associated to the mucosa, whereas percentage decreased at 42.8% in the glucan-treated group (**Figure 7D**). A slight improvement was observed concerning the severity of colitis as assessed by the disease activity index (DAI) score (**Figure 7E**) and the concentration of lipocalin-2, a fecal marker of intestinal inflammation, was significantly decreased at dpi 2 (**Figure 7F**).
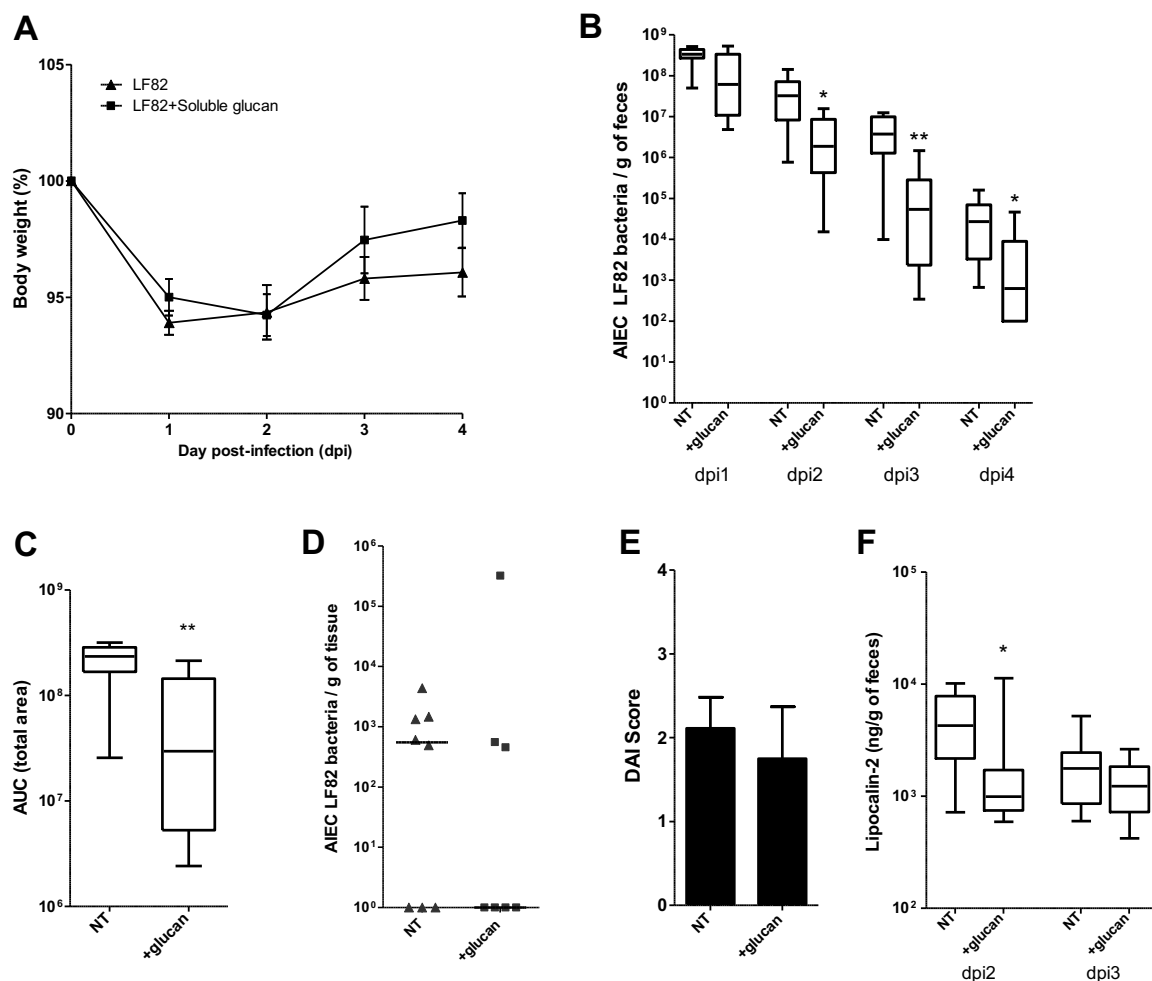
(A) Body weight in percentages (means±sem). (B) AIEC LF82 bacterial load in the feces of mice (in bacteria per gram of feces) after oral challenge with $5×10^9$ bacteria/mouse (box and whiskers-min to max). (C) Area under curve (total area) of AIEC LF82 colonization from dpi 1 to dpi 4 (box and whiskers-min to max). (D) AIEC LF82 bacteria associated to the colonic tissue (in bacteria per gram of tissue) at dpi 4. Each point represents bacterial quantification for one mouse, bars correspond to medians. (E) Disease activity Index (DAI) scores at dpi 3 (means±sem). (F) lipocalin-2 concentrations in ng/g of feces (box and whiskers-min to max). *n*=8 mice for non-treated (NT) group; *n*=7 for soluble glucan-treated group. Mann Whitney test, *p < 0.05 ; **p < 0.01.

To conclude**,** soluble glucan fraction extracted from YCW from CNCM I-5268 was highly effective to prevent AIEC LF82 attachment to intestinal epithelial cells, but also exerts beneficial properties *in vivo* in decreasing LF82 gut colonization and in reducing a fecal biomarker of intestinal inflammation, lipocalin-2. The tri-hetero partners present in soluble glucan fraction is optimal to decrease efficiently the AIEC gut colonization.

4. Discussion

The potential of probiotics in CD management still remains unclear as standard probiotic strategies have hitherto failed to demonstrate any clear benefits (Oka & Sartor, 2020). Even so, *Saccharomyces* yeast, especially *Var. boulardii* CNCM I-745*,* has been shown to decrease risks of relapse or improve intestinal permeability in inactive CD (Garcia Vilela et al., 2008; Guslandi, Mezzi, Sorghi, & Testoni, 2000), but these clinical data are rare and rely on relatively small groups of patients (32 and 25 patients for these two studies). Specific pathobiont-targeted strategies, and more precisely AIEC-targeted strategy, should be of interest to the disease management, with a requisite stratification of AIEC-carrier patients. The present work stems from the seminal observation that selected *Saccharomyces cerevisiae* CNCM I-3856

strain exhibited significant ability to prevent adhesion of AIEC to intestinal epithelial cells in culture and to human ileal biopsies isolated from CD patients abnormally expressing the mannosylated protein CEACAM6 (Sivignon, de Vallee, et al., 2015). Using a preclinical model, it is shown that CNCM I-3856 significantly decreased AIEC colonization and colitis symptoms of infected transgenic mice expressing CEACAM6 (CEABAC10 model) and restored impaired barrier function of intestinal mucosa. Both the intact yeast and the *S. cerevisiae* cell wall derivatives administered to mice showed protective properties toward AIEC, strongly suggesting that the beneficial effect was associated with a surface component. As AIEC are known to colonize the ileal mucosa of CD patients *via* the interaction of the mannose-specific adhesin FimH with CEACAM6 mannosylated protein expressed on epithelial cell surface, we hypothesized that yeast polymannosylated glycoconjugates were involved in the anti-adhesive properties by competing with natural ligand of FimH. In order to identify molecular motifs responsible for the anti-adhesive properties of the yeast, the main glycoconjugates associated with yeast cell wall were sub-fractionated to individually assay their biological properties. Both intact yeast cells and yeast cell wall (YCW) preparations were used in order to uncover potential differences in the activity of non-surface expressed molecules and to potentially improve the originally observed activity. In parallel, the impact of strain-specific structural differences was evaluated with the objective of providing insights into the structure-to-function activity of the selected molecules. Based on FimH recognition specificity, we originally expected the highly mannosylated surface glycoconjugates would present the highest anti-adhesive activity. As expected, PPM isolated from all samples exhibited good *in vitro* anti-adhesive properties toward T84 cells. On average, PPM isolated from intact yeast showed better activities (about 20 to 40% residual adhesion) at high concentration (1mg/mL) than those isolated from YCW (about 45 to 55% residual adhesion). One may speculate that pre-purification of the cell wall removes some surface components that potentiate the activity of mannan or sub-fractionates mannans with different activities. Indeed, comparative analysis showed that polymannosylated moieties of PPM from different origins exhibited variable structural features such as the extend of Manα1-3 terminal capping (Figure 2, peak II) that varies from 0 in LV08 to 20% in LV04, and the degree of Manα1-2 branching of the -6Manα1- chain (Figure 2, peak III). However, none of these structural features could be directly correlated with the anti-adhesive activity, strongly

suggesting that the linkage pattern of mannan has no influence on the overall activity of PPM. Similarly, depolymerization of the mannan chain into more homogenous smaller oligosaccharides did not improve the anti-adhesive activity. On the contrary, the maximum activity was decreased for all purified fractions, which strongly suggests that polymerization increasing avidity is important to reach optimal activity, as previously observed for numerous bacterial lectins, including FimH from uropathogenic *E. coli* and LecA from *Pseudomonas aeruginosa* (Palmioli, Sperandeo, Polissi, & Airoldi, 2019; Sauer et al., 2019). Thus, highly purified PPMs, irrespective of their origins, did not exhibit higher anti-adhesive potential than the intact yeasts (Sivignon, de Vallee, et al., 2015) as more than 80% of inhibition in the used experimental conditions could not be reached.

Unexpectedly, the highest inhibition levels were observed with soluble glucan fractions, especially those extracted from yeast cell wall (YCW) preparations. Structural analyses revealed that soluble glucan fractions, exclusively from YCW, were enriched in mannan. This mannan was covalently attached to α- and β-glucans according to previous works (Arvindekar & Patil, 2002; Kollar et al., 1997; Kwiatkowski, Thielen, Glenney, & Moran, 2009). Thus, presence of the three partners β-glucan, α-glucan and mannan in this soluble glucan fraction with a ratio 45:15:40 potentializes anti-adhesive effect, as AIEC LF82 adhesion to T84 cells was nearly abrogated. As the maximal efficiency was obtained with soluble glucans extracted from YCW CNCM I-5268, this fraction was tested in the CEABAC10 mouse model. CNCM I-5268 soluble glucans were able to accelerate AIEC elimination from mice gut, to prevent LF82 attachment to intestinal mucosa and to decrease intestinal inflammation, as demonstrates by decreased fecal lipocalin-2 level.

These data demonstrate that upstream yeast processing has a direct effect on the activity of the soluble glucan fraction but the precise role of the "strain-effect" in the anti-adhesive effects should be established. Indeed, despite a similar β-glucan/α-glucan/mannan ratio, soluble glucans from YCW CNCM I-5268 has a superior biological activity compared to those from YCW CNCM I-3856. A hypothesis should be the ligand presentation for FimH adhesin, that is different between the two yeast strains.

5. Conclusions

For better inflammatory diseases management, it seems essential to develop therapies rationally designed to manipulate dysbiotic microbiota. Here, a bioactive fraction in yeast wall responsible for competitive blocking of AIEC bacteria to intestinal epithelium through FimH was identified. This soluble glucan fraction represents a promising pathobiont-targeted tool to detach AIEC from the intestinal mucosa. To demonstrate the efficacity of such therapy in CD patients, AIEC-carriage should be considered as an essential criterion of inclusion. Use of a concomitant companion diagnostic test to rapidly guide patients towards an AIEC-targeted treatment is necessary and heptyl-mannose-grafted cellulose represents a promising method to easily detect AIEC in biological fluids (Cauwel et al., 2019). It should also be interesting to propose this soluble glucan as a post-biotic strategy in patients susceptible to be colonized by AIEC, for example after surgery to limit AIEC implantation at the ileal mucosa, in order to decrease postoperative recurrence and thus to extend periods of remission. Finally, as FimH is broadly expressed by enterobacteria, this soluble glucan fraction containing β-glucan/α-glucan/mannan with a ratio can be applied more generally in prophylactic or therapeutic treatments against FimH-expressing bacteria responsible for gastro-intestinal acute or others chronic disorders. Administration of this bioactive yeast fraction in combination with a live probiotic yeast could be considered in order to cumulate anti-adhesive properties against FimH-producers with another beneficial effect like anti-inflammatory response.

Declaration of Interest

Nathalie Ballet and Pascal Vandekerckove are full-time employed by the company Lesaffre International, Lesaffre Group, Marcq-en-Baroeul, France. All other authors declare no competing interests.

Reference

Abraham, S. N., Sun, D., Dale, J. B., & Beachey, E. H. (1988). Conservation of the D-mannose-adhesion protein among type 1 fimbriated members of the family Enterobacteriaceae. *Nature,* 336(6200), 682-684.

Agus, A., Massier, S., Darfeuille-Michaud, A., Billard, E., & Barnich, N. (2014). Understanding host-adherent-invasive Escherichia coli interaction in Crohn's disease: opening up new therapeutic strategies. *Biomed Res Int,* 2014, 567929.

Aklujkar, P. P., Sankh, S. N., & Arvindekar, A. U. (2008). A Simplified Method for the Isolation and Estimation of Cell Wall Bound Glycogen in Saccharomyces cerevisiae. *Journal of the Institute of Brewing,* 114(3), 205-208.

Arvindekar, A. U., & Patil, N. B. (2002). Glycogen--a covalently linked component of the cell wall in Saccharomyces cerevisiae. *Yeast,* 19(2), 131-139.

Bacon, J. S., Farmer, V. C., Jones, D., & Taylor, I. F. (1969). The glucan components of the cell wall of baker's yeast (Saccharomyces cerevisiae) considered in relation to its ultrastructure. *Biochem J,* 114(3), 557-567.

Barnich, N., Carvalho, F. A., Glasser, A. L., Darcha, C., Jantscheff, P., Allez, M., . . . Darfeuille-Michaud, A. (2007). CEACAM6 acts as a receptor for adherent-invasive E. coli, supporting ileal mucosa colonization in Crohn disease. *J Clin Invest,* 117(6), 1566-1574.

Baumgart, M., Dogan, B., Rishniw, M., Weitzman, G., Bosworth, B., Yantiss, R., . . . Simpson, K. W. (2007). Culture independent analysis of ileal mucosa reveals a selective increase in invasive Escherichia coli of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. *Isme J,* 1(5), 403-418.

Boudeau, J., Glasser, A. L., Masseret, E., Joly, B., & Darfeuille-Michaud, A. (1999). Invasive ability of an Escherichia coli strain isolated from the ileal mucosa of a patient with Crohn's disease. *Infect Immun,* 67(9), 4499-4509.

Bringer, M. A., Glasser, A. L., Tung, C. H., Meresse, S., & Darfeuille-Michaud, A. (2006). The Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 replicates in mature phagolysosomes within J774 macrophages. *Cell Microbiol,* 8(3), 471-484.

Carvalho, F. A., Barnich, N., Sauvanet, P., Darcha, C., Gelot, A., & Darfeuille-Michaud, A. (2008). Crohn's disease-associated Escherichia coli LF82 aggravates colitis in injured mouse colon via signaling by flagellin. *Inflamm Bowel Dis,* 14(8), 1051-1060.

Carvalho, F. A., Barnich, N., Sivignon, A., Darcha, C., Chan, C. H., Stanners, C. P., & Darfeuille-Michaud, A. (2009). Crohn's disease adherent-invasive Escherichia coli colonize and induce strong gut inflammation in transgenic mice expressing human CEACAM. *J Exp Med,* 206(10), 2179-2189.

Cauwel, M., Sivignon, A., Bridot, C., Nongbe, M. C., Deniaud, D., Roubinet, B., . . . Gouin, S. G. (2019). Heptylmannose-functionalized cellulose for the binding and specific detection of pathogenic E. coli. *Chem Commun (Camb),* 55(68), 10158-10161.

Chan, C. H., & Stanners, C. P. (2004). Novel mouse model for carcinoembryonic antigen-based therapy. *Mol Ther,* 9(6), 775-785.

Darfeuille-Michaud, A., Neut, C., Barnich, N., Lederman, E., Di Martino, P., Desreumaux, P., . . . Colombel, J. F. (1998). Presence of adherent Escherichia coli strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology,* 115(6), 1405-1413.

De la Fuente, M., Franchi, L., Araya, D., Diaz-Jimenez, D., Olivares, M., Alvarez-Lobos, M., . . . Hermoso, M. A. (2014). Escherichia coli isolates from inflammatory bowel diseases patients survive in macrophages and activate NLRP3 inflammasome. *Int J Med Microbiol,* 304(3-4), 384-392.

Dreux, N., Denizot, J., Martinez-Medina, M., Mellmann, A., Billig, M., Kisiela, D., . . . Barnich, N. (2013). Point mutations in FimH adhesin of Crohn's disease-associated adherent-invasive Escherichia coli enhance intestinal inflammatory response. *PLoS Pathog,* 9(1), e1003141.

Du, L., Zhang, X., Wang, C., & Xiao, D. (2012). Preparation of Water Soluble Yeast Glucan by Four Kinds of Solubilizing Processes. *Engineering,* Vol.04No.10, 5.

Dulary, E., Yu, S. Y., Houdou, M., de Bettignies, G., Decool, V., Potelle, S., . . . Foulquier, F. (2018). Investigating the function of Gdt1p in yeast Golgi glycosylation. *Biochim Biophys Acta Gen Subj,* 1862(3), 394-402.

Eaves-Pyles, T., Allen, C. A., Taormina, J., Swidsinski, A., Tutt, C. B., Jezek, G. E., . . . Torres, A. G. (2008). Escherichia coli isolated from a Crohn's disease patient adheres, invades, and induces inflammatory responses in polarized intestinal epithelial cells. *Int J Med Microbiol,* 298(5-6), 397-409.

Eshdat, Y., Speth, V., & Jann, K. (1981). Participation of pili and cell wall adhesion in the yeast agglutination activity of Escherichia coli. *Infect Immun,* 34(3), 980-986.

Garcia Vilela, E., De Lourdes De Abreu Ferrari, M., Oswaldo Da Gama Torres, H., Guerra Pinto, A., Carolina Carneiro Aguirre, A., Paiva Martins, F., . . . Sales Da Cunha, A. (2008). Influence of Saccharomyces boulardii on the intestinal permeability of patients with Crohn's disease in remission. *Scand J Gastroenterol,* 43(7), 842-848.

Garenaux, E., Yu, S. Y., Florea, D., Strecker, G., Khoo, K. H., & Guerardel, Y. (2008). A single step method for purification of sulfated oligosaccharides. *Glycoconj J,* 25(9), 903-915.

Gevers, D., Kugathasan, S., Denson, L. A., Vazquez-Baeza, Y., Van Treuren, W., Ren, B., . . . Xavier, R. J. (2014). The treatment-naive microbiome in new-onset Crohn's disease. *Cell Host Microbe,* 15(3), 382-392.

Glasser, A. L., Boudeau, J., Barnich, N., Perruchot, M. H., Colombel, J. F., & Darfeuille-Michaud, A. (2001). Adherent invasive Escherichia coli strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. *Infect Immun,* 69(9), 5529-5537.

Guslandi, M., Mezzi, G., Sorghi, M., & Testoni, P. A. (2000). Saccharomyces boulardii in maintenance treatment of Crohn's disease. *Dig Dis Sci,* 45(7), 1462-1464.

Klis, F. M., Mol, P., Hellingwerf, K., & Brul, S. (2002). Dynamics of cell wall structure in Saccharomyces cerevisiae. *FEMS Microbiol Rev,* 26(3), 239-256.

Kocourek, J., & Ballou, C. E. (1969). Method for fingerprinting yeast cell wall mannans. *J Bacteriol,* 100(3), 1175-1181.

Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J., Ashwell, G., Drgonova, J., . . . Cabib, E. (1997). Architecture of the yeast cell wall. Beta(1-->6)-glucan interconnects mannoprotein, beta(1-->)3-glucan, and chitin. *J Biol Chem,* 272(28), 17762-17775.

Kwiatkowski, S., Thielen, U., Glenney, P., & Moran, C. (2009). A Study of Saccharomyces cerevisiae Cell Wall Glucans. *Journal of the Institute of Brewing,* 115(2), 151-158.

Lopez-Siles, M., Martinez-Medina, M., Busquets, D., Sabat-Mir, M., Duncan, S. H., Flint, H. J., . . . Garcia-Gil, L. J. (2014). Mucosa-associated Faecalibacterium prausnitzii and Escherichia coli co-abundance can distinguish Irritable Bowel Syndrome and Inflammatory Bowel Disease phenotypes. *Int J Med Microbiol,* 304(3-4), 464-475.

Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Gloux, K., Pelletier, E., Frangeul, L., . . . Dore, J. (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. *Gut,* 55(2), 205-211.

Manners, D. J., Masson, A. J., & Patterson, J. C. (1973). The structure of a beta-(1 leads to 3)-D-glucan from yeast cell walls. *Biochem J,* 135(1), 19-30.

Manners, D. J., Masson, A. J., Patterson, J. C., Bjorndal, H., & Lindberg, B. (1973). The structure of a beta-(1--6)-D-glucan from yeast cell walls. *Biochem J,* 135(1), 31-36.

Martin, H. M., Campbell, B. J., Hart, C. A., Mpofu, C., Nayar, M., Singh, R., . . . Rhodes, J. M. (2004). Enhanced Escherichia coli adherence and invasion in Crohn's disease and colon cancer. *Gastroenterology,* 127(1), 80-93.

Meconi, S., Vercellone, A., Levillain, F., Payre, B., Al Saati, T., Capilla, F., . . . Altare, F. (2007). Adherent-invasive Escherichia coli isolated from Crohn's disease patients induce granulomas in vitro. *Cell Microbiol,* 9(5), 1252-1261.

Nelson, R. D., Shibata, N., Podzorski, R. P., & Herron, M. J. (1991). Candida mannan: chemistry, suppression of cell-mediated immunity, and possible mechanisms of action. *Clin Microbiol Rev,* 4(1), 1-19.

Oka, A., & Sartor, R. B. (2020). Microbial-Based and Microbial-Targeted Therapies for Inflammatory Bowel Diseases. *Dig Dis Sci*.

Palmioli, A., Sperandeo, P., Polissi, A., & Airoldi, C. (2019). Targeting Bacterial Biofilm: A New LecA Multivalent Ligand with Inhibitory Activity. *Chembiochem,* 20(23), 2911-2915.

Reed, G., & Nagodawithana, T. W. (1991). Yeast technology.

Sauer, M. M., Jakob, R. P., Luber, T., Canonica, F., Navarra, G., Ernst, B., . . . Glockshuber, R. (2019). Binding of the Bacterial Adhesin FimH to Its Natural, Multivalent High-Mannose Type Glycan Targets. *J Am Chem Soc,* 141(2), 936-944.

Shi, F., Shi, J., & Li, Y. (2014). Mechanochemical phosphorylation and solubilisation of beta-D-glucan from yeast Saccharomyces cerevisiae and its biological activities. *PLoS One,* 9(7), e103494.

Sivignon, A., de Vallee, A., Barnich, N., Denizot, J., Darcha, C., Pignede, G., . . . Darfeuille-Michaud, A. (2015). Saccharomyces cerevisiae CNCM I-3856 prevents colitis induced by AIEC bacteria in the transgenic mouse model mimicking Crohn's disease. *Inflamm Bowel Dis,* 21(2), 276-286.

Sivignon, A., Yan, X., Alvarez Dorta, D., Bonnet, R., Bouckaert, J., Fleury, E., . . . Barnich, N. (2015). Development of Heptylmannoside-Based Glycoconjugate Antiadhesive Compounds against Adherent-Invasive Escherichia coli Bacteria Associated with Crohn's Disease. *MBio,* 6(6), e01298-01215.

Small, C. L., Reid-Yu, S. A., McPhee, J. B., & Coombes, B. K. (2013). Persistent infection with Crohn's disease-associated adherent-invasive Escherichia coli leads to chronic inflammation and intestinal fibrosis. *Nat Commun,* 4, 1957.

Sokol, H., Brot, L., Stefanescu, C., Auzolle, C., Barnich, N., Buisson, A., . . . Investigators, R. S. G. (2019). Prominence of ileal mucosa-associated microbiota to predict postoperative endoscopic recurrence in Crohn's disease. *Gut*.

Sommer, F., Ruhlemann, M. C., Bang, C., Hoppner, M., Rehman, A., Kaleta, C., . . . Franke, A. (2017). Microbiomarkers in inflammatory bowel diseases: caveats come with caviar. *Gut,* 66(10), 1734-1738.

Stewart, T. S., & Ballou, C. E. (1968). A comparison of yeast mannans and phosphomannans by acetolysis. *Biochemistry,* 7(5), 1855-1863.

Sugawara, T., Takahashi, S., Osumi, M., & Ohno, N. (2004). Refinement of the structures of cell-wall glucans of Schizosaccharomyces pombe by chemical modification and NMR spectroscopy. *Carbohydr Res,* 339(13), 2255-2265.

Vinogradov, E., Petersen, B., & Bock, K. (1998). Structural analysis of the intact polysaccharide mannan from Saccharomyces cerevisiae yeast using 1H and 13C NMR spectroscopy at 750 MHz. *Carbohydr Res,* 307(1-2), 177-183.

Williams, D. L., McNamee, R. B., Jones, E. L., Pretus, H. A., Ensley, H. E., Browder, I. W., & Di Luzio, N. R. (1991). A method for the solubilization of a (1----3)-beta-D-glucan isolated from Saccharomyces cerevisiae. *Carbohydr Res,* 219, 203-213.

Heteropolysaccharides from *S. cerevisiae* show anti-adhesive properties against *E. coli* associated with Crohn's disease

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Supplementary methods

Monosaccharide Analysis and Linkage Analysis

For monosaccharide analysis of mannan and glucan, 100 µg of inositol and 100 µg of target sugars were taken and put in the same tube. 500 µL of 4 N trifluroacetic acid (TFA) were added and incubated at 100 °C, for 4 h to hydrolysis polysaccharides into monosaccharides, followed by drying the sample by under stream of nitrogen at room temperature. Reduction monosaccharides by 500 µL of 20 mg/mL $NaBH_4$ in 2N NH_4OH, room temperature, overnight. The reaction was quenched by adding 500 µL of 10% acetic acid in methanol, and dry under stream of nitrogen, again. Repeat 4-5 times. Then, reduced monosaccharides were peracetylated by adding 500 µL of acetic anhydride, 100°C, for 4 h, and derivatized monosaccharides were extracted by 1 mL of chloroform. 1 mL of water was added to do water-chloroform partition to remove excess salts. Repeat 5 times extraction and dry under stream of nitrogen. The derivatized monosaccharides were re-dissolved in 500 µL chloroform. 1 µL of sample was injected into GC-FID (TRACE GC Ultra, Thermo Fisher Scientific). The capillary column is SOLGEL (30m x 0.25 mm, 0.25 µm film thickness, SGE Analytical Science). The initial oven temperature was held at 120°C, increased to 230°C at 3°C /min, and then, 270°C for 10 min.

For linkage analysis of mannan and glucan, 200 µg of target polysaccharides were dissolved in 100 µL of DMSO. Polysaccharides were derivatized by modified(Hakomori, 1964). 33 mg of NaI was taken in one glass tube and washed by pentane several times to remove glycerol. NaI was dried under nitrogen and the dissolved polysaccharides were added into NaI-contained glass tube, followed by addition of 100 µL of CH_3I. Reaction was agitated for 10 minutes at room temperature. The reaction was quenched by adding 1 mL of water on ice and permethylated polysaccharides were extracted by 2 mL chloroform/water partition. The chloroform phase was dried under nitrogen. Samples were hydrolyzed by 4 N TFA at 100°C, for 4hr and reduced by 1 mL $NaBD_4$ (20 mg/mL) in 2 N NH_4. The reaction was quenched by 1 mL of 10% acetic acid in methanol. Then, sugars were peracetylated and chloroform/water extracted as described above. The derivatized monosaccharides were dissolved in 1 mL of chloroform and 1 µL were injected into

GC-MS (TRACE GC Ultra, Thermo Fisher Scientific). The initial oven temperature was held at 120°C, increased to 230°C at 2°C/min, and then, 270°C for 10 min.

Mass Spectrometry Analyses

The oligosaccharides were aliquoted for permethylation. In brief, the dried oligosaccharides in glass tube were permethylated with NaOH/DMSO and methyl iodide according to Ciucanu and Kerek method(Ciucanu & Kerek, 1984) (https://doi.org/10.1016/0008-6215(84)85242-8) extracted by chloroform/water partitioning. Repeated water-chloroform partitions were used, and the sample was dried under a stream of nitrogen. The permethylated oligosaccharides were dissolved in 100% acetonitrile and pre-mixed with 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml in 50% acetonitrile/water, v/v, Sigma), then spotted on target. MS spectra were acquired by using a MALDI-TOF/TOF 4800 mass spectrometer (Applied Biosystems) equipped with Nd:YAG laser, operated in the reflectron mode. Data acquisition was normally comprised of 40 sub-spectra of 50 laser shots, and the laser energy is set 5000-5300.
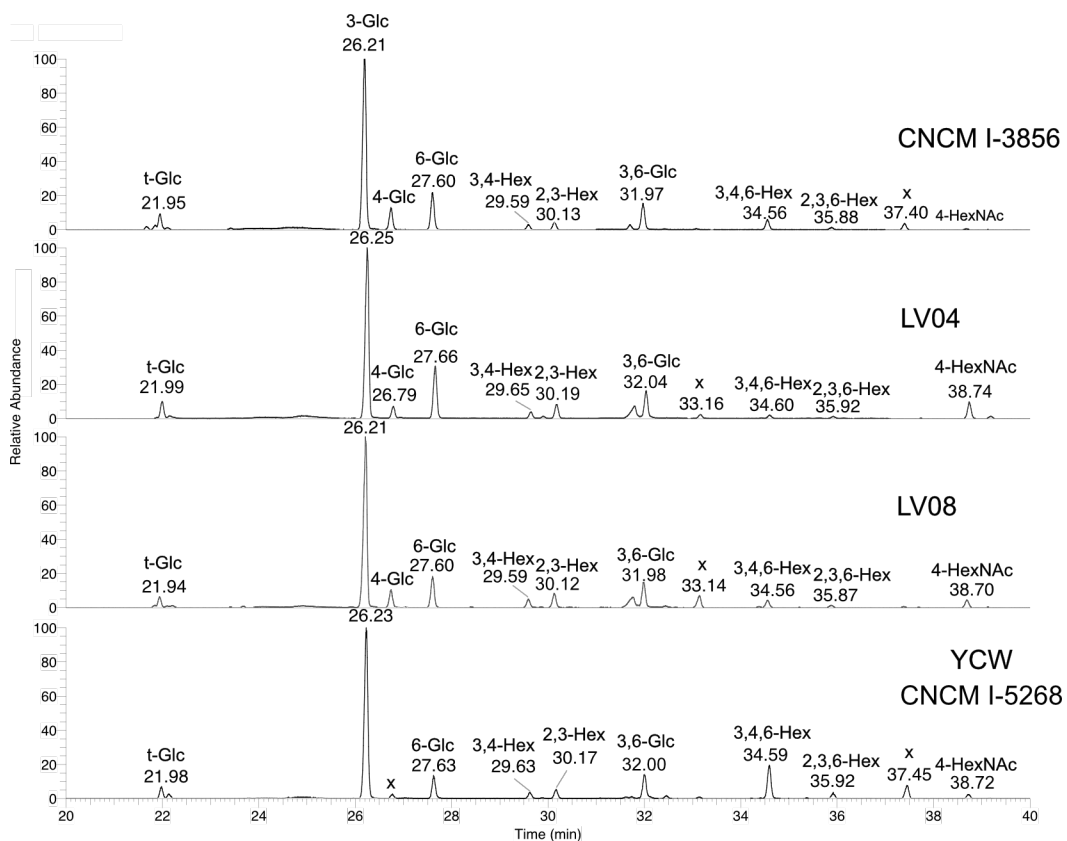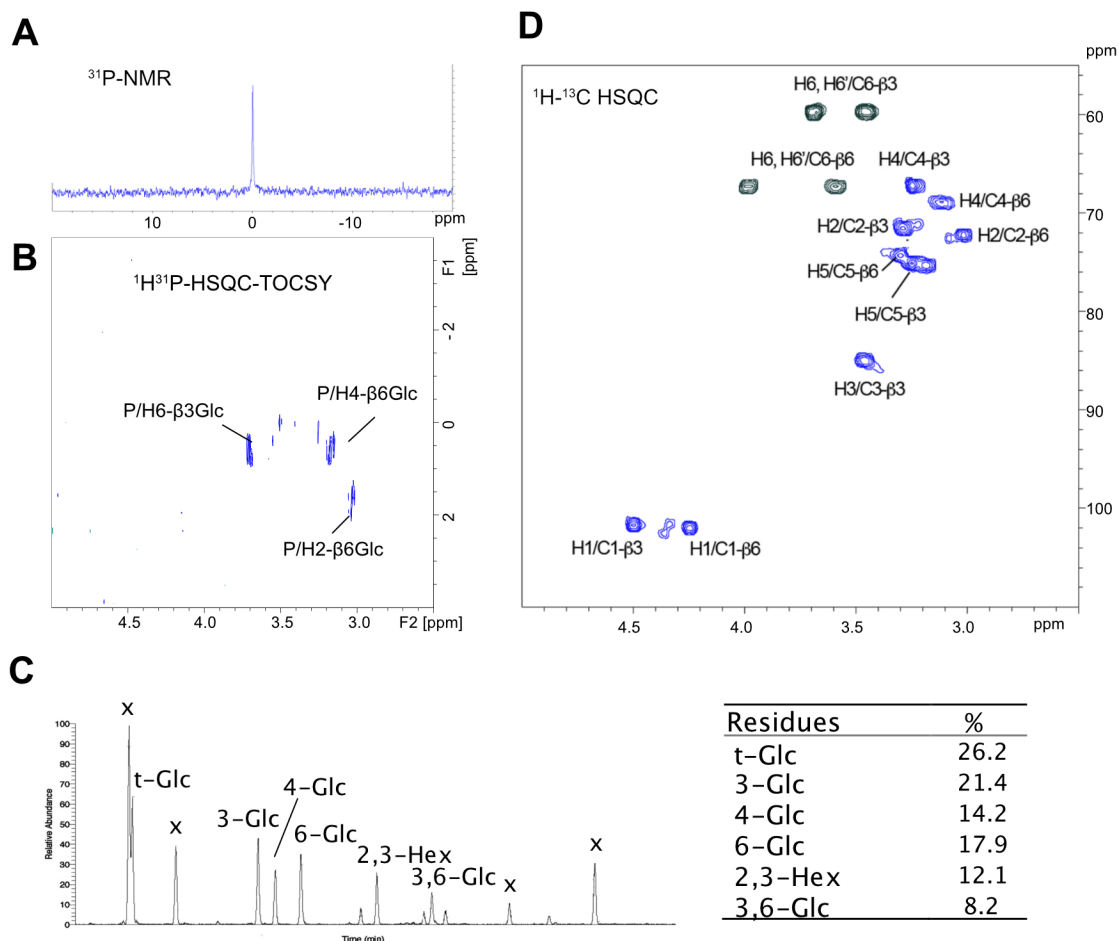
Supplementary figures
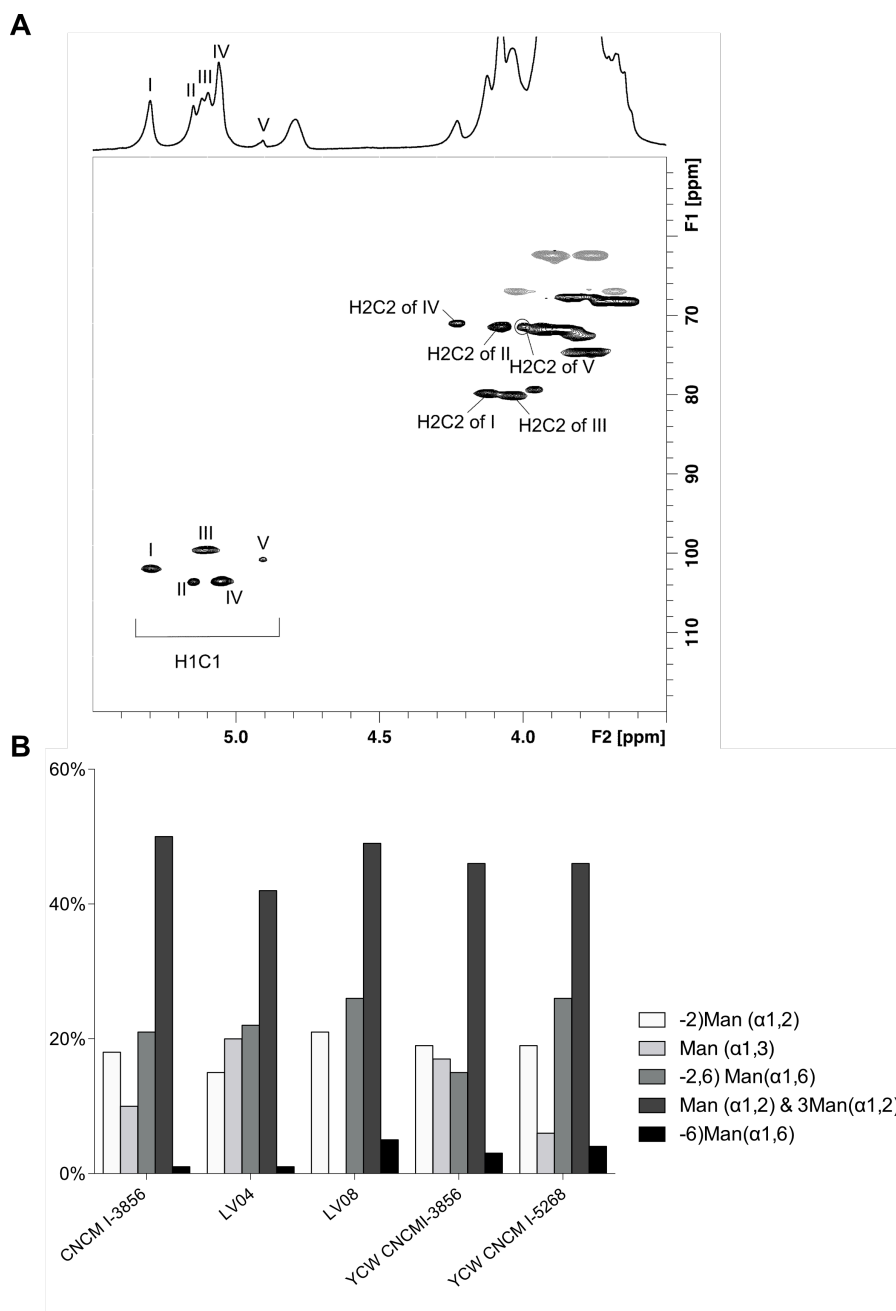
Supplementary Figure 1. ^{1}H-^{13}C HSQC NMR analysis of PPM extracted by CNCM I-3856 yeast (acquisition at 400 MHz NMR, D2O, 300K). Distinct 5 signals were shown in HSQC data, which can be used for quantification of different sugar regions in one yeast strain itself and different strains.
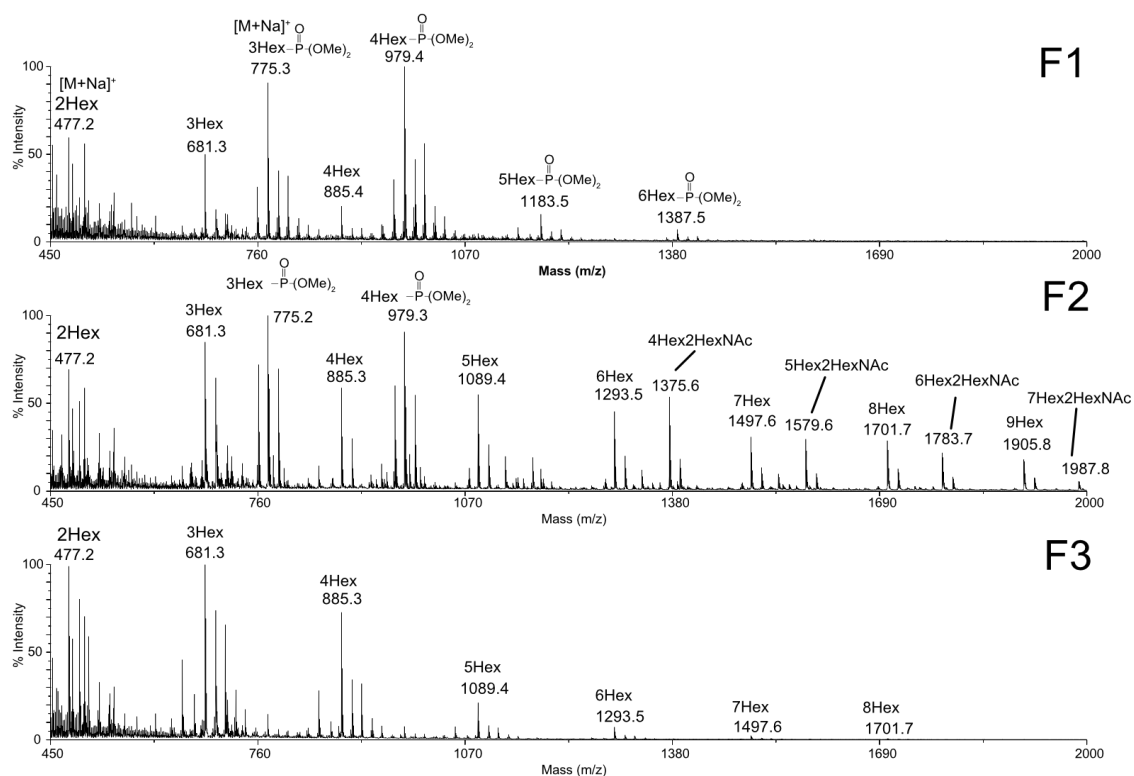
Supplementary Figure 2. Structural characterizations of three fractions containing oligomannosides separated by gel filtration. MALDI-MS analyses in the positive ion mode of permethylated oligosaccharides.

Supplementary Figure 3. Comparison of β3-glucan fractions isolated from four different yeast preparations. Linkage analysis of β3-glucan from different strains were established by GC-MS. As shown here, GC-MS chromatograms of four strains did not show obvious strain-specific differences.

Supplementary Figure 4. Structural analysis of phosphorylated β3-glucan isolated from CNCM I-3856 yeast. (A) ^{31}P-NMR spectrum and (B) ^{1}H-^{31}P-HSQC NMR spectra, (C) GC-MS linkage chromatogram, and (D) ^{1}H-^{13}C-HSQC NMR spectrum of phosphorylated β3-glucan (acquisition at 400 MHz NMR, d6-DMSO+D$_2$O, 353K). X correspond to non-carbohydrate contaminants.

A

Glycan unit	Zones of integrations (ppm)
4αglucan-linear+ terminal-4αGlc	5.45-5.30
α-mannan-I	5.3-5.25
α-mannan-II	5.18-5.14
α-mannan-III	5.13-5.09
α-mannan-IV	5.09-5.03
4,6αGlc-branching	5.01-4.94
α-mannan-V	4.92-4.90
3βglucan-linear	4.81-4.76
ter-glc β6	4.76-4.71
3,6βGlc-branching	4.59-4.55
6βglucan-linear	4.55-4.49

B
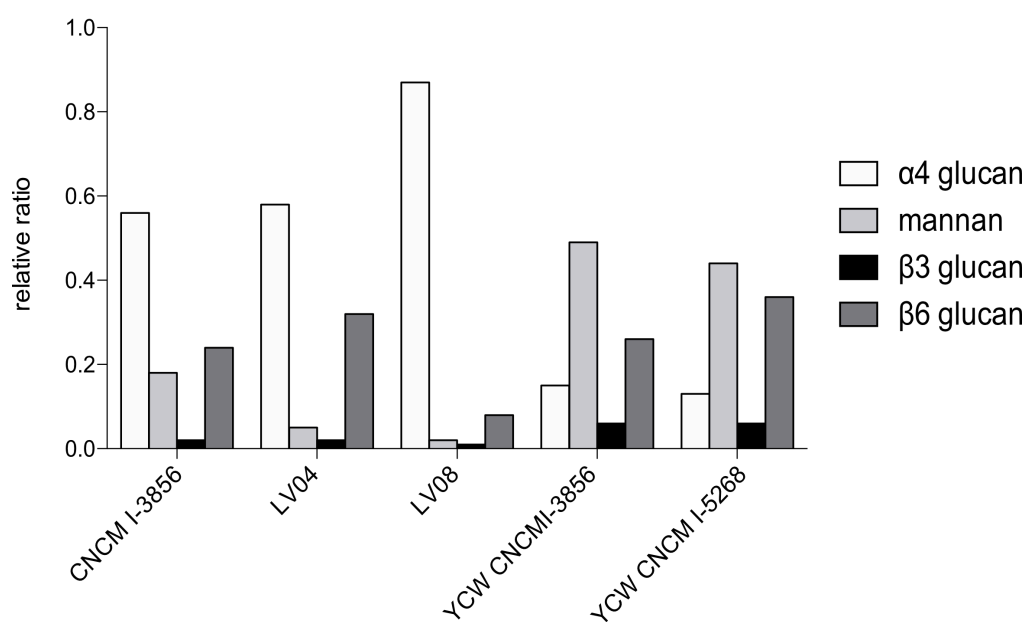
Supplementary Figure 5: Estimation of the proportions of individual polysaccharides in the soluble glucan fraction isolated from five samples. (A) Integration parameters on ^{1}H NMR spectra and (B) Calculated proportions of polysaccharides in each sample.

A

	CNCMI-3856	LV04	LV08	YCW CNCMI-5268
mannose	9.82%	11.9%	1.4%	36.1%
glucose	90.2%	88.1%	98.6%	63.9%

B
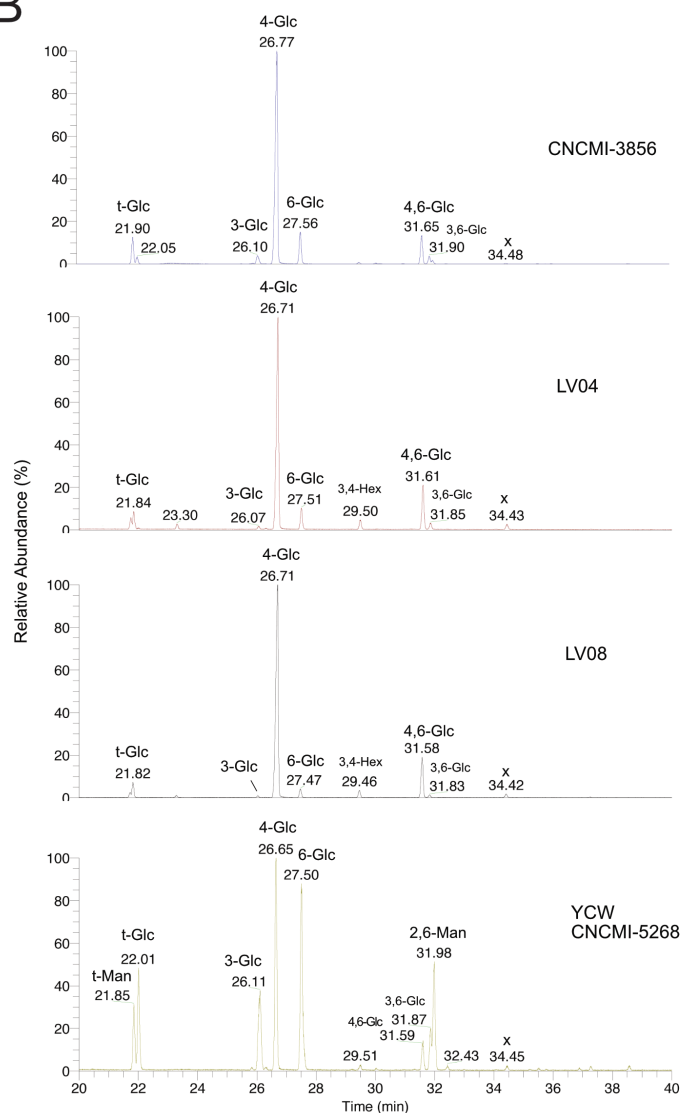
Supplementary Figure 6. Comparison of soluble glucan fractions isolated from four different yeast preparations. (A) Relative monosaccharide composition and (B) Linkage analyses of the soluble glucan fractions.

		1	2	3	4	5	6	6'
-2)Man(α1,2)	H	5.29	4.12	3.96	3.77	3.88	-	-
	C	101.77	79.85	71.76	68.07	74.65	-	-
Man(α1,3)	H	5.15	4.08	3.89	3.66	3.79	-	-
	C	103.59	71.4	71.73	68.24	-	-	-
-2,6)Man(α1,6)	H	5.11	4.03	3.94	-	-	4.03	3.68
	C	99.31	80.23	-	-	-	67.02	
Man(α1,2) & -3)Man(α1,2)	H	5.06	4.23	3.97	3.77	3.83	3.92	3.77
	C	103.41	71.09	79.21	67.51	74.61	62.21	
-6)Man(α1,6)	H	4.91	3.99	-	-	-	-	-
	C	100.77	71.59	-	-	-	-	-

"-" undetectable/undetermined value

	1	2	3	4	5	6	6'

		1	2	3	4	5	6a	6b
-3)Glc(β1,3)	H	4.53	3.31	3.48	3.27	3.27	3.71	3.49
	C	102.9	72.6	86.1	68.4	76.2	60.82	60.82
Glc(β 1,3)	H	4.39	3.12	3.26	-	-	-	-
	C	103.6	76.57	-	-	-	-	-
3,6)Glc(β 1,3)	H	4.36	3.27	3.41	-	-	-	-
	C	102.77	-	87.11	-	-	-	-
-6)Glc(β1,6)	H	4.28	3.04	3.21	3.14	3.33	4.02	3.62
	C	102.87	73.32	76.48	70.0	75.4	68.3	68.3
	C	100.77	71.59	-	-	-	-	-

"-" undetectable/undetermined value

Supplementary table 3. Chemical shifts (ppm) of ^{1}H and ^{13}C of the glycosyl residues of soluble glucan (GGM) fraction, dissolved in D_2O and measured at 431K. These values were deduced from the interpretation of the 1D-^{1}H NMR, 2D-^{1}H-^{1}H COSY, TOCSY, and ^{1}H-^{13}C HSQC spectra

		1	2	3	4	5	6a	6b
Glc(α1,4)+	H	5.37	3.64	3.97	3.65	3.75	3.89	3.77
-4)Glc(α1,4)	C	101.5	72.82	74.81	79.13	74.91	62.59	62.59
-2)Man(α1,2)	H	5.28	4.12	3.93	3.74	-	-	-
	C	102.1	79.4	-	-	-	-	-
Man(α1,3)	H	5.16	4.09	-	-	-	-	-
	C	103.51	-	-	-	-	-	-
-2,6)Man(α1,6)	H	5.11	4.03	-	-	-	-	-
	C	99.94	79.7	-	-	-	-	-
Man(α1,2)&	H	5.07	4.08	-	-	-	4.01	3.71
-3)Man(α1,2)	C	103.72	71.3	-	-	-	67.4	67.4

-4,6)Glc(α1,6)	H	4.98	-	-	-	-	-	-
	C	-	-	-	-	-	-	-
-6)Man(α1,6)	H	4.91	-	-	-	-	-	-
	C	101.2	-	-	-	-	-	-
-3)Glc(β1,3)	H	4.78	3.58	-	-	-	-	-
	C	104.19	-	-	-	-	-	-
Glc(β1,6)	H	4.73	3.37	-	-	-	-	-
	C	104.3	-	-	-	-	-	-
Glc(β1,3)	H	4.65	-	-	-	-	-	-
	C	-	-	-	-	-	-	-
-3,6)Glc(β1,6)	H	4.56	3.54	3.78	-	-	-	-
	C	103.8	74.09	86.27	-	-	-	-
-6)Glc(β1,6)	H	4.53	3.35	3.52	3.46	3.64	4.22	3.88
	C	104.2	74.7	77.35	71.25	76.6	70.59	70.59

"-" undetectable/undetermined value

Ciucanu, I., & Kerek, F. (1984). A simple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research,* 131(2), 209-217.

Hakomori, S. (1964). A Rapid Permethylation of Glycolipid, and Polysaccharide Catalyzed by Methylsulfinyl Carbanion in Dimethyl Sulfoxide. *J Biochem,* 55, 205-208.