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Composition and immunomodulatory activity of an arabinoxylan polysaccharide-rich extract of *Agaricus subrufescens* fermented rye

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ABSTRACT

This study investigated bioactive carbohydrate-related compounds in rye grain overgrown with *Agaricus subrufescens* mycelia (ROM), using cytokine induction in chicken immune cells. We focused on polysaccharides, as they have been suggested to mediate immunomodulatory effects of rye and *Agaricus subrufescens*. Fractionation of rye and ROM and subsequent testing fractions on macrophage cell line HD11 showed that IL-12p40 production was mainly induced by hot water-soluble solids (HWSS) with ROM-HWSS more active than rye-HWSS. The bioactive HWSS consisted of polymers >10 kDa. Stimulation of chicken splenocytes further confirmed their immunomodulatory capacity. Rye- and ROM-HWSS >10 kDa mainly consisted of arabinoxylan and glucan with smaller and variable amounts of protein and ferulic acid. Polymeric arabinoxylan structures were confirmed to be present by enzymatic fingerprinting using pure xylanase. NaOH treatment (0.1 M) substantially reduced IL-12p40 induction by HWSS fractions and removed some ferulic ester groups, suggesting that feruloylated arabinoxylan structures contribute to IL-12p40 induction. Degradation of arabinoxylan and small amounts of glucan and fructan in the HWSS fractions with specific enzymes did not affect IL-12p40 induction. Protease, chitosanase and Driselase treatments reduced IL-12p40 induction in ROM-HWSS, suggesting that mycelial compounds (e.g., protein, chitosan and galactan/mannan) may (also) contribute to the bioactivity.

1. Introduction

Immunomodulation is a process of modifying the immune response by e.g. feed additives containing bioactive compounds. In food-producing animals, immunomodulation is utilized aiming for enhanced the animals' resistance to infectious diseases and improved animal welfare [1]. Moreover, immunomodulating feed additives could be an alternative for antibiotic use in farm animals [2], as recent EU regulation further reduced the use of antibiotics in livestock animals, no longer allowing its routine use, such as repeated, habitual use or prophylactic application [3]. Recently, rye (*Secale cereal*) overgrown with *Agaricus subrufescens (A. subrufescens)* mycelia (ROM) has been investigated alone or as a blend with hydrolyzed copra meal rich in mannans, to stimulate gastrointestinal (GI) tract health and development, as well as enhancing immune competence and overall host health in pigs [4–6]. The blend modulated the expression of cytokines (pro- and anti-inflammatory) in intestinal tissues and in PBMCs and caused activation and proliferation of NK cells in blood [5]. Our previous research has shown that piglets that were given ROM as a feed additive (from 100 mg/day on day 2 to 1000 mg/day on day 44) showed higher ex vivo IL-10 production by mesenteric lymph node cells upon in vitro LPS restimulation, and higher activation of NK cells in blood [6]. Although the exact bioactive molecules in the complex *A. subrufescens* derived products that exert potential immunomodulatory actions have not yet

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been identified, a growing body of literature suggests that the polysaccharide fraction is the main driver for immunomodulatory effects exerted by both *A. subrufescens* and rye [7,8].

A. subrufescens, also known as *A. blazei* or *A. brasiliensis*, is considered one of the most important mushrooms for culinary as well as medicinal uses in Brazil, the United States, East Asia, and Europe [9]. Accumulating literature suggests that either the basidiome or mycelium of *A. subrufescens* may have a variety of bioactive properties, such as antitumor, antiviral, anti-inflammatory, antioxidant, and immunomodulatory activities [10]. For example, *A. subrufescens* mycelium was shown to induce many pro-inflammatory cytokines in humans and mice including IL-12p40 that can skew T helper-1 responses [11,12].These biological effects have been ascribed to the presence of bioactive molecules, including polysaccharides (especially 1 → 3–1 → 6- β-glucans), proteins, and low molecular weight (LMW) substances such as phenolic compounds, sterols, and terpenoids [13,14]. However, the exact molecules responsible for the observed bioactivity remain unidentified.

Rye is a cereal grown world-wide, containing starch, protein, and dietary fibre (DF), besides small amounts of hydroxycinnamic acid (HCA) like ferulic acid (FA) [15]. Arabinoxylan (AX) (8–12 % *w*/w) is most abundant in the DF profile of rye grain, followed by inulin-type fructan (4.5–6 % w/w) and 1,3-1,4-β-glucan (2–3 % w/w) [15]. The AX consists of a linear backbone of β-(1 → 4)-linked xylosyl units being mono-or di-substituted by arabinosyl units at O-2 and/or O-3 of the xylose residue. Some of the arabinose residues may be esterified at the O-5 position to HCA units like *p*-coumaric acid (*p*CA) and FA [16]. FA can additionally form diferulic acid moieties that can further crosslink AX chains [17]. Furthermore, FA units can covalently couple to protein [18] and to lignin [19] in rye.

During the *A. subrufescens* fermentation of rye, rye's components (especially carbohydrates) are modified by fungal extracellular hydrolytic enzymes (e.g., xylanases, endoglucanases, and α-amylases) to smaller molecules, and partially further utilized by the fungus [20,21]. For example, AXs in the rye substrate can be degraded by xylanases from *A. subrufescens* mycelium into arabinoxylo-oligosaccharides [16]. Meanwhile, a range of fungal cell components and metabolites are produced by *A. subrufescens*, such as polysaccharides (e.g., 1 → 3–1 → 6-β-glucan and chitin) and phenolic compounds (e.g., gallic acid and benzoic acid) [22,23].

To our knowledge, ROM was not tested for its ability to influence the chicken immune system. Avian immune cell lines and ex vivo derived primary immune cells can be used as in vitro models for studying the impact of feed extracts on immunomodulation [24–28]. Studying the immunomodulatory effects of ROM on chicken cells and characterization of ROM's bioactive compound(s) may show its potential as an immunomodulatory feed additive that can promote resilience of chickens against microbial infections, such as *Salmonella*, that may pose a threat to human consumers [29,30]. In this study, we assessed cytokine production by the chicken macrophage cell line HD11 and primary splenocytes induced by fractions derived from ROM using 70 % (*v*/v) ethanol, hot water and concentrated alkali extractions, with the aim to chemically and structurally characterize the immunomodulatory compounds from ROM, all compared to similar fractions produced from unfermented rye.

2. Materials and methods

2.1. Materials and chemicals

ROM, together with the parental rye grain (*Secale cereal*), were provided by Selko (Trouw Nutrition, Tilburg, the Netherlands). Rye arabinoxylan (high viscosity) (RAX), wheat arabinoxylan (medium viscosity) (WAX), barley β-glucan (low viscosity), and a fructanase mixture were purchased from Megazyme (Bray, Ireland). Frutafit® IQ (native chicory inulin, DP2–60) was provided by Sensus (Roosendaal, The Netherlands). Chitosan from crab shells (#417963), Driselase (#D8037), protease XIV (#P5147), chitosanase (#C9830), polymyxin B (PMB) (#P4932), *Salmonella enterica* serovar Typhimurium (#L6511), ionomycin (#I9657) and phorbol 12-myristate 13-acetate (PMA) (#P8139) were bought from Sigma-Aldrich (St Louis, USA). Roswell Park Memorial Institute (RPMI) 1640 (#52400–025), chicken serum (#1611008. 2), streptomycin (#15140122), 2 mM L-glutamine (#25030024), phosphate buffer saline (PBS) (#10010–015) and rabbit serum (#16120–107) were ordered from Gibco-Invitrogen (Paisley, UK). Heat-stable α-amylase (Termamyl 120 L) and amyloglucosidase (AMG 300 L) were bought from Novozymes (Bagsvaerd, Denmark). Beta-mercaptoethanol (#1610710) and Ficoll-Paque (#17–1440-02) were ordered from Bio-Rad (Hercules, USA) and Cytiva (Freiburg im Breisgau, Germany), respectively. Endo-(1,4)-β-D-xylanase (Endoxylanase I, GH 10) was purified from *Aspergillus awamori* CMI 142717 as described elsewhere [31]. Endoglucanase I was purified and derived from *Trichoderma viride* as described previously [32]. All other chemicals were obtained from commercial suppliers and used without further purification. Milli-Q water (Millipore, Billerica, MA, USA) was used in all experiments.

2.2. Sequential extraction and fraction preparation

Whole rye grains and ROM were milled with an ultra-centrifugal mill (ZM 200, Retsch, Haan, Germany) using a 0.5 mm sieve, followed by sequential extraction with 70 % (*v*/v) ethanol, hot water, and finally concentrated alkali (**Supplementary Fig. 1**).

First, four equal weights (5 g/bottle) of milled rye and ROM were individually extracted with 200 mL 70 % (v/v) ethanol at 50 °C for 1 h. This step was repeated with the residues obtained by centrifugation (15 min, 20 °C, 30,000 x g) until the supernatants were free of sugar according to the phenol–sulphuric acid assay [33]. The supernatants were combined and filtered with a Büchner funnel using Whatman filter paper (no. 5951/2 filter paper, Schleicher & Schuell GmbH, Dassel, Germany), followed by a rotary vacuum evaporation to remove all ethanol. Afterwards, the ethanol soluble solids (ETOHS) and one quarter of the alcohol insoluble solids (AIS) were freeze-dried.

Next, the remaining AIS was subsequentially enzyme treated with heat-stable α-amylase and amyloglucosidase to hydrolyse the starch. In brief, each bottle of AIS was first suspended with 50 mL 0.1 M sodium acetate buffer (NaOAc) pH at 5.2 and then pre-incubated with 30 μL thermostable α-amylase at 80 °C for 30 min. Afterwards, samples were cooled down to 50 °C and another 30 μL thermostable α-amylase and 50 μL amyloglucosidase were added and samples were incubated at 40 °C. After 2.5 h of incubation, 120 mL 96 % (*v*/v) ethanol was added to each bottle to extract the sugar residues formed during the enzymatic degradation of starch. The obtained pellets were washed twice with 200 mL 70 % (v/v) ethanol. The degradation of starch with both steps mentioned above was repeated one more time and the final residue was washed with 200 mL 70 % ethanol. One third of all material was freeze-dried to yield Destarched AIS (D-AIS).

The remaining D-AIS was sequentially extracted with 200 mL hot water at 70 °C for 1 h. This extraction was repeated twice, the obtained hot water-soluble solids (HWSS) were filtered using Whatman filter paper and the filtrate was combined. The combined filtered HWSS fraction and part of the water unextractable solids (HWUS) fraction were freeze-dried. The remaining HWUS was extracted with 200 mL concentrated alkali (4 M NaOH with 0.02 M $NaBH_4$) at 4 °C for 1 h and this was repeated twice. Both the residue and supernatant from alkali treatment were neutralized (pH 6) with acetic acid. Then the extract and residue were separately dialysed against demineralized water using cellulose dialysis membranes (molecular weight cut off 12–14 kDa for proteins; Visking, Medicell International, London, UK). After dialysis, the concentrated alkali soluble solids (CASS) and the concentrated alkali unextractable solids (CAUS) were freeze-dried.

Additionally, HWSS fractions from a new batch of ROM, as well as cereals of maize and wheat were prepared using the same procedures as

above described.

2.3. Constituent anhydromonosaccharide content and composition

To determine the constituent anhydromonosaccharide content and composition of the milled rye and ROM and their derived fractions (ETOHS, AIS, D-AIS, HWSS, HWUS, CASS and CAUS), 10 mg of each sample (in duplicate) was pre-hydrolysed with 72 % (*w*/w) H_2SO_4 at 30 °C for 1 h, followed by hydrolysis with 1 M H_2SO_4 at 100 °C for 3 h. The detailed procedures for measuring monosaccharides [34] by GC as alditol acetates and uronic acid (UA; colour assay) [35] in the hydrolysate was performed as previously described [36].

2.4. Molecular weight analysis

High performance size exclusion chromatography (HPSEC) was performed to analyse molecular weight (Mw) distribution of the soluble fractions derived from rye and ROM on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA). Elution was monitored by refractive index detection (Shodex RI-101, Showa Denko K.K., Kawasaki, Japan) and UV detection at two wavelengths ($\lambda = 280$ nm and 320 nm). Calibration of the column was performed with pullulan standards (Supelco, Bellefonte, USA). The detailed procedure has been previously described [37].

2.5. Starch and nitrogen content analysis

Total starch contents in raw materials of milled rye, ROM and their derived ETOHS, AIS, and D-AIS fractions were analyzed according to the AOAC method 996.11 using the total starch assay kit (Megazyme, Bray, Ireland) as described elsewhere [38]. The total nitrogen content (N * 6.25) of the milled rye, ROM, and the derived fractions was determined by the combustion method using a Flash EA 1112 Nitrogen Analyzer (DUMAS) (Thermo Scientific, Sunnyvale, CA, USA) [39].

2.6. Centrifugal ultrafiltration of selected fractions

Rye- and ROM-HWSS fractions with a concentration of 5 mg/mL were first centrifuged (20 min, 20 °C, 30,000 x g) as they could not be completely resolubilized in water. The obtained supernatants were further sequentially separated using centrifugal ultrafiltration units with 3 kDa and 10 kDa cut-off membranes (Millipore centrifugal filter units, Merck, Billerica, Massachusetts, United States). The resulting subsamples with different molecular size (< 3 kDa, 3–10 kDa, and > 10 kDa) of HWSS were freeze-dried and used for stimulation of the HD11 chicken macrophage cell line, to explore which molecular size in HWSS fractions were (primarily) responsible for the bioactivity. HPSEC was performed on the generated subsamples to confirm the ultrafractionation efficiency.

2.7. Quantitative pyrolysis-GC–MS with ^{13}C lignin as internal standard

The >10 kDa HWSS fractions of rye and ROM were characterized by analytical pyrolysis coupled to gas chromatography with high-resolution mass spectrometric detection (Exactive Orbitrap, Thermo Scientific, Waltham, MA, USA) as previously described [40]. Uniformly ^{13}C-labeled lignin (97.7 atom % ^{13}C), isolated from ^{13}C wheat straw (IsoLife BV, Wageningen, The Netherlands), was used as an internal standard (^{13}C-IS) [41]. To each accurately weighed sample (90 μg, XP6 excellence-plus microbalance, Mettler Toledo, Columbus, OH, USA), 10 μL of a ^{13}C-IS solution (1 mg/mL ethanol/chloroform 50:50 *v*/v) was added and dried prior to analysis. All samples were prepared and analyzed in duplicate. Lignin contents and relative abundances of lignin-derived pyrolysis products were calculated as previously described [40], excluding p-hydroxyphenolic pyrolysis products that are mainly derived from aromatic amino acids. The tryptophan pyrolysis marker indole (Rt 18.31 min, *m/z* 117.05723), the chitin pyrolysis markers 3-acetamidofuran (Rt 18.86 min, *m/z* 83.0661) and acetylpyridone (Rt 14.91 min, *m/z* 81.05731), glucan marker levoglucosan (Rt 25.87 min, *m/z* 60.02077) and xylan marker xylosan (Rt 16.56 min, *m/z* 57.03346) and glycerol (Rt 11.39 min, *m/z* 61.02838) were also monitored in full MS mode on the most abundant fragment per compound but conversely to lignin without applying response correction.

2.8. Enzyme and mild alkali treatments, and subsequent chemical analysis

2.8.1. Enzyme- and mild alkali treatment

Endoxylanase and/or endoglucanase hydrolysis were performed to check whether the presence of AX and/or glucan polymer structures in rye-and ROM-HWSS could be responsible for the immunomodulatory effect, i.e. the IL-12p40 induction [31,32]. HWSS fractions, together with controls of RAX, WAX, and barley β-glucan (5 mg) were solubilised in 50 mM NaOAc buffer (pH 5.0). Ten μL endoxylanase and/or endoglucanase (1.0 % *v*/v) were added and incubated in an Eppendorf Thermomixer® Comfort at 35 °C for 24 h at 500 rpm, followed by heating (5 min, 100 °C) to inactivate the enzymes.

Fructanase hydrolysis was performed to check whether the presence of fructan polymers could be involved in the bioactivity. HWSS fractions and Frutafit® IQ (5 mg) were suspended in 750 μL water, followed by heating at 85 °C for 15 min under constant shaking. After incubation, samples were cooled and mixed with 250 μL sodium acetate buffer pH 4.5 (200 mM). Next, 8 μL fructanase was added and the mixture was incubated in an Eppendorf Thermomixer® Comfort at 60 °C for 1 h at 500 rpm, followed by inactivation (5 min, 100 °C) of the enzyme.

Driselase hydrolysis was performed to assess whether the bioactivity of HWSS was caused by polysaccharide-related polymer structures. HWSS fractions (5 mg) were suspended with 850 μL 50 mM NaOAc buffer (pH 5.0), mixed with 150 μL freshly prepared Driselase solution (10 mg/mL) and incubated in an Eppendorf Thermomixer® Comfort at 37 °C for 24 h at 500 rpm, followed by inactivation (5 min, 100 °C) of the enzyme.

Chitosanase hydrolysis was performed to investigate whether the presence of chitosan structures could be involved in bioactivity. HWSS fractions, together with chitosan control (5 mg), were incubated with 0.1 unit of chitosanase in 1 mL 50 mM NaOAc buffer (pH 5.0) in an Eppendorf Thermomixer® Comfort at 37 °C for 24 h at 500 rpm, followed by heating (5 min, 100 °C) to inactivate the enzyme.

Protease hydrolysis was performed to investigate whether the presence of protein related structures could be involved in bioactivity. HWSS fractions (5 mg) were first solubilised with 990 μL water and incubated with 10 μL protease solution (10 mg/mL) in an Eppendorf Thermomixer® Comfort at 37 °C for 24 h at 500 rpm, followed by heating (5 min, 100 °C) to inactivate the enzyme.

Last, mild alkali treatment was performed to explore the effect of ester-linked moieties to AX structures in HWSS fractions on IL-12p40 induction. HWSS fractions (5 mg) were dissolved in 500 μL water and then mixed with 500 μL 0.1 M NaOH and incubated for 24 h at 4 °C, followed by adding 500 μL 0.1 M acetic acid.

The supernatant was obtained by centrifugation (10 min, 20 °C, 30,000 x g) for all above-mentioned enzymes or 0.1 M NaOH treated samples. One aliquot of the supernatant was freeze-dried and used for further biochemical analysis, while the remainder was taken for HPSEC and High-Performance Anion-Exchange Chromatography (HPAEC) analysis.

2.8.2. Enzymatic fingerprinting of polysaccharides in rye-and ROM-HWSS based on oligosaccharide profiles

Oligomers generated by the enzymatic degradation with endoxylanase and/or endoglucanase, as well as fructanase were analyzed by an ISC5000 HPAEC (Dionex, Sunnyvale, CA, USA) equipped with a Pulsed Amperometric Detector (PAD) as previously described [42,43].

2.8.3. Determination of free and bound HCAs

The amount of free and bound HCAs in milled rye, ROM, and their derivatives (i.e., HWSS with or without mild alkali treatment) was determined as described elsewhere [44] as single measurement and repeated under slightly different conditions. Although results were very similar, statistical evaluation would not be fully correct.

The extracted HCAs were analyzed using reverse phase ultra high performance liquid chromatography coupled to UV detection and mass spectrometry (**RP-UHPLC-UV-ESI-MS/MS**) as described previously [45] with minor changes. In brief, a Thermo Accela UHPLC system (Thermo Scientific, San Jose, CA, United States) equipped with a photodiode array (PDA) was used. Detection wavelengths were set to the range of 200–600 nm and quantification was performed at 320 nm. Samples (1 μL) were injected onto an Acquity UPLC BEH C18 (150 mm × 2.1 mm i.d., 1.7 μm) column connected to a VanGuard (5 mm × 2.1 mm i.d., 1.7 μm) guard column of the same material (Waters, Milford, MA). Column temperature was set to 30 °C and the flow rate was 0.4 mL/min. Eluents used were (A) water and (B) acetonitrile both with 0.1 % (*v*/v) formic acid. The elution profile was adjusted as follows: started running for 4.39 min isostatically at 0 %, followed by linear gradient 4.39–22.68 min to 50 % B, 22.68–23.78 min linear gradient to 100 % B and 23.78–29.27 min isostatically. The mobile phase was adjusted to starting conditions within 29.27–30.36 min, followed by equilibration for 5.5 min. Detection wavelengths were set to the range of 200–600 nm and UV–Vis was recorded at 285 nm, 310 nm and 320 nm. Mass spectrometric data were acquired using an LTQ Velos Pro ion trap mass spectrometer (Thermo Scientific) equipped with a heated ESI probe coupled in-line to the RP-UHPLC system as described above. Minor changes to the procedure included changing the ion transfer tube temperature to 263 °C, and changing the source heater temperature to 425 °C. Data were processed using Xcalibur 4.0, instead of Xcalibur 2.2 (Thermo Fisher Scientific).

Quantification of FA and *p*CA was based on the response factor of *trans*-FA and *trans*-*p*CA at 320 nm as it provided the highest signal. FA and *p*CA standard curves showed linearity at 320 nm and resulted in R2-values of 99.99 % and 100.00 %, respectively. Quantities of the bound HCAs were calculated by subtracting free HCAs from the total HCAs. Dihydroferulic acid (DiFA) was quantified as described by van Dinteren et al. (2022) [46] using a molar extinction coefficient of FA of 16,203 $L{\cdot}mol^{-1}{\cdot}cm^{-1}$ by Kaeswurm et al. (2021) [47] and molecular weight correction of 386 g/mol representing the molar mass of DiFA.

2.9. Assessment of bioactivity

2.9.1. HD11 cell culture and media

The chicken monocyte/macrophage cell line HD11 was derived from chicken bone marrow cells that were immortalized by transformation with the MC29 avian leukaemia virus [48]. HD11 cells were stored in liquid nitrogen, and after thawing the cells were used between the 5th and 18th passage. The cells were cultured in complete medium RPMI 1640 supplemented with 10 % heat inactivated chicken serum, 100 units/mL penicillin and 100 μg/mL streptomycin, 2 mM L-glutamine and 0.05 mM beta-mercaptoethanol in 150-cm^2 cell culture flasks (#430823, Corning Inc., Corning, NY, USA) at 41 °C, 5 % CO_2 and were passaged 2–3 times a week. Cells were harvested when confluency was at 80–90 %. Subsequently, HD11 cells were washed with PBS and incubated with 5 mM EDTA in PBS for 10 min at 41 °C to detach the cells. The cells in EDTA/PBS solution were transferred to a 50 mL tube. The flask was washed with PBS to collect all the remaining cells that were subsequently added to the same tube. The tube was centrifuged at 210 x*g* for 5 min at room temperature (RT), and the cells were resuspended in complete medium. Subsequently, the cells were counted and diluted to a concentration of 2×10^6 cells/mL.

2.9.2. Preparation of samples for cell stimulation

The milled rye, ROM, and derived fractions (except for HWSS) after freeze drying were further fine milled in a PM100 planetary ball mill (Retsch, Haan, Germany) after which each rye or ROM derived freeze-dried extract was dissolved (soluble solids) or suspended (insoluble/unextractable solids) in PBS and stored at a concentration of 10 mg/mL at −20 °C. On the stimulation day, extracts were thawed, diluted in complete medium with and without 75 μg/mL PMB at concentrations ranging from 0.7 to 500 μg/mL and incubated for 4 h at room temperature (RT). PMB was used to block lipopolysaccharide (LPS) that could be present as a contamination in the sample and could otherwise stimulate the cells. Only samples that did not contain LPS or of which its effect was fully inhibited by PMB, assessed by the absence of nitric oxide (NO) induction in HD11 cells, were included in further analyses (for detailed procedure, see section 2.9.7).

Controls for cell stimulation included 10 μg/mL LPS from *Salmonella enterica* serovar Typhimurium or 100 ng/mL PMA + 500 ng/mL ionomycin as a positive control, complete media without PMB (CTRL-) as negative control, and complete media with 75 μg/mL PMB (CTRL+) as negative control for samples incubated in the presence of PMB.

2.9.3. Stimulation of chicken macrophage cell line HD11

The chicken macrophage cell line HD11 cells was seeded at 200,000 cells/well in 100 μL volume of complete media in 96-well flat bottom cell culture plates (#3596, Corning Inc., Corning, NY, USA) and incubated for 2 h at 41 °C, 5 % CO_2 to let the cells adhere to the plate. Subsequently, freshly prepared extracts at concentrations ranging from 0.7 to 500 μg/mL and controls were added to the cells at a volume of 100 μL, followed by incubation for 24 h at 41 °C, 5 % CO_2. The next day, plates were centrifuged at 210 *g* for 2 min at RT. Cell culture supernatant was collected, aliquoted, and stored for later testing either at −20 °C for Griess assay or at −80 °C for quantification of cytokines by capture ELISA [49].

2.9.4. Isolation and stimulation of chicken splenocytes

Spleens from four 42-days old Ross 308 broilers were obtained from slaughterhouse Plukon Dedemsvaart BV (Dedemsvaart, the Netherlands). In a laminar flow cabinet, capsids were removed from the spleens, and the spleens were gently pushed through 100 μm strainers to obtain single cell suspensions. Subsequently, 10 mL Ficoll-Paque was carefully placed under 20 mL splenocytes suspension and tubes were centrifuged at 1017 x*g* for 20 min at 21 °C. Next, the interphase containing the leukocytes was collected and transferred to a new tube. Leukocytes were washed two times with 50 mL PBS, followed by centrifugation at 355 x*g* for 5 min at 21 °C. Next the pelleted splenocytes were resuspended in complete culture media and seeded at 200,000 cells/well in 120 μL volume in flat bottom 96-well cell culture plates. The cells were immediately stimulated with 120 μL of rye/ROM-HWSS and their >10 kDa sub-fractions at concentrations ranging from 50 to 200 μg/mL for 24 h at 41 °C, 5 % CO_2, and cell culture supernatant was harvested as described above.

2.9.5. Griess assay

Nitric oxide (NO) formation was measured indirectly by quantifying the concentration of NO_2^- in cell culture supernatant with the Griess assay [50] within 3 days after supernatant collection as previously described [51] and measured in a FilterMax F5 (Molecular Devices, San Jose, CA, USA) plate reader at OD_{548}. Nitrite concentration of the samples was calculated in SoftMax Pro v.6.2.2 and v.7.1.0 (Molecular Devices) using linear regression.

2.9.6. Capture ELISA

Release of cytokines to cell culture supernatant was measured by in house developed capture ELISAs for chicken IL-6, IL-10, and IL-12p40 as previously described [49] with a minor change for the dilution of the supernatants. The supernatant samples from splenocytes were diluted in the dilution buffer with 1 % rabbit serum for IL-6, and IL-10 ELISAs to block unspecific binding. Other supernatant samples were tested

without rabbit serum.

2.9.7. Statistical analyses

The effects of PMB on induction of NO within a sample were analyzed (**Supplementary Fig. 2 and 3**). When (1) NO induction by the sample in the presence of PMB was <1.56 μM (lowest point on standard curve) or less than mean CTRL +2 x SD (when CTRL+ value was higher than 1.56 μM); or (2) NO induction was reduced by <15 % by the presence of PMB, then samples were apparently not contaminated with LPS or its effect could nearly completely be inhibited. These samples were thus selected for further analyses for the cytokine inducing capacity of the samples.

Least square means of production of IL-12p40 induced by the different fractions at the various concentrations (0.7–500 μg/mL) for samples with PMB were compared with the negative control with PMB using SAS 9.4. Variances were estimated by concentration to account for heteroscedasticity, and if necessary, data were square root or logarithm10 transformed to meet normality assumptions. For rye-HWSS <3 kDa, rye-HWSS 3–10 kDa, rye-HWSS >10 kDa, ROM-HWSS <3 kDa, and ROM-HWSS 3–10 kDa from the first stimulation test, model assumptions were not met, and these data were analyzed using a non-parametric test of Dwass, Steel, Critchlow-Fligner (DSCF) multiple comparison analysis. Effects of non-treated extract vs enzyme/mild alkali treated extract and rye-HWSS > kDa vs ROM-HWSS >10 kDa (at 2 concentrations) were analyzed using GraphPad Prism 9.3.1 and were tested by ANOVA with concentration as fixed effect (at 500 μg/mL and 100 μg/mL or 500 μg/mL, 166.6 μg/mL and 55.5 μg/mL, respectively) using uncorrected Fisher's LSD test with a single pooled variance or by unpaired *t*-test assuming the same SD for both populations. When only one concentration was tested, one-way ANOVA was performed. Differences among means with $p < 0.05$ were considered statistically significant. Main figures only contain samples and CTRL tested with PMB. Data from samples and CTRL with and without PMB can be found in supplementary data. Data are presented as raw means and SD.

3. Results

3.1. Chemical characteristics and bioactivity screening of rye and ROM fractions

Rye grain, ROM, and fractions from their sequential extractions with ethanol (70 % (*v*/v), hot water, and 4 M NaOH were analyzed for monosaccharide content and composition, starch and protein content (Table 1), and the mass balances for monosaccharides and protein are presented in **Supplementary Table 1**. As expected, a large part of all sugars was present as starch, accounting for 48.3 % in rye and 33.0 % in ROM. Other main sugars in ROM and rye were arabinose, xylose, and non-starch glucose. Fermentation of rye with *A. subrufescens* reduced the total sugar content (70.6 % (*w*/w) in rye versus 51.5 % (w/w) in ROM (Table 1). However, there was an increase in protein content in ROM (9.3 %, w/w) compared to rye (7.3 %, w/w) (Table 1). We found that 10.49 % (w/w) and 16.47 % (w/w) of small molecules were recovered by 70 % (*v*/v) ethanol extraction from rye and ROM, respectively. Sequential extractions from D-AIS by hot water and 4 M NaOH were performed to obtain different hemicellulose populations (e.g., AX and β-glucan). Most of AX and β-glucan ended up in CASS fractions for both rye and ROM, while protein was mostly solubilised in ETOH fractions, with only a small part being present in HWSS fractions (**Table 1 and Supplementary Table 1**).

Rye and ROM and their derived fractions were tested on the chicken macrophage cell line HD11 to assess whether they were able to induce secretion of the pro-inflammatory cytokines IL-12p40 and IL-6 and the

Table 1
Chemical composition and yield of rye and ROM, and fractions derived from rye and ROM.

Raw materials and fractions	Sugar composition (mol%)[a]								Content (*w*/w%)			Yield [g per 100 g starting material]
	Fuc	Ara	Xyl	Man	Gal	Glc	UA	A/X[b]	Total sugars	Starch	Protein	
Rye	0	6 ± 0	9 ± 1	1 ± 0	1 ± 0	82 ± 1	2 ± 0	0.67 ± 0.0	70.6 + 0.6	48.3 ± 0.8	7.3 ± 0.0	100
Rye-ETOHS	0	2 ± 0	1 ± 0	20 ± 1	5 ± 0	68 ± 1	4 ± 1	2.10 ± 0.2	25.7 ± 0.2	10.6 ± 0.5	22.6 ± 0.0	10.5
Rye-AIS	0	6 ± 0	9 ± 0	1 ± 0	0 ± 0	82 ± 0	2 ± 0	0.60 ± 0.0	83.4 ± 1.3	58.9 ± 0.2	5.9 ± 0.0	78.2
Rye-D-AIS	0	23 ± 0	40 ± 0	4 ± 0	2 ± 0	29 ± 0	2 ± 0	0.57 ± 0.0	57.7 ± 0.6	12.9 ± 0.3	21.5 ± 0.0	21.6
Rye-HWSS	0	29 ± 0	49 ± 0	3 ± 0	1 ± 0	18 ± 0	1 ± 0	0.60 ± 0.0	74.7 ± 2.4		2.3 ± 0.0	3.8
Rye-HWUS	0	23 ± 0	40 ± 0	3 ± 0	3 ± 0	29 ± 1	2 ± 0	0.57 ± 0.0	54.1 ± 0.0		24.7 ± 0.0	17.8
Rye-CASS	0	16 ± 0	45 ± 0	8 ± 0	2 ± 0	27 ± 1	1 ± 0	0.36 ± 0.0	65.0 ± 0.9		12.8 ± 0.2	9.0
Rye-CAUS	0	24 ± 0	39 ± 1	5 ± 0	3 ± 0	26 ± 1	3 ± 0	0.61 ± 0.0	49.4 ± 1.6		24.4 ± 0.2	9.0
ROM	0	4 ± 1	7 ± 0	5 ± 0	1 ± 0	80 ± 2	2 ± 0	0.64 ± 0.0	51.5 ± 2.1	33.0 ± 0.2	9.3 ± 0.1	100
ROM-ETOHS	0	4 ± 0	5 ± 0	44 ± 2	1 ± 0	42 ± 2	3 ± 0	0.85 ± 0.1	30.9 ± 0.8	12.9 ± 0.4	21.5 ± 0.2	16.5
ROM-AIS	0	4 ± 0	7 ± 0	1 ± 0	1 ± 0	84 ± 1	2 ± 0	0.63 ± 0.0	58.9 ± 0.7	41.5 ± 0.0	6.4 ± 0.2	78.3
ROM-D-AIS	1 ± 0	13 ± 0	20 ± 1	4 ± 0	3 ± 0	54 ± 1	4 ± 0	0.62 ± 0.0	39.8 ± 1.0	13.4 ± 0.3	13.4 ± 0.1	34.5
ROM-HWSS	3 ± 0	8 ± 0	16 ± 0	5 ± 0	13 ± 0	53 ± 0	4 ± 0	0.54 ± 0.0	46.6 ± 0.4		12.3 ± 2.2	4.8
ROM-HWUS	0	14 ± 0	22 ± 1	4 ± 0	2 ± 0	53 ± 1	5 ± 0	0.64 ± 0.0	33.1 ± 0.0		12.3 ± 1.4	32.8
ROM-CASS	0	14 ± 0	30 ± 0	6 ± 0	2 ± 0	45 ± 0	2 ± 0	0.45 ± 0.0	56.5 ± 2.7		11.50 ± 0.1	10.1
ROM-CAUS	0	9 ± 0	16 ± 1	3 ± 0	2 ± 0	59 ± 0	11 ± 0	0.54 ± 0.0	38.7 ± 0.9		20.98 ± 0.3	8.3

[a] Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acids (UA). [b]A/X: ratio of arabinose to xylose.

anti-inflammatory cytokine IL-10. Firstly, the contamination by LPS of rye, ROM and their corresponding fractions was assessed. We found that rye-AIS, rye-D-AIS and rye-HWUS (**Supplementary Fig. 2 A and 2B**) were LPS contaminated and hence these fractions were excluded from further analysis. The other (i.e. non-contaminated) fractions were tested for their immunomodulating potential on HD11 macrophages. Only the HWSS fractions induced production of IL-12p40 (Fig. 1**A&B, Supplementary Fig. 4 A&B**) by HD11 cells, showing immunomodulatory properties. None of the tested fractions induced the other tested cytokines, i.e. IL-6 or IL-10 (**Supplementary Fig. 4C&D and E&F**).

The IL-12p40 inducing effect of the HWSS-fractions of ROM and rye was subjected to further tests to confirm its reproducibility. First, HWSS fractions were compared directly to each other (Fig. 1C). Both fractions induced at all concentrations a significant (all $p < 0.0001$) increased production of IL-12p40 when compared to the negative control, and ROM-HWSS induced significantly higher IL-12p40 levels than rye-HWSS at 500 μg/mL ($p = 0.0036$) and 166.6 μg/mL ($p = 0.0348$) (Fig. 1C). Next, we tested reproducibility of the ROM-HWSS effect, since ROM is obtained by fermentation with *A. subrufescens* and this process might result in variable properties for different batches. The newly obtained ROM-HWSS fraction from a new batch of ROM product showed some LPS contamination as indicated by the induced NO-production, but this could be fully neutralized by the addition of PMB (**Supplementary Fig. 5 A**). The ROM-HWSS fraction produced from a new batch of ROM did induce a clear IL-12p40 production ($p < 0.0001$), albeit somewhat lower than the first ROM-HWSS, confirming the immune-stimulatory effect also observed for the first batch (Fig. 1D**, Supplementary Fig. 5B**).

The chemical analysis revealed that the polysaccharides in rye-HWSS were mainly composed of xylose (34.5 %, *w*/w), arabinose (20.8 %, w/w) and non-starch glucose (15.3 %, w/w), while these values were changed in ROM-HWSS with reduced levels of xylose (6.2 %, w/w) and arabinose (3.3 %, w/w), and increased non-starch glucose (25.7 %, w/w) (**Supplementary Table 1)**. The protein content was five times higher in ROM-HWSS (12.3 %, w/w) than in rye-HWSS (2.3 %, w/w) (Table 1).

To summarize, fermentation by *A. subrufescens* reduced the total

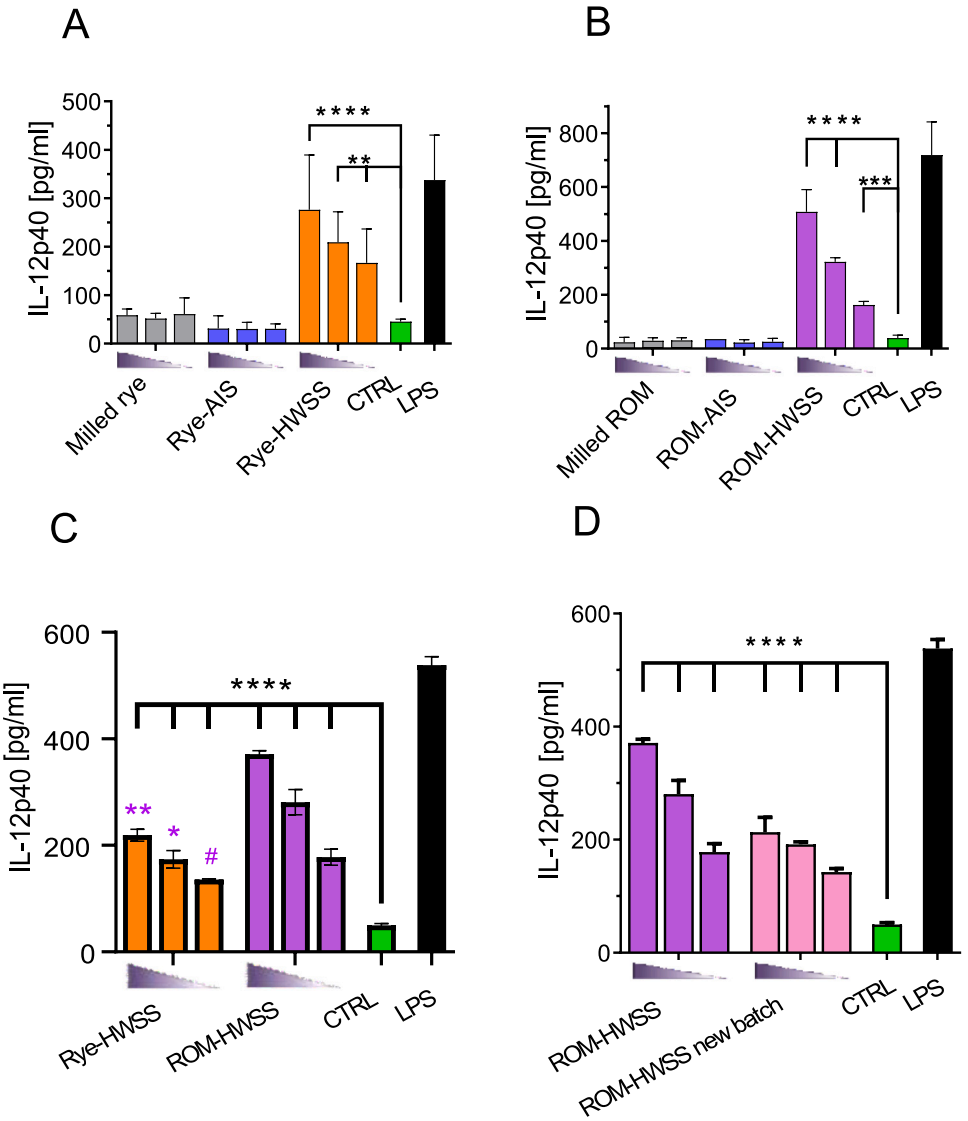

Fig. 1. Levels of IL-12p40 produced by HD11 cells after stimulating with rye and ROM extracts. The cell line HD11 was incubated with **A**) rye-derived extracts, **B**) ROM-derived extracts, **C**) re-tested rye- and ROM-HWSS, and **D**) ROM-HWSS of the old and a new batch of ROM. All samples were tested at concentrations 500, 166.6 and 55.5 μg/mL (from left to right, represented by ◣) in the presence of 75 μg/mL PMB. Cells were also incubated with 10 μg/mL LPS or 75 μg/mL PMB (CTRL) as positive and negative controls, respectively. The bars represent mean ± SD. Black asterisks indicate that mean levels of IL-12p40 induced by samples significantly differs from CTRL. Purple asterisks indicate significant differences of IL-12p40 levels for rye-HWSS vs. ROM-HWSS at the corresponding concentrations (* <0.05, ** <0.01, *** <0.001, **** <0.0001).

sugar content and led to much more small molecules and proteins in ROM. Out of all tested extracts, only the HWSS fraction of both rye and ROM reproducibly induced IL-12p40 production by HD11 cells, but ROM-HWSS seemed to induce even higher levels than rye-HWSS. The polysaccharides in HWSS fractions were predominately composed of arabinose, xylose and glucose, but Mw distribution of HWSS from rye and ROM was substantially different despite the similar sugar composition.

3.2. *High molecular weight fractions of HWSS are responsible for the IL-12p40 inducing effect*

Further analysis of immunostimulatory fractions by HPSEC revealed that multiple Mw populations were present in rye- and ROM-HWSS (Fig. 2A). Therefore, a size fractionation of rye- and ROM-HWSS was performed by centrifugal ultrafiltration using 3 and 10 kDa cut-off membranes. The predominant sub-fraction in both rye- and ROM-HWSS was >10 kDa in size with a lower recovery for ROM-HWSS (36.9 %, *w*/w) than for rye-HWSS (59.3 %, w/w), while the total recovery for ROM-HWSS (89.7 %, w/w) was higher than for rye-HWSS (71.1 %, w/w) (**Supplementary Table 2**).

The cell assay showed that production of IL-12p40 was clearly induced by the >10 kDa sub-fraction of ROM ($p = 0.0034$ for 500 μg/mL, $p = 0.0073$ for 100 μg/mL, $p = 0.092$ for 20 μg/mL) and was observed for rye ($p = 0.3069$ for 500 and 100 μg/mL, $p = 0.9389$ for 20 μg/mL) (**Supplementary Fig. 6 A&B**). IL-12p40 production was not induced by sub-fractions of <3 kDa and 3–10 kDa (**Supplementary Fig. 6 A&B**), nor did any of the sub-fractions induce IL-6 or IL-10 production (**Supplementary Fig. 6C—F**). Direct comparison of the induction by>10 kDa fractions of rye- and ROM-HWSS showed significant IL-12p40 production by both fractions at all tested concentrations, with ROM-HWSS inducing higher levels than rye-HWSS (Fig. 3).

As both rye- and ROM-HWSS >10 KDa fractions demonstrated biological activities, they were further characterized to examine which compounds could be responsible for the IL-12p40 inducing activity. HPSEC was performed to monitor the Mw distribution of HWSS >10 kDa using RI detection to monitor all compounds as well as monitoring compounds providing signals at UV 280 nm and 320 nm, more specifically indicating the presence of protein and phenolic compounds, respectively (Fig. 2A-C). Whereas RI detection showed rather similar signals for both fractions, much higher UV signals at UV_{280} and UV_{320} were observed in HWSS >10 kDa for ROM than for rye (Fig. 2B**&C**). The saccharide content and composition of rye- and ROM- HWSS >10 kDa is shown in Fig. 2D **and Supplementary Table 3**. The predominant sugars in HWSS >10 kDa from rye were arabinose (15.2 %, *w*/w), xylose (27.3 %, w/w) and non-starch glucose (11.9 %, w/w), while the major sugars derived from ROM were xylose (11.0 %, w/w), arabinose (6.4 %, w/w), galactose (10.8 %, w/w) and non-starch glucose (7.4 %, w/w). Even though the levels of arabinose and xylose, as well as the Mw distribution were lower in HWSS >10 kDa from ROM than HWSS >10 kDa from rye, the arabinose to xylose (A/X) ratio was comparable in both cases, with values of 0.56 for rye-HWSS >10 kDa and 0.59 for ROM-HWSS >10 kDa. Additionally, the amounts of mannose (4.9 %, w/w) and uronic acid (3.1 %, w/w) were higher in HWSS >10 kDa from ROM than rye, which contained 2.5 % (*w*/w) mannose and 0.8 % (w/w) uronic acid. The percentage of total sugars was higher in HWSS >10 kDa from rye (58.7 % w/w) than ROM (45.9 % w/w).

Pyrolysis-GC–MS analysis was performed to further screen for compounds beyond carbohydrates in rye- and ROM-HWSS >10 kDa. This analysis showed that both rye- and ROM-HWSS >10 kDa fractions contained low amounts of lignin and derived products (~0.5 % (w/w), which were, however, negligible when 4-vinylguaiacol as mainly derived from FA, was excluded from content calculations (Fig. 4A, **Supplementary Table 4**). Despite the low lignin contents, it is worth noting that the relative abundance of the lignin-derived pyrolysis products from the ROM extracts was substantially different when compared to the rye extracts (**Supplementary Table 4**). ROM-HWSS >10 kDa showed a clearly higher accumulation of Cα-oxidized substructures (e.g., diketones) indicative of oxidative lignin degradation through fungal fermentation (Fig. 4B **and Supplementary Table 4**). Py-

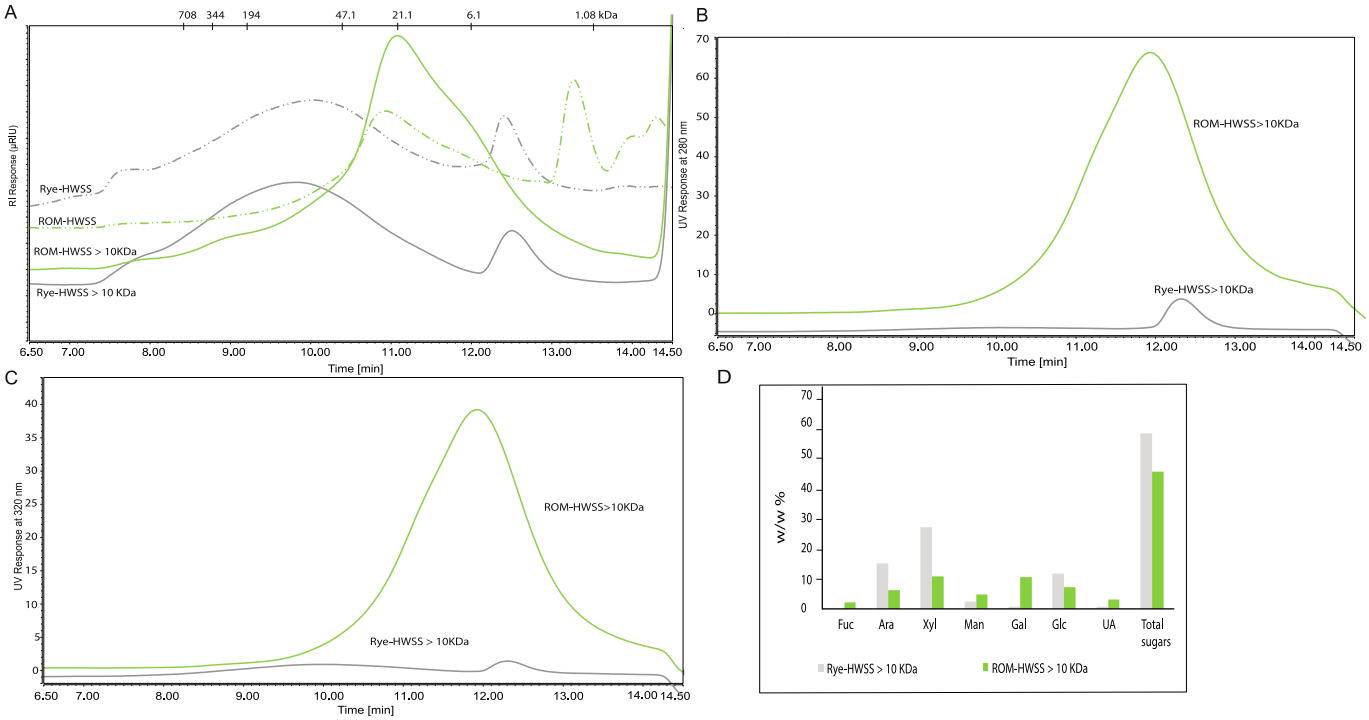

Fig. 2. HPSEC elution patterns of rye- and ROM-HWSS, along with subfractions >10 KDa using RI detection (**A**), UV detection at 280 nm (**B**), and UV detection at 320 nm (**C**), as well as bar plots illustrating the sugar composition of sub-fractions >10 KDa (**D**). The pullulan is used for Mw calibration. Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acids (UA).

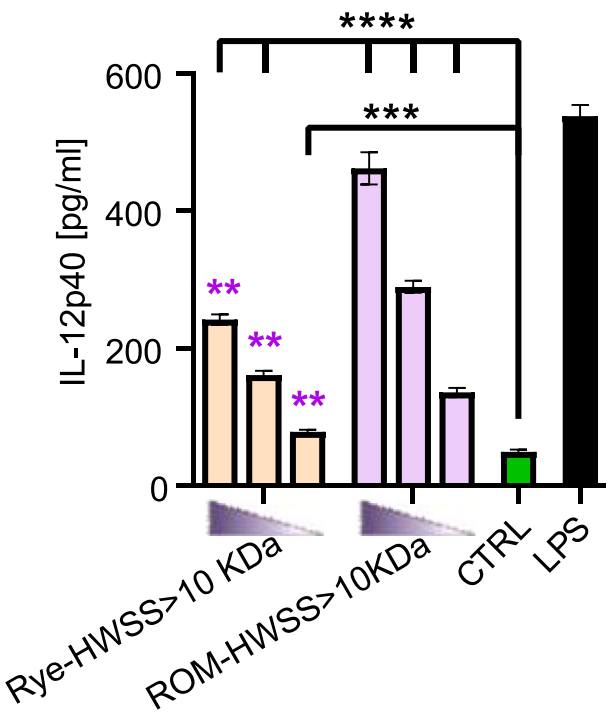

Fig. 3. Levels of IL-12p40 produced by HD11 cells after stimulating with rye- and ROM-HWSS >10 KDa. The cell line HD11 was incubated with 500, 100 and 20 μg/mL (from left to right, represented by ◣) of rye-and ROM-HWSS >10 KDa, in the presence of 75 μg/mL PMB. Cells were also incubated with 10 μg/mL LPS or 75 μg/mL PMB (CTRL) as positive and negative controls, respectively. The bars represent mean ± SD. Black asterisks indicate that mean levels of IL-12p40 induced by samples significantly differs from CTRL. Purple asterisks indicate significant differences of IL-12p40 levels for rye-HWSS >10 KDa vs. ROM-HWSS >10KDa at the corresponding concentrations (* <0.05, ** <0.01, *** <0.001, **** <0.0001).

GC–MS also revealed that HWSS >10 kDa from rye had an approximately three times higher abundance of 4-vinylguaiacol than the fraction derived from ROM (Fig. 4C), indicative for the presence of FA [52]. On the contrary, ROM-HWSS >10 kDa showed a 3.7 x higher abundance of indole as tryptophan marker pyrolysis product, compared to rye-HWSS >10 kDa (Fig. 4D). ROM-HWSS >10 kDa showed a substantially higher abundance of chitin-derived pyrolysis products (3-acetomidofuran and acetylpyridone) [53] (Fig. 4E**&F**). Additionally, HWSS >10 kDa from rye had higher abundance of levoglucosan, xylosan and glycerol than HWSS >10 kDa from ROM (Fig. 4G-I). Levoglucosan and xylosan are markers for pyrolysis products originating from glucan and xylan, respectively. These latter observations thus confirm the above-mentioned analysis of constituent anhydromonosaccharide content and composition.

To summarize, the >10 kDa sub-fraction was most predominant in rye and ROM, and only this sub-fraction showed the ability to induce IL-12p40 production. Rye-HWSS and ROM-HWSS >10 kDa had different sugar compositions, and consisted consequently of different polysaccharide structures. Beyond carbohydrates, pyrolysis-GC–MS analysis revealed notable differences between rye-HWSS and ROM-HWSS >10 kDa regarding a relatively small proportion of compounds, such as amino-acids, FA and chitin-derived substructures.

3.3. HWSS and HWSS >10 KDa also stimulate IL-12p40 production by chicken splenocytes

Immunomodulating rye- and ROM-HWSS and their >10 kDa sub-fractions were also tested on chicken splenocytes to confirm if the IL-12p40 induction capacity observed on the chicken macrophage cell line HD11 is also observed for primary immune cells present in the chicken spleen. All the tested samples induced significantly higher levels of IL-12p40 (all $p < 0.0001$) in a dose-dependent manner (Fig. 5) than the unstimulated control. The full HWSS fraction and its >10 kDa sub-fraction of ROM induced IL-12p40 levels more than twice as high as the levels induced by the corresponding rye fractions ($p < 0.0001$). Therefore, rye- and ROM-HWSS and their >10 kDa sub-fractions were also able to induce IL-12p40 production by primary immune cells, with ROM-HWSS being a stronger inducer than rye-HWSS. For comparison of samples with and without PMB see **Supplementary Fig. 7**, where no difference in IL-12p40 induction was observed between the two conditions, indicating that the IL-12p40 induction was not caused by LPS contamination.

3.4. Mild alkali treatment of rye- and ROM-HWSS inhibited stimulation of IL-12p40 production more efficiently than the enzymatic treatments

To further characterize in more detail the potential IL-12p40 inducing compound(s), rye- and ROM-HWSS were used in place of the >10 kDa sub-fractions, due to the lack of sufficient materials for the subsequent test. To this end, we assumed that the <10 kDa compounds in HWSS fractions will not impact the results as they were shown to lack bioactivity in HD11 cells. HWSS fractions were treated with enzymes including endoxylanase, endoglucanase and protease to investigate whether the degradation of compounds with a certain structure could influence or completely eliminate the IL-12p40 inducing capacity. Furthermore, hydrolysis with fructanase targeting fructan was also included as rye grain is known to contain a small amount of the fructan polysaccharide [15]. Chitosanase hydrolysis, as well as mild alkali treatment using 0.1 M NaOH were performed to explore the potency of chitosan, and of ester-linked moieties of AX structures in HWSS fractions on the bioactivity since their presence was suggested by pyrolysis-GC–MS analysis and literature [54,55]. Additionally, Driselase (a complex mixture of carbohydrases targeting a very wide range of polysaccharide structures) hydrolysis was performed to assess whether the treatment would result in an extensive degradation and loss of activity.

HPSEC was used to display the effects of above mentioned enzymatic or chemical treatments on polysaccharides, and the Mw distributions of the digests together with the parental samples are shown in Fig. 6. The activity and efficiency of the enzymes was demonstrated on their pure polymeric substrates using HPSEC, which showed a completed shift to smaller compounds (**Supplementary Fig. 8**). With endoxylanase and/or endoglucanase digestion, the populations were clearly degraded to small molecules in rye-HWSS (Fig. 6A), indicating the degradation of AX and/or glucan structures. The Mw profile of ROM-HWSS (Fig. 6B) did not change as much as rye-HWSS upon the xylanase treatment. Using HPAEC, we found that digestion with endoxylanase generated a wide range of products including xylose, xylobiose, xylotriose, as well as single, double and multiple arabinose-substituted xylo-oligomers from rye-HWSS (Fig. 7). Arabinoxylan oligosaccharides could be annotated on bases of earlier enzymatic degradation studies of wheat [56] and rye arabinoxylan (**Fig. 7**). HPAEC also showed arabinoxylan degradation products for ROM-HWSS, although in lower abundance and in lower amounts for the higher arabinose-substituted xylo-oligomers than rye-HWSS (Fig. 7). In the case of endoglucanase treatment, HPAEC also clearly demonstrated glucan degradation for both rye- and ROM-HWSS fractions (**Supplementary Fig. 9 A**). After fructanase incubation, no clear changes in Mw distribution on HPSEC were observed for either rye- or ROM-HWSS (Fig. 6A**&B**), while clear fructan degradation products in both rye- and ROM-HWSS fractions were observed on HPAEC (**Supplementary Fig. 9B**). After Driselase treatment, the population was almost completely depolymerised into small fragments for rye-HWSS (Fig. 6C). However, only a slight change in Mw pattern was observed for ROM-HWSS (Fig. 6D), emphasizing the difference in structures present in the two HWSS extracts. No changes in Mw distribution on HPSEC were observed for HWSS fractions treated with protease,

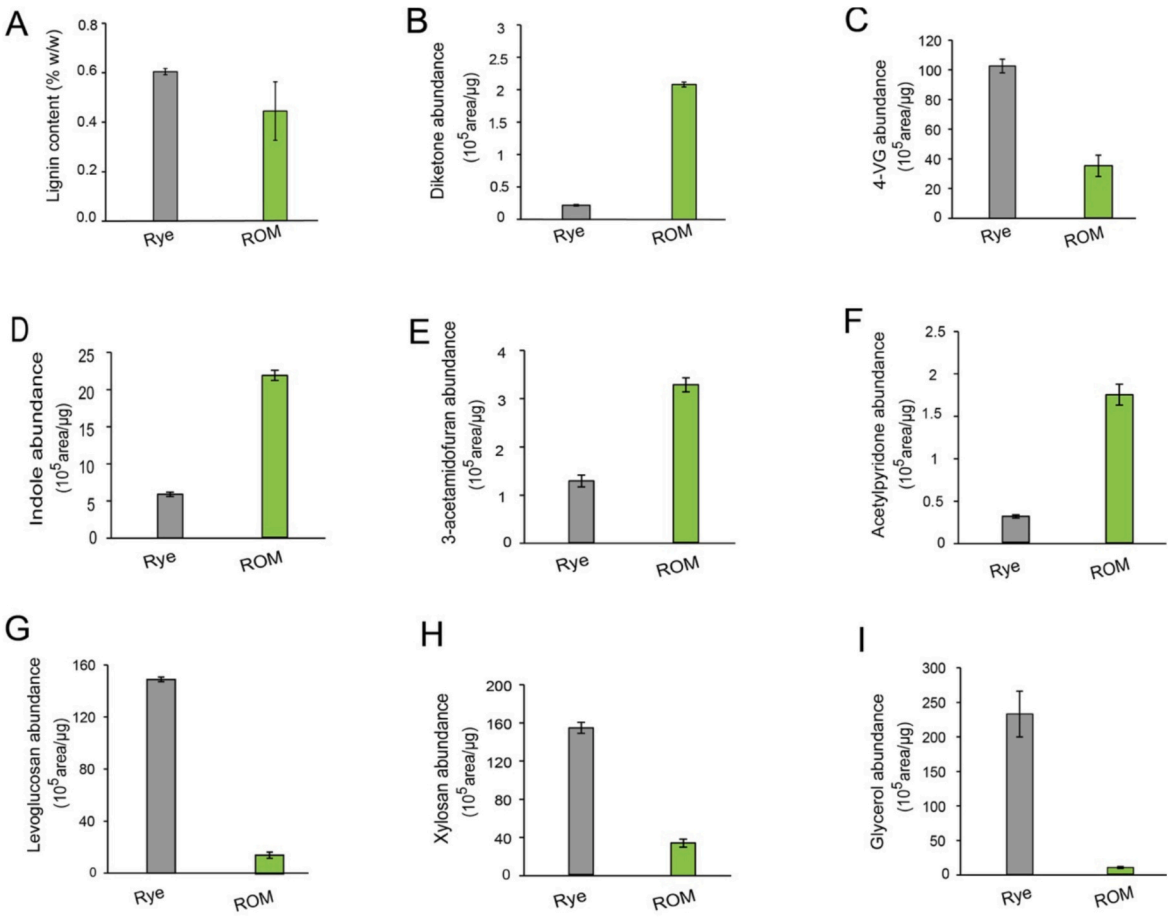

Fig. 4. Pyrolysis-GC–MS of rye- and ROM fractions HWSS>10 KDa: lignin content (**A**), abundance of diketones **(B)**, 4-vinylguaiacol (4-VG) (**C**), indole (**D**), 3-acetamidofuran (**E**), acetylpyridone (**F**), levoglucosan (**G**), xylosan (**H**) and glycerol (**I**).

chitosanase and 0.1 M NaOH (Fig. 6C**&D**).

The cell assay revealed that enzymatic treatments of arabinoxylan and/or beta-glucan (Fig. 8A**&B**), or fructan (Fig. 8C**&D**) in rye- and ROM-HWSS did not reduce the IL-12p40 inducing capacity of the corresponding digesta. Enzymatic treatments of rye-HWSS (Fig. 8E) with protease and Driselase+chitosanase did significantly reduce IL-12p40 production by a modest 10 % ($p = 0.0044$ and 0.0129, respectively), while 0.1 M NaOH treatment reduced IL-12p40 production by 50 % ($p < 0.0001$). For ROM-HWSS, the 0.1 M NaOH treatment reduced its IL-12p40 inducing capacity most (68 %; $p < 0.0001$) (Fig. 8F). However, other treatments also considerably reduced the cytokine induction: protease by 44 % ($p < 0.0001$), Driselase+chitosanase by 35 % ($p < 0.0001$), Driselase by 28 % ($p < 0.0001$), and chitosanase by 14 % ($p = 0.0004$). Comparison of IL-12p40 induction by samples with and without PMB is presented in **Supplementary Fig. 10**. The reference compounds, WAX, RAX, barley β-glucan, Frutafit® IQ and a chitosan from crab shells, did not induce IL-12p40 production before and after corresponding enzyme treatment (**Supplementary Fig. 10**).

To summarize, endoxylanase, endoglucanase and Driselase treatments substantially degraded the polysaccharide structures for rye-HWSS, while only slight degradation was observed for these enzymes treated ROM-HWSS. This suggests that, despite a similar sugar composition, different polysaccharide structures were present in rye-and ROM-HWSS fractions. The cell assay revealed that 0.1 M NaOH treatment for both rye- and ROM-HWSS caused the strongest reduction in the IL-12p40 inducing capacity. Additionally, components in ROM-HWSS sensitive to protease, Driselase, and Driselase+chitosanase could also considerably contribute to its IL-12p40 inducing capacity.

3.5. Analysis of HCAs in rye- and ROM-HWSS

As mild alkali treatment caused the strongest reduction in the IL-12p40 inducing capacity for both rye- and ROM-HWSS, RP-UHPLC-UV-ESI-MS was performed to measure esterified HCAs present in in the initial materials of milled rye and ROM, as well as in rye- and ROM-HWSS before and after the 0.1 M NaOH treatment.

The results, as shown in Table 2, demonstrated that FA in esterified form was the primary HCA (indicated as bound FA and *p*CA). The esterified FA for rye-HWSS (2.98 µg/mg) showed higher levels than for ROM-HWSS (0.31 µg/mg), corroborating the findings obtained by pyrolysis-GC–MS analysis. We also found a higher concentration of esterified FA in milled rye (2.32 µg/mg) than in ROM (0.28 µg/mg). The esterified DiFA, potentially crosslinking polymers within the lignocellulose matrix, was exclusively found in rye-HWSS at a level of 0.15 µg/mg, while it was not detected in ROM-HWSS. For *p*CA, a higher concentration of esterified forms was present in rye-HWSS (0.12 µg/mg) compared to ROM-HWSS (0.05 µg/mg) fractions.

Mild alkali treatment resulted in a marked reduction of esterified FA in rye-HWSS (from 2.98 µg/mg to 0.35 µg/mg) and ROM-HWSS (from 0.31 µg/mg to 0.11 µg/mg). Furthermore, this treatment eliminated *p*CA in both rye-and ROM-HWSS, and nearly all the esterified DiFA was removed from rye-HWSS, decreasing from 0.15 µg/mg to 0.02 µg/mg.

4. Discussion

In the current study, we demonstrated for the first time that hot water-soluble solids (HWSS) from rye grains and rye overgrown with *A. subrufescens* mycelia (i.e., ROM) can induce IL-12p40 production by

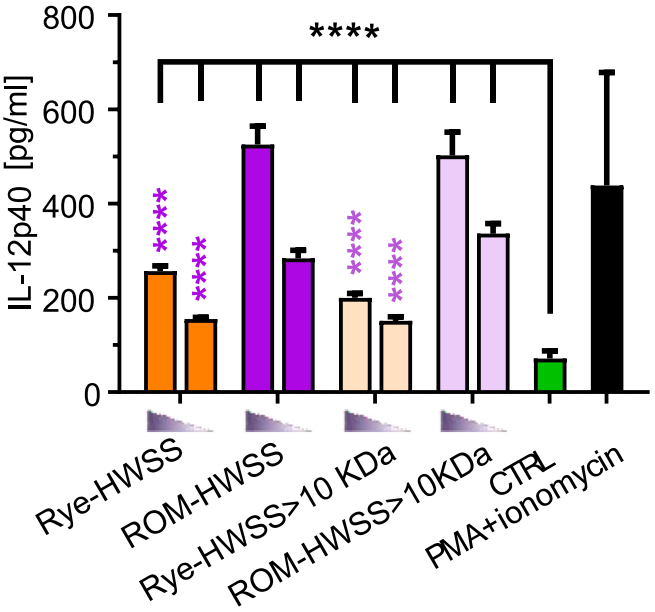

Fig. 5. Induction of IL-12p40 by splenocytes (n = 4) after stimulating with HWSS samples. Splenocytes were incubated with 200 and 66.6 μg/ml rye/ROM-HWSS, as well as 100 and 50 μg/ml of rye/ROM-HWSS >10KDa in the presence of 75 μg/ml PMB (from left to right, represented by ◣). Cells were also incubated with 100 ng/ml PMA + 500 ng/ml ionomycin or 75 μg/ml PMB (CTRL +) as controls. The bars represent mean ± SD. Black asterisks indicate that mean levels of IL-12p40 induced by samples significantly differs from CTRL. Purple asterisks indicate significant differences of IL-12p40 levels for rye-HWSS VS. ROM-HWSS, or rye-HWSS >10 KDa VS. ROM-HWSS >10KDa at the corresponding concentrations (* <0.05, ** <0.01, *** <0.001, **** <0.0001).

the chicken macrophage cell line HD11 and chicken splenocytes, and that the bioactive molecule(s) were polymers (> 10 kDa). Chemical and structural characterization for HWSS fractions >10 kDa revealed that the polysaccharides mainly consisted of AX and glucan with small amounts of protein and FA, although in different relative amounts and/or structures. The contents of AX, glucan and FA were higher in rye-HWSS, while protein, and some other polysaccharides (e.g., chitosan, galactan and mannan) were much higher in ROM fractions. Even though the levels of AX and the Mw distribution were lower in HWSS >10 kDa from ROM than rye, the A/X ratio was comparable in both cases, indicating similar AX structures in these two fractions. Enzymatic hydrolysis of rye-and ROM-HWSS by endoxylanase, endoglucanase, and fructanase did not reduce their IL-12p40 inducing capacity in the chicken macrophage cell line HD11, indicating that bioactivity is not restricted to these polymers. Treatment with 0.1 M NaOH substantially reduced the IL-12p40 production by 50 % for rye- HWSS and by 68 % for ROM-HWSS. This treatment cleaved almost all FA, DiFA and *p*CA units in rye-HWSS, while it cleaved around one third of FA units and all *p*CA in ROM-HWSS, suggesting that the structure of these HCA units linked to AXs could be involved in the bioactivity. Furthermore, protease treatment reduced IL-12p40 induction by 10 % for rye-HWSS and 44 % for ROM-HWSS, which suggests that the protein related structures in HWSS fractions contributed to the bioactivity. Additionally, chitosanase and Driselase treatments considerably lowered the IL-12 p40 production, but only for ROM-HWSS. This may suggest that *A. subrufescens* mycelia compounds, such as chitosan and other polysaccharide structures like galactan and mannan may also play a role in IL-12 p40 production for the case of ROM-HWSS.

We found that ethanol (70 % *v*/v) extracts from either rye or ROM did not induce cytokine production in the chicken macrophage cell line HD11. Our findings may indirectly be supported by a study where the ethanol extracts from mycelium of *A. subrufescens* demonstrated inhibitory instead of stimulatory effects on NK cells [57]. It was reported that both water and concentrated alkali extracts from *A. subrufescens* had

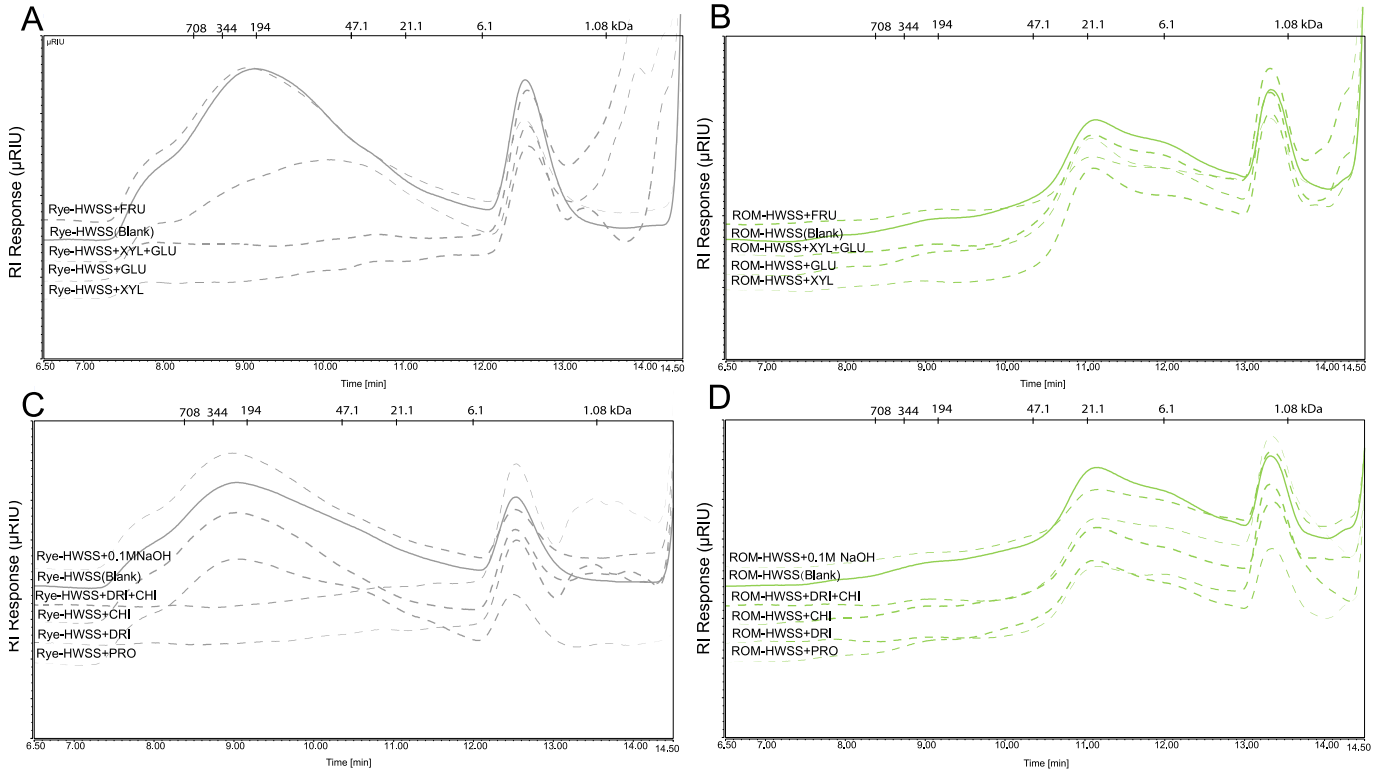

Fig. 6. HPSEC elution patterns of rye-and ROM-HWSS before and after endoxylanase (XYL), endoglucanase (GLU) and fructanase (FRU) treatments (**A&B**), as well as protease (PRO), driselase (DRI), chitosanase (CHI) and mild alkali (0.1 M NaOH) treatments (**C&D**). The pullulan is used for Mw calibration.

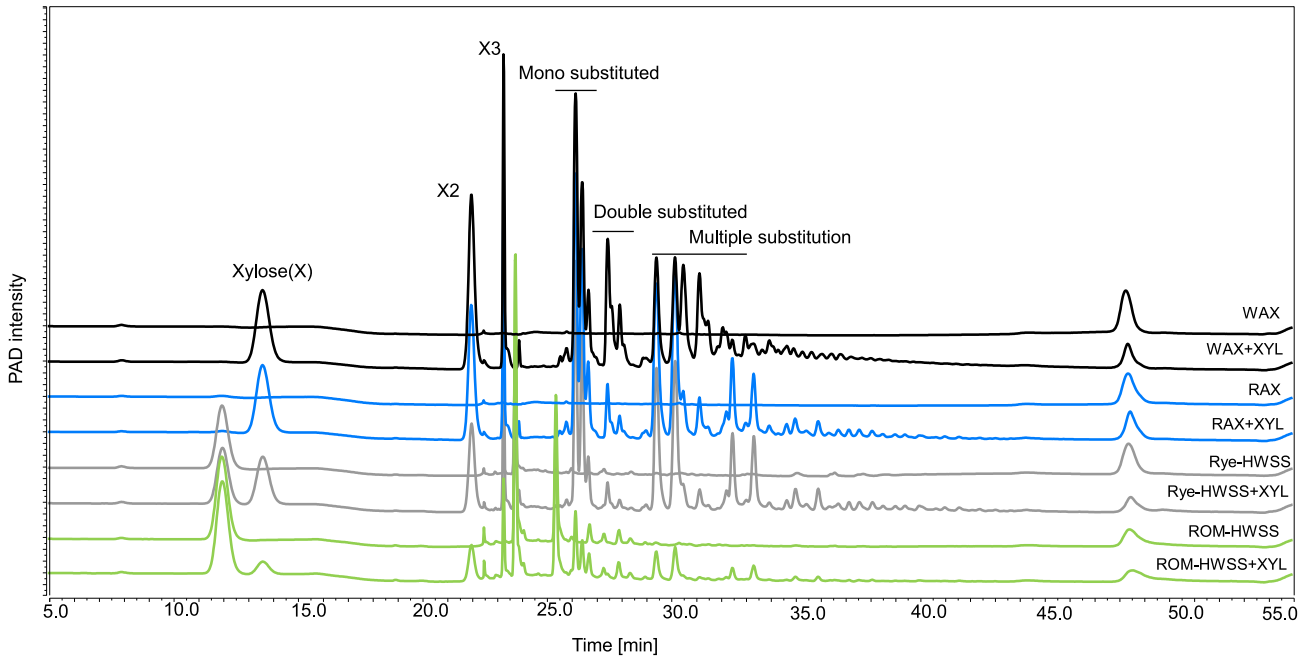

Fig. 7. HPAEC elution pattern of rye and ROM-HWSS before and after endoxylanase I (XYL) treatment. Rye arabinoxylan (high viscosity) (RAX), wheat arabinoxylan (medium viscosity) (WAX).

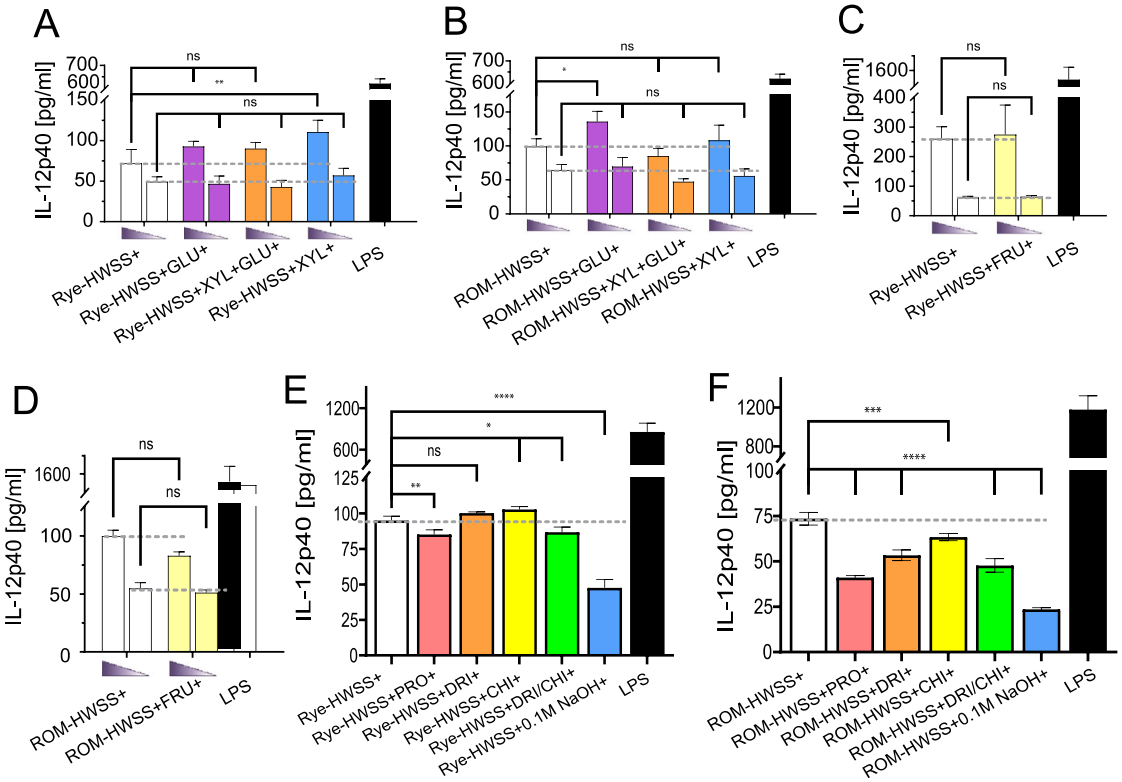

Fig. 8. Inhibition of the IL-12p40 inducing capacity of rye- and ROM-HWSS by different enzymatic and chemical treatments. Graphs present the production levels of IL-12p40 by HD11 cells after stimulation with untreated or treated HWSS of rye (**A, C, E**) or ROM (**B, D, F**) after subtracting mean of negative control with PMB (CTRL+). Untreated HWSS was a reference for HWSS treated with endoxylanase I (XYL) and/or endoglucanase I (GLU) (**A, B**), fructanase (FRU) (**C, D**), protease (PRo), driselase (DRI), chitosanase (CHI) and 0.1 M NaOH (**E, F**). Samples were used at a final concentration of 500 μg/mL or 500 and 100 μg/mL (from left to right, represented by ◣) with 75 μg/mL PMB (+), and control included 10 μg/mL LPS. Dotted lines indicate the level of IL-12p40 induction of non-treated HWSS. The bars represent mean + SD. Asterisks indicate that mean levels of IL-12p40 induced by enzymatically or chemically treated HWSS were significantly different from untreated HWSS at each corresponding concentration (* <0.05, ** <0.01, *** <0.001, **** <0.0001).

Table 2
Free and bound hydroxycinnamic acids in milled rye and ROM, as well as rye and ROM-HWSS before and after 0.1 M NaOH treatment.

Samples	FA (μg/mg sample)		*p*CA (μg/mg sample)		DiFA (μg/mg sample)	
	free	bound	free	bound	free	bound
Milled rye	0.03	2.32	0.00	0.42	0.00	0.46
Rye-HWSS	0.02	2.98	0.00	0.12	0.00	0.15
Rye-HWSS+0.1 M NaOH	1.55	0.35	0.00	0.00	0.11	0.02
Milled ROM	0.00	0.28	0.00	0.01	0.05	0.08
ROM-HWSS	0.00	0.31	0.00	0.05	0.00	0.00
ROM-HWSS+0.1 M NaOH	0.11	0.11	0.00	0.00	0.00	0.00

Quantity of the bound HCAs were calculated by subtracting free HCAs from the total HCAs. Quantity of (FA, *p*CA) and estimated free and bound HCAs content is presented in mg/mg sample (dry matter). diFA, diferulic acid; FA, ferulic acid; *p*CA, p-coumaric acid.

immunomodulatory activity [58,59]. However, in the present study, results from HD11 stimulation showed that only HWSS fractions induced the production of IL-12p40. This could be ascribed to AX differences in molecular weight and degree of branching, which have been suggested as major determinants of their biological functionalities (e.g., antioxidant activity and immune modulation) [8,17]. For example, concentrated alkali-based extraction can cleave FA substitutions that are naturally present in cereal AX, which could have inflammatory activity and antioxidant potential [60,61].

We found that molecules in size >10 kDa in HWSS fractions were responsible for IL-12p40 production, with HWSS >10 kDa from ROM inducing higher levels than from rye upon stimulation of HD11 cells and chicken splenocytes. This may indicate that compounds derived from *A. subrufescens* mycelium (e.g., protein, 1 → 3–1 → 6- β-glucan and chitosan) or the modified AX from rye have contributed to the higher induction of IL-12p40. Our findings are in line with other studies where the water extract from hemicellulase-digested compounds of *A. subrufescens* mycelia induced IL-12 production in human peripheral mononuclear cells (PBMCs) [11] and in murine splenic DCs [12]. However, no follow-up studies were performed to identify the effective components present in that *A. subrufescens* mycelia extract. In the present study, we observed that HWSS from ROM had a five times higher level of protein than the corresponding fraction from rye, and protease treatment caused a significant reduction of IL-12p40 production by 10 % for rye-HWSS and 44 % for ROM-HWSS. This could indicate that part of the IL-12p40 inducing capacity was present in protein structures of rye- and ROM-HWSS. In case of ROM-HWSS, it has been reported that polysaccharides obtained by hot water extraction either from *A. subrufescens* mycelia are composed of β (1 → 6) and α (1 → 4) glucan–protein complexes, constituted mainly of β-glucan [62,63]. The immune response-stimulating activity of *A. subrufescens* mycelia has been widely attributed to this glucan–protein complex [59,62,64]. Therefore, we speculate that such glycan-protein structures should also exist in ROM-HWSS and play an important role on IL-12p40 induction on chicken immune cells. In addition, chitosan-derived pyrolysis products were observed in ROM-HWSS >10 kDa, and chitosanase hydrolysis only significantly reduced the IL-12p40 production for ROM-HWSS. It was reported that phagocytosable small (1 to 10 μm) chitin particles induced IL-12 production in murine spleen cells [65,66], suggesting that the presence of chitosan in ROM-HWSS could also contribute to the IL-12p40 production.

In the present study, we found that the degradation of the AX backbone, and structures of glucan and fructan of rye- and ROM-HWSS did not reduce the level of IL-12p40, and Driselase treatment lowered IL-12p40 production only for ROM-HWSS. This suggests that the bioactivity is not restricted to AX, glucan and fructan polymers, and other polysaccharide structures (e.g., galactan and mannan) in ROM-HWSS derived from *A. subrufescens* mycelium could be involved in the bioactivity. To the best of our knowledge, the immunomodulatory effects of individual mannan or galactan derived from *A. subrufescens* have not yet been studied. In contrast, other fungi, such as *Amanita muscaria* and *Hypsizigus marmoreus* have been reported to produce an α-D-galactan [67] and a heavily branched α-mannan [68], respectively, both having shown immunomodulating activities. It was reported that hydrolysis with mild alkali (i.e., 0.1 M NaOH) can cleave the linkage with ester bonds [69,70]. In our study, upon stimulation of HD11 cells, treatment with 0.1 M NaOH showed strongest inhibition of the IL-12p40 induction among all selected enzyme treatments for HWSS fractions. The alkali treatment cleaved nearly all esterified FA, DiFA and *p*CA units in rye-HWSS, while around one third of esterified FA and all *p*CA present in ROM-HWSS was cleaved off. This strongly suggests that structures of phenolic esters linked with AX in HWSS fractions were involved in stimulating IL-12p40 production by chicken macrophages. In case of rye-HWSS, phenolic acids (mainly FA) ester-linked to AX are widely reported to be enriched in water extracts from rye [71,72]. Therefore, this suggests that structures of FA ester-linked to AXs in rye-HWSS play a key role on IL-12p40 induction. In terms of ROM-HWSS, the effect of 0.1 M NaOH treatment on the bioactivity may not only be based on the presence of the ester-linked FA and *p*CA residues. This is because the amount of HCAs is around 10 times lower in ROM-HWSS than in rye-HWSS, yet the 0.1 M NaOH treatment led to a comparable reduction in IL-12p40 inducing capacity. Characterization of individual compounds from *A. subrufescens* remains at a relatively preliminary stage. Other phenolic compounds (e.g., gallic acid and benzoic acid) have been reported from dried fruiting bodies of *A. subrufescens* [73,74]. However, to our knowledge, there are no reports regarding the potential phenolic acids ester-linked with polysaccharides that can be obtained from mycelia of *A. subrufescens* under solid-state fermentation on rye.

Since we clearly showed that HWSS from rye grains had immunomodulatory properties, we additionally tested HWSS extracts from wheat and maize to check whether such a bioactive water extract could be generic for other cereals besides rye. We found the IL-12p40 inducing capacity of HWSS obtained from rye was ~40 % higher than that from maize- and wheat-HWSS (**Supplementary Fig. 11**). The observed IL-12p40 induction by HWSS of maize and wheat was at least partly caused by contamination with LPS. The contamination could not be fully neutralized by PMB, as was evident by the NO levels in the presence of PMB, which although reduced, were not entirely eliminated. It has been reported that there are variations in functionality of water extractable AX derived from different cereals due differences in AX structures, such as branching degree, the substitution position, FA content, and chain length of the side chains [8,71,75]. Our data suggested that the IL-12p40-inducing effect might be unique to rye when compared to other cereals.

Some dietary interventions with rye, maize, and wheat in livestock animals also suggest that rye may be associated with potential health benefits. An experiment with broilers compared inclusion of 0, 5, or 10 % (*w*/w) of rye, substituting for maize, in a maize-based diet [76]. Broilers fed diets containing 5 % or 10 % (w/w) rye showed down-regulation of the immunosuppressive TGF-beta pathway compared with broilers fed 0 % rye [76] while it is known that TGF-beta is responsible for suppressing differentiation of Th1 cells [77]. Together with our data where rye-HWSS, and ROM-HWSS, induced IL-12p40, we could hypothesize that the mode of action of those extracts would be via inducing a Th1 type of response through IL-12 induction by the innate immune cells. It is well established that IL-12p40, in combination with IL-12p35, skews Th0 to a Th1 type of response, which is the type of immune response that is most effective against intracellular pathogens [78]. Th1 has been shown to be a dominant response in chickens to control infections with (facultative) intracellular bacteria, such as enteric *Salmonella* [79,80], through induction of IL-12p40 from splenocytes and macrophages [81,82]. In pigs, Th1 response and macrophage activation are also crucial in the immune response against *Salmonella* [83] and elevated IL-12p40 was observed in pigs with persistent shedding of *Salmonella Typhimurium* [84]. Therefore, the IL-12p40 inducing rye- or

ROM-HWSS fractions could be tested as immunomodulating feed additives that may promote resistance to (facultative) intracellular pathogens, like *Salmonella*, as both fractions were active, and various compounds in those extracts were responsible for the bioactivity and they could be (partly) different for rye and for ROM.

5. Conclusion

We demonstrated for the first time that hot water extractions (HWSS) from ROM and rye can both induce IL-12p40 production by chicken HD11 cells and splenocytes, with ROM-HWSS inducing higher levels than rye-HWSS. The compounds and mechanisms underlying the bioactivity of rye- and ROM-HWSS could be different. Chemical and structural characterization of HWSS fractions revealed that AX and HCA units (e.g., FA) were present in rye-HWSS which seemed to have been degraded/modified during the fermentation process as also new compounds (e.g., protein and galactan) were present in ROM-HWSS. The esterified HCA esters linked to AX could be involved in stimulating IL-12p40 production for both rye- and ROM-HWSS fractions on chicken immune cells. Interestingly, ROM-HWSS demonstrated a more complicated profile; treatment with 0.1 M NaOH cleaved about ten times more HCAs from rye-HWSS than ROM-HWSS, yet rye-HWSS showed a smaller reduction in IL-12p40 induction (50 %) compared to ROM-HWSS (68 %). Further research is necessary to explore phenolic acid esters linked to polysaccharides and their role in ROM-HWSS's immunomodulatory effects. Furthermore, structures derived from *A. subrufescens* mycelia, such as glucan–protein complex, chitosan and galactans/mannans may also promote IL-12p40 production. Future research should focus on isolating and characterizing these compounds to fully unlock their potential immunomodulatory effects. Overall, our findings suggest that ROM-HWSS, but also rye-HWSS, as a feed additive for immunomodulatory strategies might help reduce susceptibility and improve resilience of animals to bacterial infections.

CRediT authorship contribution statement

Caifang Wen: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paulina Krzysica:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Gijs van Erven:** Writing – review & editing, Methodology, Data curation. **Coen H. Smits:** Writing – review & editing, Supervision, Project administration. **Sonja de Vries:** Writing – review & editing, Supervision, Formal analysis. **Huub F.J. Savelkoul:** Writing – review & editing, Supervision, Project administration. **Hauke Smidt:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Edwin Tijhaar:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Henk A. Schols:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

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Declaration of competing interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.144861.

Data availability

No data was used for the research described in the article.

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