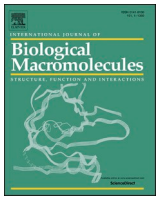
International Journal of Biological Macromolecules 286 (2025) 138344

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Citrus pectins impact the function of chicken macrophages

Adil Ijaz [a], Noah Pols [a], Kahlile Youssef Abboud [b], Victor P.M.G. Rutten [a,c], Femke Broere [a], Henk Schols [d], Edwin J.A. Veldhuizen [a,*,1], Christine A. Jansen [e,1]

[a] Division Infectious Diseases and Immunology, Department Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[b] Center for Healthy Eating and Food Innovation (HEFI)- Maastricht University, Campus Venlo, the Netherlands
[c] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa
[d] Laboratory of Food Chemistry, Wageningen University & Research, Wageningen, the Netherlands
[e] Cell Biology and Immunology group, Department of Animal Sciences, Wageningen University & Research, Wageningen, the Netherlands

ARTICLE INFO

Keywords:
HD11
Chicken toll-like receptors
Pectin
Phagocytosis
Nitric oxide
Macrophages

ABSTRACT

The restrictions on excessive use of antimicrobials in the poultry industry have led to the search for alternative strategies including nutritional interventions to enhance gut health with the ultimate aim to prevent gut infections. Pectins as prebiotics have shown beneficial effects on gut health in humans and mice by improving the gut barrier function, altering the gut microbiota, and by modulating the gut immune response. However, little is known about immunomodulatory properties of pectins in chickens. The present *in vitro* study assessed the effect of three pectins (SPE6, SPE7, SPE8) differing in methyl esterification, on responsiveness of the chicken macrophage cell line HD11 cells and primary monocyte derived macrophage from the blood, through interaction with chicken TLRs. All three pectins increased gene expression of *iNOS* and *IL10* in chicken macrophages. Differences in immunomodulatory activity between the three pectins were observed in other assays. The low methoxyl pectin (SPE8) interacted with TLR4 leading to the production of NO, but also to increased phagocytosis of *E. coli*, while high methoxyl pectins SPE6 and SPE7 did not activate TLR4. All three pectins were able to attenuate PAM3CSK4 induced activation of chicken macrophages as measured by decreased NO production and phagocytosis. Additional studies using ITC and flow cytometry suggest that the inhibiting properties of pectins (SPE6, SPE7) on macrophages are due to pectins occupying TLR2 and blocking PAM3CSK4 to activate chicken macrophages, whereas SPE8 actually binds to the TLR2 ligand and that way attenuates the PAM3CSK4 induced activation. Based on these immunomodulatory properties observed in this study, these pectins may in the future be suitable as feed additive for the treatment and prevention of inflammatory disorders in poultry.

1. Introduction

Poultry farming is an intensive industry responsible for 37 % of the global meat production [1]. Intestinal infections in chickens like salmonellosis and coccidiosis not only affect production performance but can also cause food-borne zoonoses in humans [2]. Restriction to the use of antimicrobials due to antimicrobial resistance often leads to destruction of the entire flock to control the spread of disease [3,4]. To overcome the huge economic losses, alternative strategies aiming at strengthening the host immune system are warranted [5]. These include nutritional interventions since feed components in the gut can affect the gut microbiota as well as intestinal immune cells [6,7].

The gut associated lymphoid tissue (GALT) along with gut epithelial cells help to prevent gut infections [8]. The cellular composition of the GALT includes intraepithelial lymphocytes (IELs) and phagocytes such as macrophages and dendritic cells that are present in the lamina propria [9]. Macrophages are among the early responders against microbial invasions of the intestine, and key players of the innate immune system [9]. They contribute directly to clearing the infection by production of nitric oxide (NO) and by phagocytosis and subsequent intracellular killing of bacteria [10,11]. Moreover, macrophages can produce both pro- or anti-inflammatory cytokines to attract and activate other immune cells, like T- and B cells, to the site of infection [12].

Macrophages recognize microbial agonists by pattern recognition receptors, including Toll like receptors (TLRs) which recognize a variety of microbial agonists [13]. For example, TLR4 interacts with

* Corresponding author.
E-mail address: e.j.a.veldhuizen@uu.nl (E.J.A. Veldhuizen).
[1] Authors contributed equally to this manuscript

https://doi.org/10.1016/j.ijbiomac.2024.138344
Received 22 December 2023; Received in revised form 13 November 2024; Accepted 2 December 2024
Available online 3 December 2024

lipopolysaccharide (LPS), TLR5 with flagellin and TLR2 interacts with multiple ligands including small bacterial lipopeptides and lipoteichoic acid of Gram positive bacteria [14]. Like mammals, chicken immune cells also contain a variety of TLRs with some avian specific characteristics [15,16]. Most of the chicken TLRs (chTLR) actually share functional homology with their mammalian counterparts [17–19] except chTLR15 and chTLR21 that are exclusively expressed in avian species [20]. Among 10 different TLRs that have been described in chickens [21], chTLR2 is a complex receptor since there are several isoforms due to gene duplication and it forms heterophilic dimers with other receptors, such as two isoforms of chTLR1 [22]. In general, chicken TLR2 is functionally comparable to its mammalian orthologue, recognizing a variety of microbial components for example peptidoglycan, lipoproteins, lipotechoic acid, glycolipids, and zymosan [17], but has a broader ligand specificity, possibly due to the presence of the isoforms [23].

Dietary fibers have gained substantial attention due to their health benefits on host [24]. Dietary fibers withstand enzymatic digestion in the host gut, and modulate immune response through microbiota dependent and microbiota independent manner [24]. Dietary fibers are fermented by the gut microbiota producing short chain fatty acid, which subsequently can interact with host (immune) cells [25–27]. Moreover, dietary fibers can also pass the gut barrier and directly interact with the resident immune cells that are embedded between the epithelial cells as well as in lamina propria [24,28]. This interaction of dietary fibers with the intestinal epithelial and resident immune cells can modulate immune response resulting into health promoting effects in humans and animals [29].

Supplementation with dietary fibers as feed additives in poultry results in beneficial effects on gut health, disease resistance, and production performance [30]. Similarly, different pre- and probiotic supplements in poultry feed have shown to increase the expression of genes related to innate and adaptive immune responsiveness in the chicken gut [31–33]. For example, *in vivo* studies have shown that long chain glucomannan used as feed additive not only enhanced immune responsiveness [34], but also impacted *Salmonella* infection in broiler chickens that might be due to higher relative abundance of *Streptococci* [35].

Pectins are complex polysaccharides, structural components of cell walls of vegetables, plants, and fruits [36]. The most dominant structure of pectin consists of a polymeric homogalacturonan backbone of α-1,4 linked of galacturonic acid (GalA) residues [37]. Due to their established health benefits and prebiotic properties, pectins are widely used as dietary fiber both in human and animal diet [38]. Apart from modulating immune responsiveness, they also alter the gut microbiota composition, and improve the gut barrier function, which gets impaired in inflammatory conditions [39]. Functional properties of pectins mostly depend on the degree of methyl esterification (DM), degree of blockiness (DB), and neutral side chain structures attached to the rhamnogalacturonan segment of the polymer [40–45]. The immunomodulatory activity of pectins in mammalian species is often related to the interaction of pectins with TLRs on host cells. For example, a low DM citrus pectin has been shown to block the TLR2/1 receptor on blood derived dendritic cells in humans and murine RAW 264.7 macrophages *in vitro* resulting in anti-inflammatory effects, and was able to reduce doxorubicin induced inflammation of the ileum *in vivo* in mice [43]. In addition, citrus pectins with different DM were able to activate human THP1 monocytes and were interacting with TLR2 and TLR4 in a DM and methyl ester distribution dependent manner [46].

In chickens only a few studies have described the direct effects of polysaccharides on chicken immune cells. The activation of chicken monocytes, and heterophils upon binding to TLR2 and TLR4 has been described in an *in vivo* study [47]. Moreover, ulvan extract, a complex sulphated polysaccharide has been shown to activate chicken gamma-delta T cells [48]. Glucose oligosaccharides, and glucomannan supplementation in broiler chickens not only modulated immune responsiveness as well as enhanced activation of intraepithelial NK cells, but also altered gut microbiota composition [34,35]. Additionally, reduction in chemotactic and phagocytic activity of chicken monocytes, and NK cell activation [49], after citrus pectin stimulation *in vitro* has been described [50], but no mechanism of activation of NK cells after citrus pectin stimulation was explained. In general, little is known about the immunomodulatory properties of pectins on any chicken immune cells. Innate immune cells are abundantly present in the intestinal epithelium and the lamina propria of the chicken gut [9], and act as a potential target of immunomodulation as they can interact with pectins, used as feed additives, through immune receptors. The current study focusses on chicken macrophages as they are one of the early responders in chicken gut in case of infection [51].

The purpose of the present study was to determine the immunomodulatory properties of three pectins (SPE6, SPE7, and SPE8), that differ in chemical composition and have shown to affect the cecal microbiota composition in the *in vitro* chicken intestinal gut model called Cecal Chicken ALIMEntary tRact mOdel-2 (CALIMERO-2) (Oost et al., manuscript in preparation). The study showed that one pectin (SPE8) is able to activate macrophages to produce more nitric oxide, while pre-incubation of macrophages with SPE6 and SPE7 was shown to inhibit subsequent activation of macrophages by the TLR2 ligand PAM3CSK4.

2. Materials and methods

2.1. Pectin samples

Three pectin samples (SPE6, SPE7, and SPE8) were obtained from Agrifirm (Apeldoorn, the Netherlands). Pectins were dissolved in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS) and 200 U/mL each of penicillin and streptomycin (P/S) (all from Gibco, Life Technologies Limited, Paisley, UK) at a stock concentration of 10 mg/mL for experiments with HD11 cells; and in DMEM +10 % FCS + P/S for experiments with the HEKBlue TLR reporter cell line. Dissolved pectins were stored at −20 °C for future use. Endotoxin level in the pectin samples was determined using the Endozyme II endotoxin detection kit (Biomereux Benelux BV, Amersfoort, the Netherlands) and LPS content was found to be below the detection limit of the kit. Galacturonic acid content of pectins was determined spectrophotometrically using the *m*-hydroxydiphenyl color assay [52,53]. Degree of methyl esterification was determined by saponification of pectins and measuring the methanol released by head-space gas chromatography (GC) [54]. The molecular weight of the pectins were determined using high performance size exclusion chromatography. Oost et al. (manuscript in preparation) have described the methods for the structural properties and composition of pectins in detail.

2.2. Cell lines

To study the interaction between toll like receptors (TLR) present on immune cells and three pectins, human reporter cell lines, HEK-Blue™, were used: HEKBlue TLR2, HEKBlue TLR4, and HEKBlue Null1 (Invivogen, Toulouse, France). HEKBlue reporter cell lines are adherent cells and stably express the soluble embryonic alkaline phosphatase gene that is under control of an NF-κB/AP-1 responsive promoter. The stimulation of HEK-Blue TLR reporter cell lines with a specific agonist leads to production of secreted embryonic alkaline phosphatase (SEAP) that can be quantified using detection solution called QUANTI-Blue (Invivogen). The HEKBlue Null1 cell line is the parental cell line of the different HEKBlue TLR cell lines and lacks a TLR expression construct. Frozen stocks (−140 °C) of HEKBlue TLR2, TLR4 and Null1 cells were thawed and cultured in 75 cm^2 flasks in DMEM supplemented with 10 % FCS and 200 U/mL P/S at 37 °C incubator. All the cell lines were passaged twice before maintaining the cell lines in culture medium containing selection antibiotics (Invivogen). For HEKBlue TLR2 and 4, HEKBlue selection antibiotics (stock concentration 250×) 1× was used, while for

HEKBlue Null1 cells Normocin (100 μg/mL) was added in the culture medium.

HD11, a chicken macrophage like cell line [55], frozen at −140 °C in RPMI-1640 (Gibco, Life Technologies Limited, Paisley, UK) was maintained in a 25 cm^2 or 75 cm^2 cell culture flask (Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C and 5 % CO_2 in complete RPMI 1640 medium supplemented with 10 % FCS and 200 units/mL penicillin, and 200 mg/mL streptomycin (P/S) (Gibco, Life Technologies Limited, Paisley, UK). The cells were passaged twice a week upon reaching 90 % confluency using 0.05 % trypsin/EDTA (Gibco, Life Technologies Limited, Paisley, UK). The cells were used between passages 2 and 15. HD11 cells have been described to express a range of chicken TLR, such as TLR1/6/10, TLR2 Type1, TLR2 Type 2, TLR3, TLR4, TLR5, and TLR7 [19].

2.3. Blood monocyte derived macrophages (MDMs)

Peripheral blood mononuclear cells (PBMCs) were isolated using a protocol described by Peng et al., [56] from fresh blood anticoagulated with heparin (Leo Pharma B.V., Amsterdam, the Netherlands) of 36 weeks old healthy layer chickens (Novogen white) using Ficoll density separation and stored at −140 °C until further use. Briefly, to isolate primary monocytes, PBMCs (1×10^6 cells) were seeded in a 96 well plate per well in 200 μL of RPMI-1640 culture medium supplemented with 10 % FCS, and P/S. After 6 h of incubation at 41 °C, non-adherent cells were removed. The cells were washed 3 times with PBS to remove remaining non-adherent cells. Subsequently, the adherent cells, which are monocytes, were maintained in 200 μL RPMI medium with FCS and P/S additionally supplemented with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) at a concentration of 1:1600 for 3 days at 41 °C to differentiate monocytes to macrophages [56]. Recombinant GM-CSF was produced by transfecting COS-7 cells with pCI-neo (Promega Corporation, Madison, Wisconsin, USA) that expresses the relevant cytokine, which were a kind gift from P. Kaiser and L. Rothwell (The Roslin Institute, Edinburgh, UK). Next, macrophages were stimulated with pectins and activation was assessed.

2.4. Alamar blue cell viability assay

Effects of pectin stimulations on viability of HD11 and HEKBlue TLR2 cells were determined by the Alamar blue viability assay. For that, cells were stimulated with different pectins (0.6 to 2.5 mg/mL) for 24 h. An unstimulated control with only cells and culture medium, and a positive control with cells and 1 % Triton X-100 (Sigma-Aldrich, Merck, St. Louis, MO, USA), was included. After 24 h, supernatant was removed and 100 μL of 10 % Alamar blue reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in RPMI medium was added to each well and incubated at 37 °C, 5 % CO_2 for 4 h. The absorbance of each well was measured at a wavelength of 570 nm with a reference at 600 nm, using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany).

2.5. TLR-mediated activation of HEKBlue reporter cell lines

Briefly, 5×10^4 HEKBlue TLR2, HEKBlue TLR4, and Null1 cells per well were seeded in a 96 well plate in 200 μL complete DMEM culture medium and stimulated with pectins (0.6–2.5 mg/mL) for 24 h at 37 °C, 5 % CO_2. Following incubation, 20 μL supernatant from each well was collected in a 96 well plate and mixed with 180 μL HEK-Blue detection medium (Invivogen) to measure SEAP production. The amount of SEAP was quantified by measuring the optical density (OD) at 650 nm using iMark microplate absorbance reader (Bio-Rad Laboratories, Hercules, California, United States).

2.6. Nitric oxide (NO) production assessed by Griess assay

NO production by HD11 cells after stimulation with different pectins was measured using the Griess assay. Briefly, in a 96-well Flat-bottom plate, 5×10^4 cells in 200 μL of RPMI-1640 culture medium were seeded per well. The next day, supernatants were removed, and cells were stimulated with pectins (0.6–2.5 mg/mL). PAM3CSK4 (20 ng/mL) was used as positive control, and only RPMI-1640 medium as negative control. After 24 h of incubation, 50 μL of supernatant was pipetted in a new 96-well flat-bottom plate and then 50 μL of 10 mg/mL sulfanilamide (2.5 % phosphoric acid) (Sigma, Missouri, USA) was added. The mixture was incubated for 5 min at room temperature in the dark. After that 50 μL of 3 mg/mL N-(1-napthyl) ethylenediamine dihydrochloride (Sigma, Missouri, USA) was added and incubated again in the dark for 5 min at room temperature. Finally, the absorbance was measured at a wavelength of 550 nm using an iMark microplate absorbance reader. The concentration of NO was determined using a standard of sodium nitrite (3.13–100 μM).

To determine the NO production by MDMs, 100,000 cells were stimulated with pectins (2.5 mg/mL), PAM3CSK4 (100 ng/mL), and only medium for 24 h. The next day, 50 μL of supernatant was taken in a 96 well flat bottom plate and a Griess assay was performed as described above.

2.7. Expression of IL10, iNOS, and TNFα genes as measured by real-time qPCR

The gene expression of specific cytokines and immune-related genes in HD11 cells was measured using real-time qPCR (RT-qPCR). Briefly, in a 12-wells plate 5×10^5 cells per well were seeded and incubated at 37 °C, 5 % CO2 overnight. The next day, cells were stimulated with 2.5 mg/mL of pectins, PAM3CSK4 (20 ng/mL) as positive control, or medium as negative control for 24 h at 37 °C. Following stimulation, the supernatant was removed and HD11 cells were harvested using 0.05 % trypsin/EDTA. The cells were then centrifuged at 208 ×*g* for 5 min and the pellet was lysed in 350 μL RLT-buffer (Qiagen, the Netherlands) containing 1 % β-mercaptoethanol after centrifugation. RNA was extracted using RNeasy mini kit (Qiagen, 74,106) according to the manufacturer's description, and quantified on a Nanodrop spectrophotometer (Isogen, the Netherlands). Subsequently, 500 ng of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to manufacturer's guidelines. Finally, the 500 ng of cDNA was diluted 1:1 using ultrapure water.

RT-qPCR was performed on a CFX384 Connect qPCR system (Bio-Rad). The primer sequences of target genes and house-keeping gene are depicted in Table 1. For each reaction 12.5 μL SYBRgreen (Bio-Rad, Veenendaal, the Netherlands), 1 μL (400 nM) of forward primer, 1 μL (400 nM) of reverse primer and 5.5 μL ultrapure water along with 5 μL of cDNA sample was added per well. The reaction was performed using the following conditions: initial denaturation cycle of 5 min at 95 °C; followed by 40 cycles of 10 s at 92 °C, 10 s at 55 °C, and 30 s at 72 °C. This was followed by the last step of 1 min at 95 °C and 2 min at 65 °C. Target gene expression levels were normalized against the expression levels of the house keeping gene GAPDH.

Table 1
Primer sequences used for real time RT-qPCR.

Gene	Accession number		Sequence: 5′- 3′
IL10	AJ621614	Forward	CATGCTGCTGGGCCTGAA [57]
		Reverse	CGTCTCCTTGATCTGCTTGATG
iNOS	U46504	Forward	TGGGTGGAAGCCGAAATA [58]
		Reverse	GTACCAGCCGTTGAAAGGAC
TNFα	MF000729	Forward	CGCTCAGAACGACGTCAA [59]
		Reverse	GTCGTCCACACCAACGAG
GAPDH *(house-keeping gene)*	K01458	Forward	GTGGTGCTAAGCGTGTTATC [60]
		Reverse	GCATGGACAGTGGTCATAAG

2.8. PAM3CSK4 binding assessed by flow cytometry

To determine the binding of PAM3CSK4 to TLR2 on HD11 cells, FITC labelled PAM3CSK4 was used. Briefly, 2.5×10^4 HD11 cells were seeded in a 24 well plate, supernatant was removed the following day and cells were preincubated with pectins for 1 h at 37 °C and 5 % CO_2. Subsequently, the cells were treated with PAM3CSK4-FITC (500 ng/mL) for 30 min at 37 °C. After PAM3CSK4-FITC treatment, cells were washed with PBS and detached with 0.05 % trypsin/EDTA. Cells were centrifuged twice in PBS and then stained with viakrome808 (Beckman Coulter Life Sciences, Indianapolis, USA) for 30 min at RT. After that, the cells were centrifuged and resuspended in 200 uL of FACS buffer and analysed on a Cytoflex flow cytometer (Beckman Coulter, Woerden, the Netherlands). At least 100,000 events were recorded.

2.9. Phagocytosis assessed by flow cytometry

The phagocytotic capacity of HD11 cells after pectin stimulation was determined using a Fluorescence-activated Cell Sorting (FACS) based method. For that, 2.5×10^4 HD11 cells per well were seeded in a 24 well plate in complete RPMI-1640 medium. After overnight incubation, supernatant was removed and HD11 cells were stimulated with either pectins only (0.6–2.5 mg/mL) for 24 h, or 1 h pre-incubation with pectins followed by treatment with PAM3CSK4 (with the pectin still present) at 37 °C and 5 % CO_2 for 23 h. After the incubation, supernatants were removed and the HD11 cells were incubated with log-phase Green Fluorescent Protein-labelled (GFP) *Escherichia coli (source)* with a Multiplicity of Infection (MOI) of 50 for 1 h at 37 °C and 5 % CO_2. After 1 h of incubation supernatants were removed and the cells were washed three times with ice-cold PBS. The cells were washed and detached using 0.5 % trypsin/EDTA, and transferred to a 96 well U-bottom plate and centrifuged for 3 min at 200 x*g*. The supernatants were discarded, and the cells were then washed 2 times with 200 μL ice-cold PBS. After the last washing step, cells were stained with live/dead marker viakrome808 (1:400) in PBS for 30 min at room temperature in the dark. After live/dead staining, cells were washed twice with FACS buffer and resuspended in 200 μL of FACS buffer. The cell suspensions were analysed for bacterial uptake using the Cytoflex flow cytometer and at least 100,000 events were recorded.

2.10. Interaction between pectins and PAM3CSK4 assessed by isothermal titration calorimetry (ITC)

Interaction between pectins and PAM3CSK4 was studied using isothermal titration calorimetry (ITC). Binding between PAM3CSK4 and chicken Cathelicidin-2 (CATH-2) was used as positive control [61]. Briefly, CATH-2 was diluted to 400 μM, PAM3CSK4 was diluted to a concentration of 330 μM, and Pectins were diluted to a concentration of 25 μM in 50 % PBS. The syringe of the ITC was filled with 50 μL of PAM3CSK4 (330 μM) solution and the chamber was filled with 169 μL Pectin solution. For interaction between CATH-2 and PAM3CSK4, syringe was filled with CATH-2 (400 μM) and titrated against PAM3CSK4 (165 μM). After baseline was determined, 2 μL injections were performed at 300 s intervals. All experiments were executed on a NANO ITC (TA instruments – Waters LLC, New Castle, USA). The experiments were performed at 37 °C and the data was analysed using the Nano Analyze software (TA instruments – Waters LLC, New Castle, USA).

2.11. Statistical analysis

Statistical analysis of the results was performed using Graphpad Prism program version 9 (San Diego, CA, USA). The data are represented as mean ± SEM. Statistical significance was determined using one-way ANOVA with Dunnett's multiple comparisons test. The statistical significance of phagocytosis assay was determined by two-way ANOVA, while $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Structural characteristics of pectins

The pectins SPE6 and SPE7 are characterized as high methoxyl pectin (HMP), while SPE8 clearly represented a low methoxyl pectin (LMP) as based on galacturonic acid (GalA) content and DM (Table 2). In addition to the DM, also the distribution of methyl esters over the homogalacturonan backbone has been described with descriptive parameters [62] (Table 2). Both HMPs have a similar, rather random distribution of methyl esters over the pectin backbone. Despite the low DM, the LMP had about a similar degree of blockiness value as of DM. The molecular weight distribution of the three pectins are rather similar (Table 2).

3.2. Effect of Pectins on viability of HEKBlue TLR2 cells and HD11 cells

To exclude possible toxic effects of pectins on the cell lines used in the study, the viability of HEKBlue TLR2, and HD11 cells was determined using the Alamar blue viability assay upon culture with the different pectins. Alamar blue reagent is a resazurin based, ready to use solution that uses the reducing power of live cells to measure cell viability [63]. The concentrations of the pectins used in this study (0.6–2.5 mg/mL) did not affect the viability of HEKBlue TLR2 or HD11 cells as compared to unstimulated control as shown in Fig. 1A and B respectively. Treatment of HEKBlue TLR2, and HD11 cells with Triton X-100 resulted in a reduced cell viability.

3.3. SPE6 and SPE7 bind and activate hTLR2, while SPE8 activates both hTLR2 and hTLR4

To determine whether pectins are able to interact with TLRs, the human TLR reporter cell lines HEKBlue Null1, HEKBlue TLR2, and HEKBlue TLR4 were stimulated with the pectins SPE6, 7 and 8. Stimulation of HEKBlue Null1 cells for 24 h at 37 C did not release SEAP, as shown in Fig. 2A. In contrast, all three pectins stimulated the HEKBlue TLR2 to produce SEAP, while only SPE8 stimulation resulted in SEAP production in HEKBlue TLR4 cells (Fig. 2B-C). Stimulation of HEKBLUE TLR2 with the pectins resulted in similar SEAP levels as stimulation with the TLR2 agonist, PAM3CSK4. These experiments show that pectins SPE6,7 and 8 can indeed bind and crosslink human TLR2 and 4.

3.4. Effect of Pectins on NO production and cytokine gene expression in chicken HD11 cells

In the next set of experiments, chicken HD11 cells were stimulated with pectins SPE6, SPE7, and SPE8 (0.6–2.5 mg/mL) to determine whether pectins can activate HD11 cells. Stimulation with SPE6 and SPE7 did not affect NO levels, while stimulation with pectin SPE8 resulted in a moderate but significant increase in NO production compared to unstimulated control (Fig. 3A). Next, the effect of pectins on cytokine production by HD11 cells was determined at gene expression level, using *iNOS* and *TNFα* as representative proinflammatory genes, and *IL10* as a representative anti-inflammatory gene. Incubation of HD11 cells with either of the three pectins resulted in a significant upregulation of *iNOS* and *IL10* m RNA expression (4–16 fold) while there was no effect on *TNFα* gene expression levels (Fig. 3B-D).

3.5. SPE6 significantly reduced PAM3CSK4 induced NO production by HD11 cells

To determine the affinity of chicken TLR2 for the SPE6, SPE7, and SPE8 pectins and PAM3CSK4, HD11 cells were stimulated with the TLR2 ligand PAM3CSK4, and different pectins in three slightly different competition experimental setups. Stimulation of HD11 cells with PAM3CSK4 alone resulted in a significant increase in NO production

Table 2
Descriptive parameters of pectins.

Pectins	GalA (w/w%)[a]	M_w (kDa)[b]	DM (%)[c]	DB (%)[d]	DB_{abs} (%)[e]	DB_{PGme} (%)[f]	DB_{PLme}(%)[g]
SPE6 (HMP)	68.2	131	63	22	8	22	31
SPE7 (HMP)	72	135	63	20	7	22	35
SPE8 (LMP)	76.9	115	26	25	19	45	0.6

[a] Determined by the automated colorimetric *m*-hydroxydiphenyl assay.
[b] Molecular weight distribution (Mw) in kDa determined by HPSEC based on the pectin standards.
[c] Degree of methyl-esterification (DM): mol of methanol per 100 mol of the total GalA in the sample. Pectin presenting DM lower than 50 % is considered low methyl-esterified and DM above 50 % is considered highly methyl-esterified.
[d] Degree of blockiness: amount of non-esterified mono-, di-, and triGalA per 100 mol of the non-esterified GalA in the sample.
[e] Absolute degree of blockiness: amount of non-esterified mono-, di-, and tri-GalA per 100 mol of total GalA in the sample.
[f] Degree of blockiness by *endo*-PG (DB_{PGme}): amount of saturated methyl-esterified galacturonic residues per 100 mol of total galacturonic acid in the sample.
[g] Degree of blockiness by PL (DB_{PLme}): amount of unsaturated methyl-esterified galacturonic oligomers per 100 mol of total galacturonic acid in the sample.

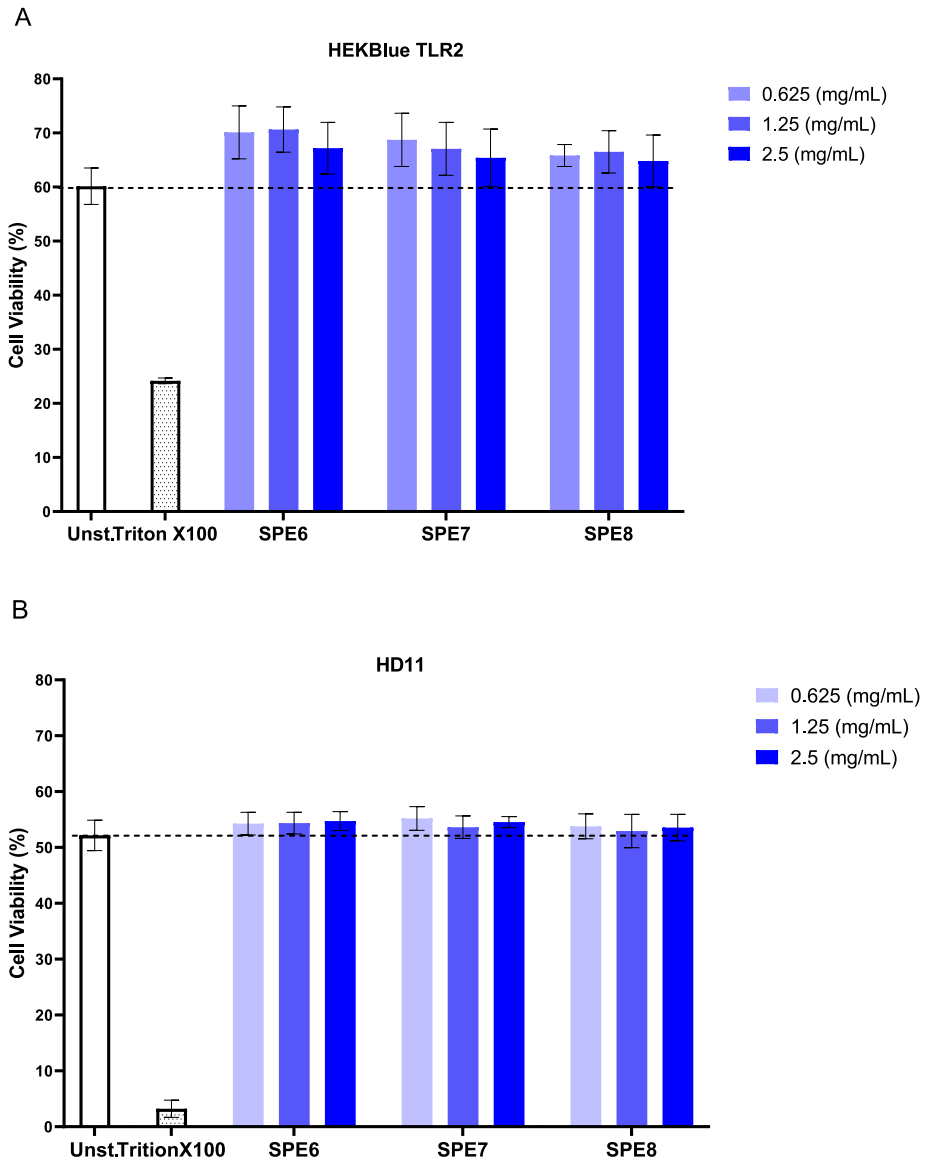


Fig. 1. Pectins are not toxic to HEKBlue TLR2 and HD11 cells. A) Cell viability of HEKBlue TLR2 cells and HD11 cells (B) after 24 h stimulation with pectins (SPE6, SPE7, SPE8) from 0.625 to 2.5 mg/mL. Unstimulated control cells are used as negative control while 1 % solution of Triton X100 was used as positive control. Data are represented as means ± SEM of 3 independent experiments done in duplicate.

(±70 μM) compared to unstimulated control as shown in Fig. 4A. When both PAM3CSK4 and pectin were added together (co-incubation) no significant reduction in the NO production by HD11 cells (Fig. 4A) was observed compared to PAM3CSK4. However, for all pectins, a tendency towards lower PAM3CSK4-induced NO production at higher pectin concentrations was present. In the second setup (1 h pretreatment), HD11 cells were pre-treated with pectins for 1 h, after which the pectins were washed away and the HD11 cells were subsequently incubated

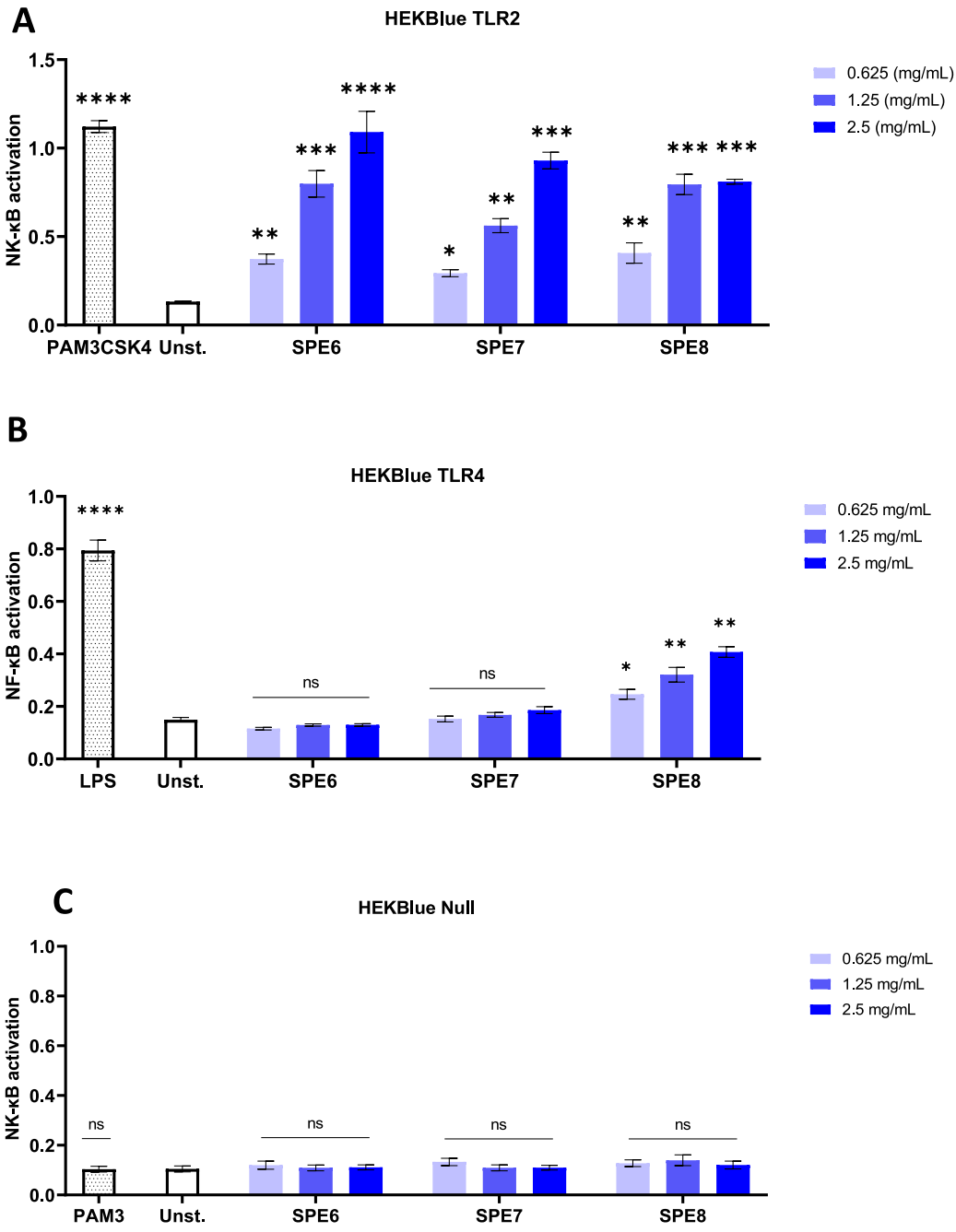


Fig. 2. Pectins stimulate human TLR2 and TLR4. A) Activation of HEKBlue TLR2 cells after 24 h stimulation with pectins (SPE6, SPE7, SPE8) (0.6–2.5 mg/mL), PAM3CSK4 (10 ng/mL), and with medium only. B) Activation of HEKBlue TLR4 cells after 24 h stimulation with pectins (0.6–2.5 mg/mL), LPS (10 ng/mL), and with medium only. C) NF-κB activation of HEKBlue Null cells after 24 h stimulation with pectins (0.6–2.5 mg/mL), PAM3CSK4 (10 ng/mL), and medium only. Data are means ± SEM of 3 independent experiments done in duplicate. Significance compared to the unstimulated control was declared when $P < 0.05$ (*).

with PAM3CSK4 for the next 23 h. This also did not result in a significant reduction in NO production (Fig. 4B). In the final set-up (1 h pre-incubation), HD11 cells were preincubated with pectins for 1 h followed by treatment with PAM3CSK4 in the presence of pectins for the following 23 h. In this setup, co-incubation of SPE6 and PAM3CSK4 significantly reduced NO production by HD11 cells compared to PAM3CSK4 only (Fig. 4C). Co-incubation of PAM3CSK4 with SPE7 and SPE8 showed a tendency to reduce NO production, but this did not reach statistical significance.

3.6. Pectins differentially reduce the attachment of FITC labelled PAM3CSK4 to HD11 cells

The binding of PAM3CSK4 to TLR2 on HD11 cells and the effect of pectins on this process was assessed by flow cytometry. The representative gating strategy to determine the binding of FITC conjugated PAM3CSK4 to HD11 cells is shown in Fig. 5A. HD11 cells preincubated with SPE6, SPE7 and SPE8 for 1 h followed by 30 min treatment with PAM3CSK4-FITC (in the presence of the pectins) showed a significantly reduced attachment of PAM3CSK4-FITC to the cells for all three pectins compared to unstimulated HD11 cells (Fig. 5B). Among different pectins, SPE6 showed the strongest effect compared to the other two

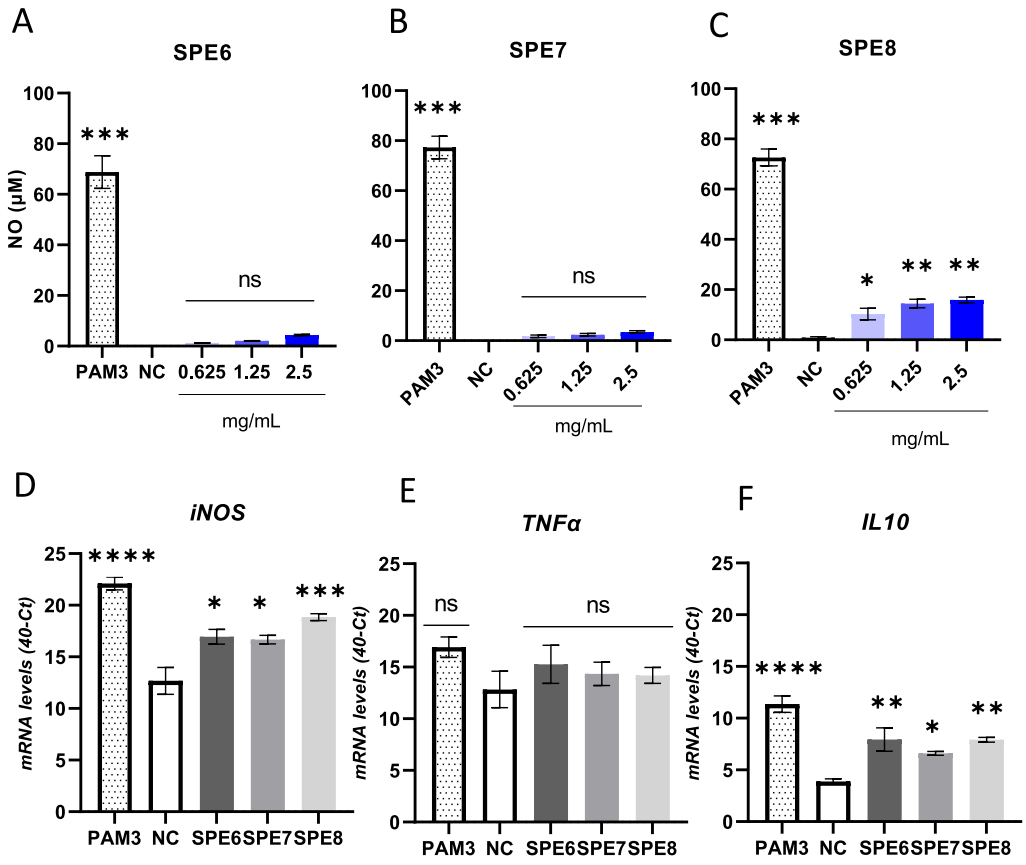


Fig. 3. NO production and changes in gene expression of *IL10*, *iNOS*, and *TNFα* by HD11 cells upon stimulation with pectin SPE6, SPE7 and SPE8. A-C) NO production by HD11 cells after stimulation with SPE6, SPE7, SPE8, PAM3CSK4 (20 ng/mL), and unstimulated control for 24 h. D—F) *iNOS*, *TNFα,* and *IL10* mRNA expression in HD11 cells after stimulation with SPE6, SPE7, SPE8 (2.5 mg/mL), PAM3CSK4 (20 ng/mL), and unstimulated control for 24 h. Data are means ± SEM of 3 independent experiments each was done in duplicate. Significance compared to the unstimulated control was declared when $P < 0.05$ (*).

pectins.

3.7. Pectin SPE8 binds to PAM3CSK4, while SPE6 and SPE7 do not

The interaction between different pectins and PAM3CSK4 was determined by isothermal titration calorimetry. SPE8 showed binding to PAM3CSK4 with a dissociation constant (Kd) of 7.72 μM (Fig. 6A) comparable to PAM3CSK4 to chicken Cathelicidin-2 (Kd = 1.83 μM) for which binding to PAM3CSK4 has been described before [61] (Fig. 6D). A negative enthalpy was observed upon binding of SPE8 to PAM3CSK4, indicating a mostly electrostatic interaction between both components. No binding to PAM3CSK4 was observed for the pectins SPE6 and SPE7 (Fig. 6B-C).

3.8. SPE8 increases the phagocytosis of GFP-E. coli by HD11 cells and decreases the PAM3CSK4 induced phagocytosis of GFP-E. coli

The uptake of *GFP-E. coli* by HD11 cells upon pectin stimulation was assessed by flow cytometry. The gating strategy for uptake of *GFP-E. coli* is shown in Fig. 7A. Stimulation of HD11 cells by SPE8 showed a significant increase in phagocytosis of *E. coli* from 20 to 30 % positive cells compared to the unstimulated control. Stimulation with PAM3CSK4 increased phagocytosis to almost 45 % GFP positive cells. SPE6 and SPE7 did not show any difference compared to the unstimulated control (Fig. 7B). In the pre-incubation setup, all three pectins actually showed significant reduction in PAM3CSK4 induced phagocytosis of *E. coli* (Fig. 7C).

3.9. SPE6 significantly reduces NO production by MDMs

In order to confirm that the observed effects of pectins were not limited to HD11 cells, stimulations with pectins and PAM3CSK4 were performed with MDMs. When MDMs were stimulated with pectin SPE8 an increase in NO production was observed, while no increase in NO production was seen with SPE6 and SPE7, compared to the unstimulated control (Fig. 8A). Moreover, 1 h pre-incubation of MDMs with pectins followed by 23 h stimulation with PAM3CSK4 (100 ng/mL) in the presence of pectins, resulted in a significant reduction in NO production for SPE6. In the case of SPE7 and SPE8, the NO production was reduced but this decrease did not reach significance as compared to PAM3CSK4 stimulated MDMs (Fig. 8B).

4. Discussion

Pectins are well known for prebiotic properties and, more recently, also for their immunomodulatory activity, as they can escape enzymatic digestion in the small intestine and reach to the colon in chicken gut. In the colon pectins are degraded by the gut microbiota and used as energy source to produce short chain fatty acids [64]. During the retention time in the colon pectins can directly interact with intestinal epithelial as well as resident immune cells in the intestinal epithelium and lamina propria as they can pass through the gut barrier [28]. Therefore, it is important to determine the direct effects of pectins or other dietary fibers with immune cells, like macrophages, that are well represented among the phagocytes present in the lamina propria of the chicken gut associated lymphoid tissue.

In this study the immunomodulatory properties of pectins on chicken

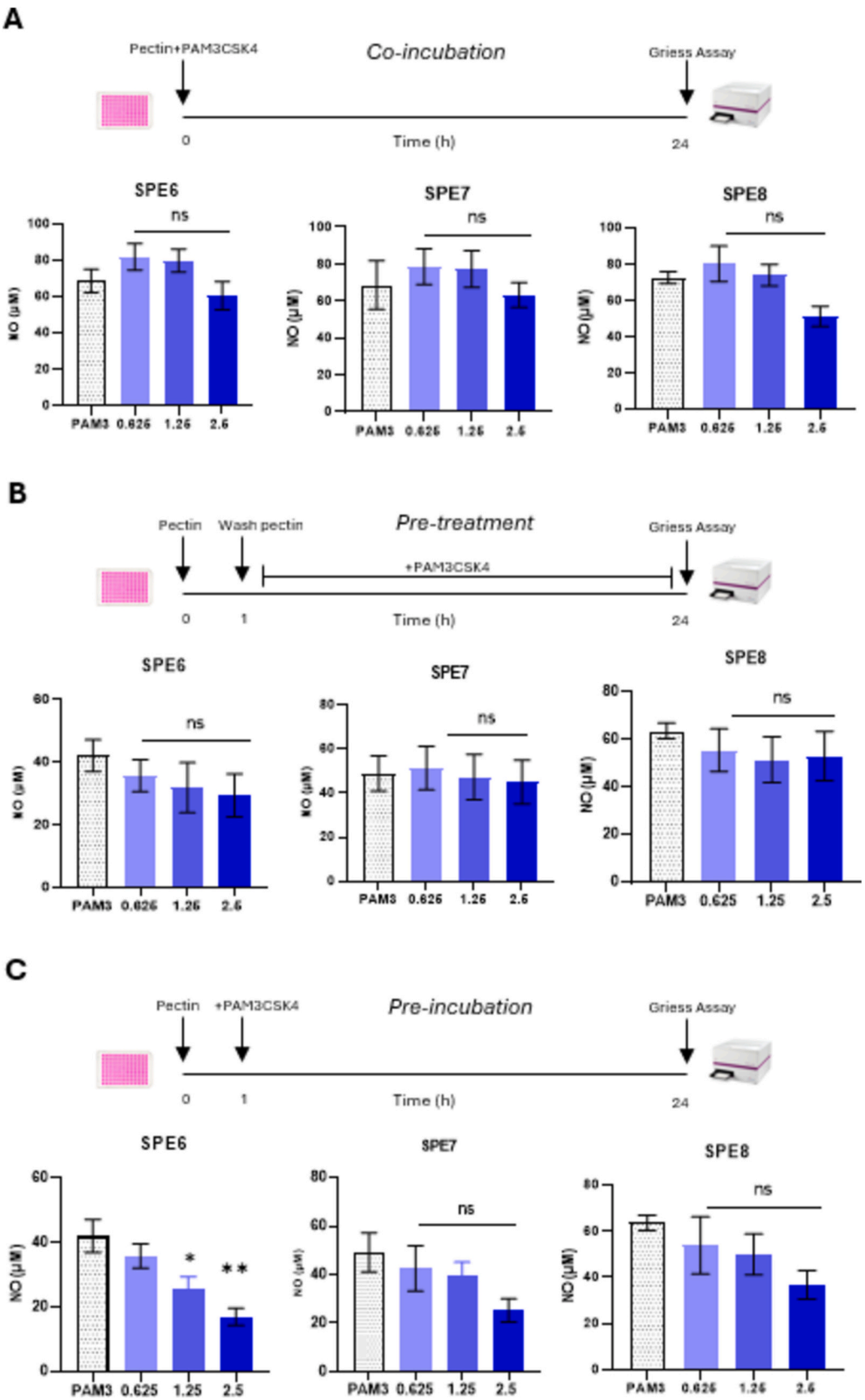


Fig. 4. Nitric oxide (NO) production by HD11 macrophages in three experimental setups after stimulation with pectins. A) NO production by HD11 macrophages after stimulation with SPE6, SPE7, SPE8 and PAM3CSK4 (20 ng/mL) in co-incubation setup for 24 h. B) NO production by HD11 macrophages after 1 h pre-treatment with pectins SPE6, SPE7, SPE8 and then 23 h stimulation with PAM3CSK4 (20 ng/mL) in the absence of pectins. C) NO production by HD11 macrophages after 1 h pre-incubation with pectins SPE6, SPE7, SPE8 and then 23 h stimulation with PAM3CSK4 (20 ng/mL) in the presence of pectins. Data are means ± SEM of 3 independent experiments done in duplicate. Significance compared to PAM3CSK4 only was declared when $P < 0.05$ (*).

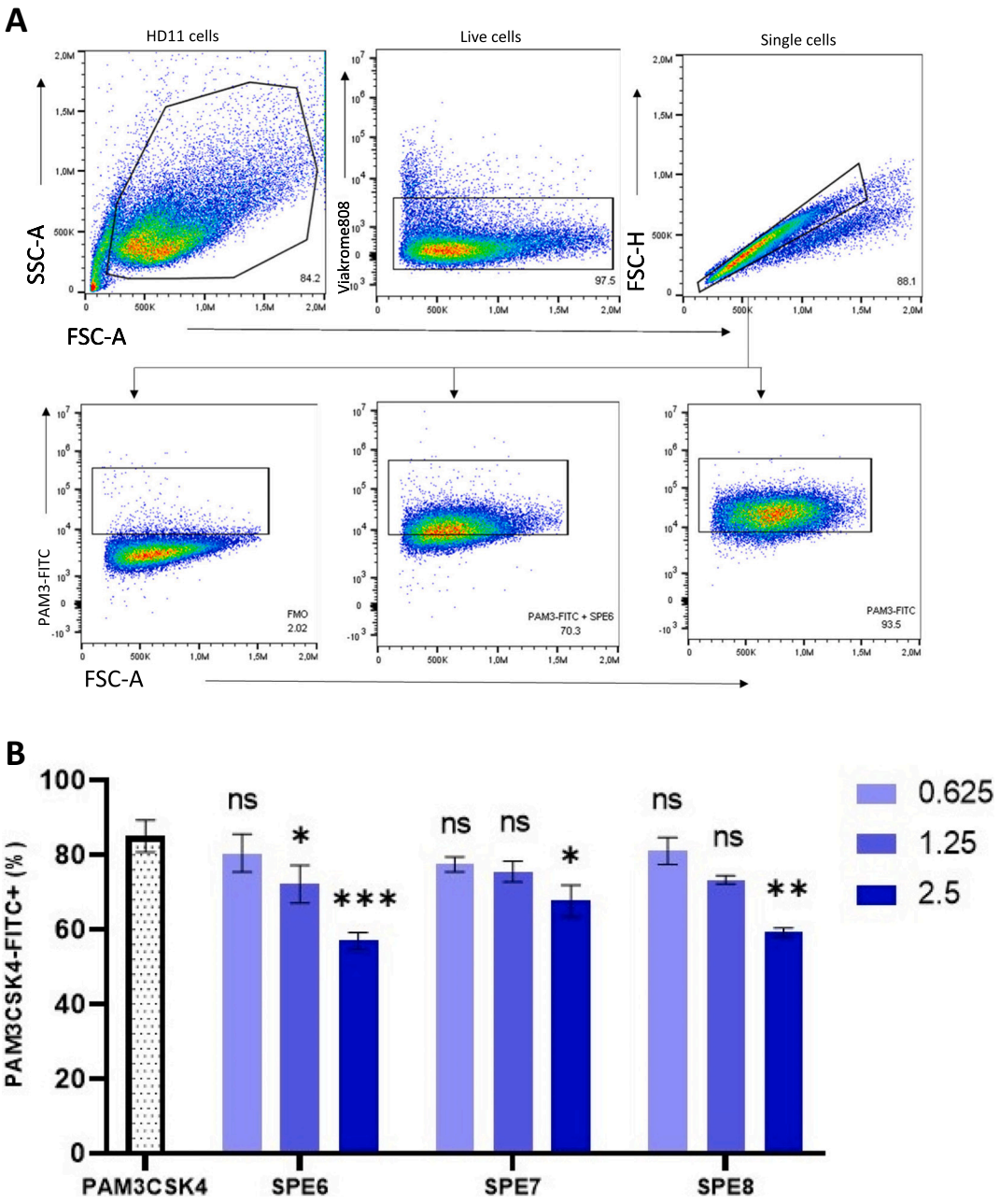


Fig. 5. Binding of FITC-conjugated PAM3CSK4 to HD11 cells determined by flow cytometry. A) Representative gating strategy to determine binding of PAM3CSK4-FITC to HD11 cells after 1 h pre-incubation with and without pectin SPE6 (2.5 mg/mL). B) Graph shows the binding of FITC conjugated PAM3CSK4 (500 ng/mL) to HD11 cells after 1 h pre-incubation with SPE6, SPE7, SPE8 (0.6–2.5 mg/mL). Data are means ± SEM of 3 independent experiments done in duplicate. Significance was declared when $P < 0.05$ (*).

monocyte derived macrophages, and HD11 cells, a macrophage cell line, are reported. The main findings are that the studied pectins can indeed interact with chicken TLRs and depending on the specific pectin can activate chicken TLR4 or interact with TLR2, not leading to activation of the TLR but competitively inhibiting the binding of activating ligands. The pectins used in this study may be nominated as feed additives in poultry. The chemical composition of pectins is described in a recent study which showed differences in degree of methylation (DM), monosaccharide composition, and molecular weight among these pectins (Oost et al., manuscript in preparation). Pectin SPE6 and SPE7 are regarded as high DM pectins (DM 63) and have high molecular weights (around 135 kDa) compared to SPE8 which is low DM pectin (DM 26) and also has a lower molecular weight (115 kDa). It has also been described that these pectins differentially affected short chain fatty acid production and microbiota composition in the *in vitro* chicken gut model CALIMERO-2 (Oost et al., manuscript in preparation). However, how and if these pectins can affect chicken immune cells is still unknown.

TLRs are the most defined Pattern recognition receptors (PRR) and multiple studies have described TLR dependent interaction of immune cells with pectins in mammals [43,65,66]. Therefore, to investigate potential interaction of pectins with chicken TLRs, initially a human TLR reporter cell line, HEKBlue, is used as *in vitro* tool to determine if the pectins has affinity for (human) TLR2 and 4. Pectin SPE6 and SPE7 stimulated cells *via* TLR2 while having no effect on TLR4, whereas SPE8 activated both TLR2 and TLR4. Previous studies have shown activation of immune cells *via* TLR2 and TLR4 in humans and mice by pectins derived from different sources [46,65,67]. None of the pectins activated the HEKBlue Null1 cells, which rules out the possibility of a TLR-

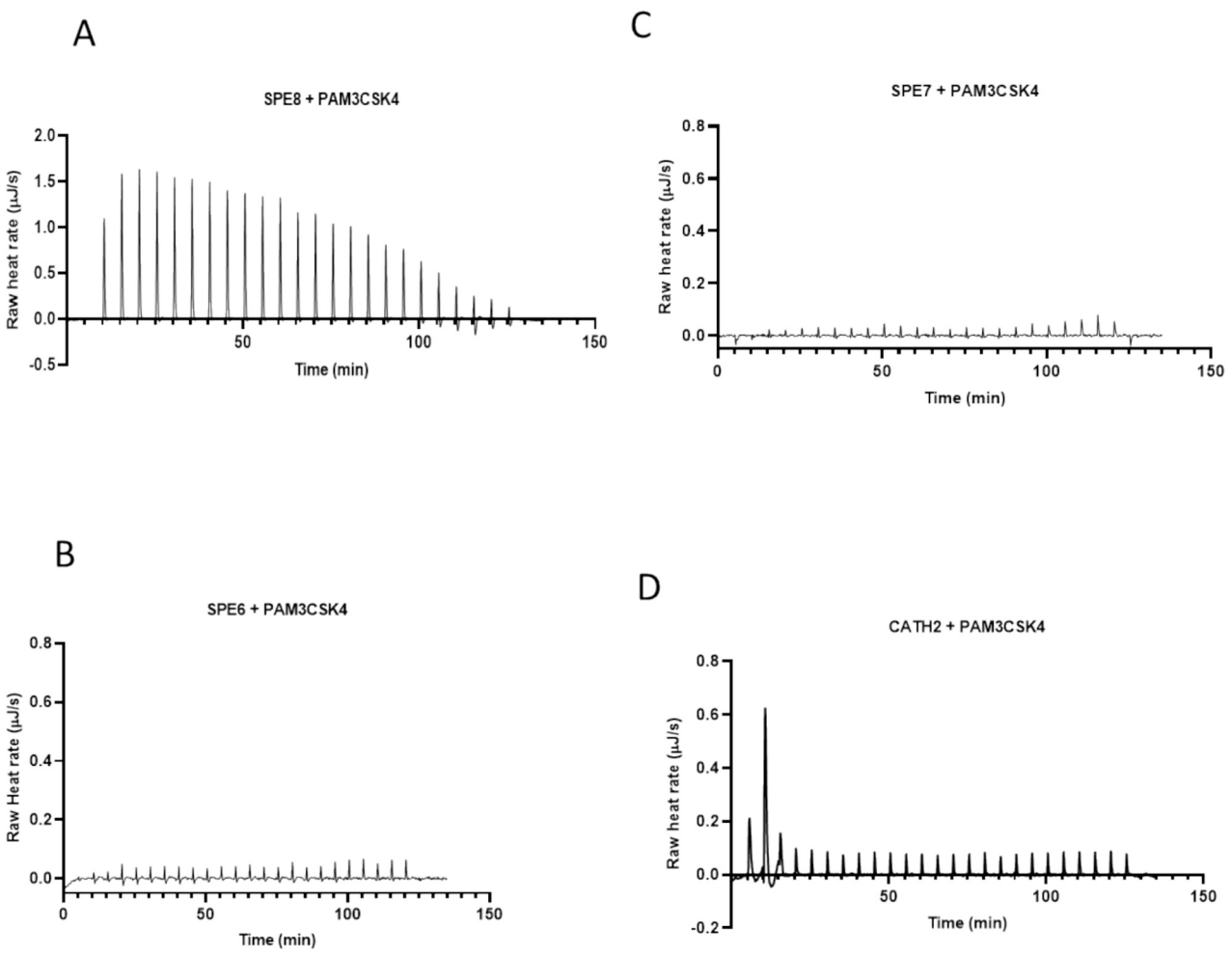


Fig. 6. Interaction between SPE6, SPE7, SPE8, and PAM3CSK4 determined by isothermal titration calorimetry (ITC). Heat production upon interaction between SPE8 (A), SPE6 (B), SPE7 (C), CATH-2 (D) and PAM3CSK4; Graphs are representative of 3 independent experiments for each compound.

independent process of activation of these human cells *via* TLRs. The difference in the degree of activation of TLR2 by pectins or activating a different TLR (TLR2 or TLR4) may be due to variation in chemical composition, for example the degree of methylation (DM) but our set of pectins is too small to determine proper structure activity correlations and other factors than DM cannot be ruled out. However, since SPE8 is a low DM pectin, this might be the reason that TLR4 might be involved behind activation. A previous study has described the activation of TLR4 by low DM pectins [46].

After the initial activity observations using human TLRs, the effect of pectin on chicken macrophages was determined. Stimulation of chicken derived HD11 cells with the three pectins significantly upregulated *IL10* and *iNOS* gene expression, while having no effect on *TNFα* gene expression. The effect of the pectins on the *IL10* and *iNOS* was comparable to PAM3CSK4, a TLR2 agonist. This observation is in agreement with a previous study which has described TLR2-induced upregulation of *IL10* by human THP1 macrophages after stimulation with pectins *in vitro* [42]. Interestingly, activation of HD11 cells after interaction with pectins, leading to increased *iNOS* gene expression did not result into actual nitric oxide production except for SPE8. It is unclear whether the difference in NO production (positive for PAM3CSK4 and SPE8, none for SPE6 and SPE7) is related to the extent of upregulation of the *iNOS* gene, which was highest for SPE8, (Fig. 3B) or whether SPE6 and SPE7 somehow block the gene translation pathway of *iNOS*, by interfering with one of the signaling molecules in the pathway, a phenomenon which has been described in earlier studies for guluronate oligosaccharides [68], but this is obviously highly speculative. A similar outcome is observed in term of NO production when chicken MDMs were stimulated with pectins that only SPE8 stimulated MDMs produced NO.

We also determined the phagocytic activity of chicken MDMs after stimulation with pectins. SPE6 and SPE7 did not increase phagocytosis of GFP labelled *E. coli* compared to the unstimulated control. In contrast, pectin SPE8 increased phagocytosis of *E. coli*, but to a lower extent than PAM3CSK4. This effect of SPE8 is likely explained by the fact that it can stimulate TLR4 (as shown in Fig. 2) which is known to increase phagocytosis. The effect of pectins on phagocytosis has been studied before with mixed outcomes depending on the pectin and macrophages used [50,69–71], hence no consensus on the effect of pectins on levels of phagocytosis. Most likely the varying chemical and structural characteristics of pectins from different sources play a big role in phagocytic capacity.

To further investigate whether that the pectins used in this study may be ligands for chicken TLR2, competition studies with PAM3CSK4 were performed. We hypothesized that since the pectins seem to have lower affinity than PAM3CSK4 to activate cells *via* TLR2, competition between pectins and PAM3CSK4 should result in a lower activation than PAM3CSK4 alone. Our observation in the pre-incubation experiments that resulted in lower NO production of HD11 cells and MDMs, and reduced phagocytic capacity of HD11 cells confirms the hypothesis. Pre-exposure of the macrophages to the pectins resulted in less PAM3CSK4 induced activation of HD11 cells, most likely due to decreased binding of PAM3CSK4 to TLR2. The hypothesis is confirmed by the flow cytometry analysis that showed that less binding of fluorescently

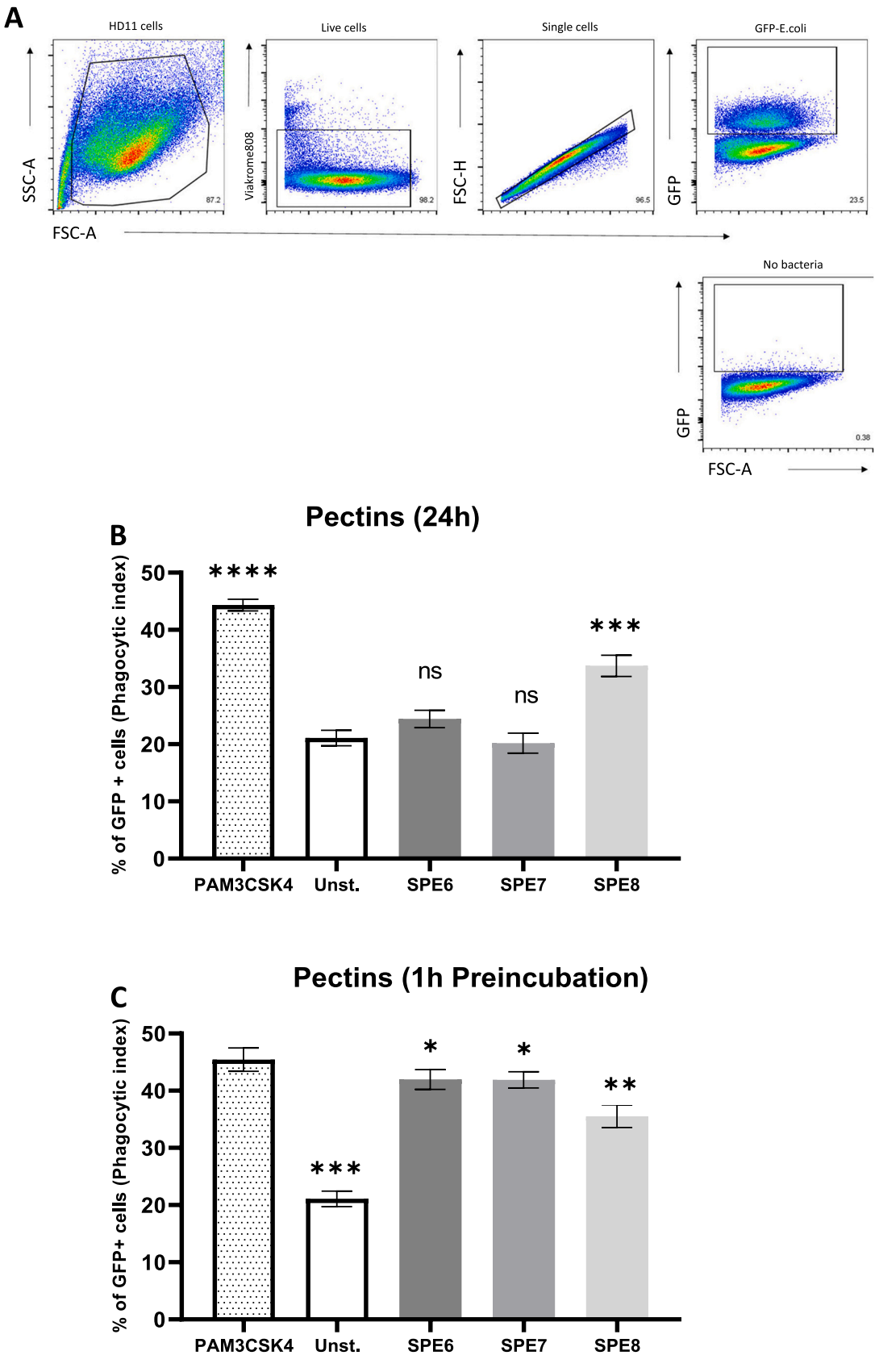


Fig. 7. Phagocytosis of GFP labelled *E. coli* by HD11 cells. A) Representative gating strategy to determine uptake of GFP-*E. coli* by HD11 cells. B) Phagocytosis of GFP labelled *E. coli* by HD11 cells after 24 h stimulation with SPE6, SPE7, SPE8 (2.5 mg/mL). C) Phagocytosis of GFP labelled *E. coli* by HD11 cells after 1 h pre-incubation with SPE6, SPE7, SPE8 (2.5 mg/mL) and then 23 h co-incubation PAM3CSK4 (20 ng/mL) for 23 h. Data are means ± SEM of 3 independent experiments done in duplicate. Significance was declared when $P < 0.05$ (*).

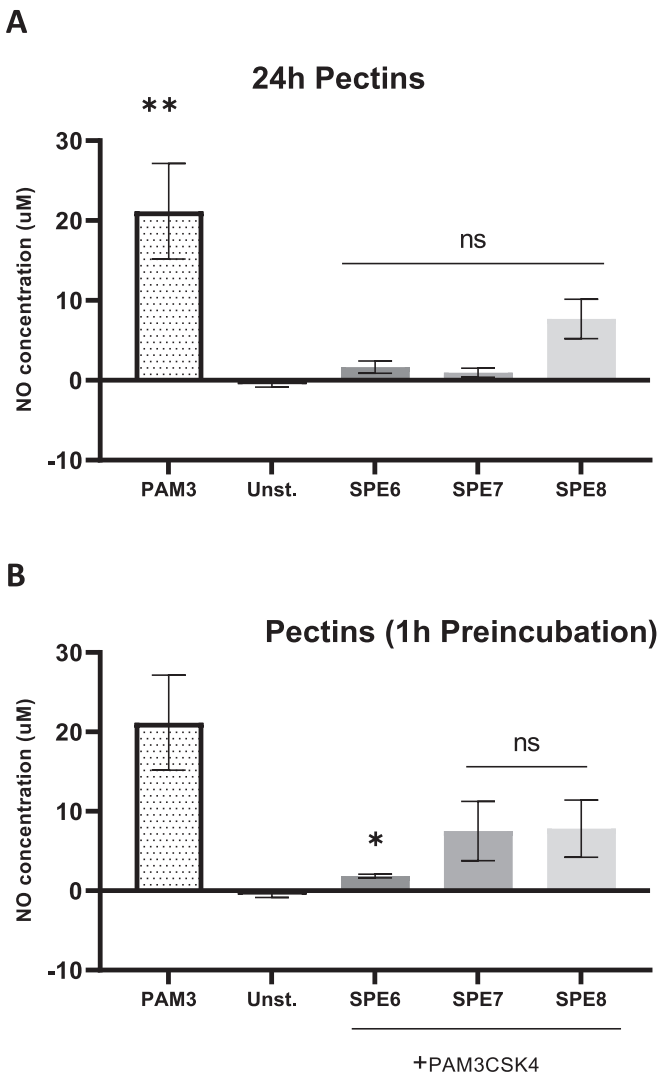


Fig. 8. NO production by MDMs. A) NO production by MDMs upon stimulation with pectins, and PAM3CSK4 (100 ng/mL) for 24 h. B) NO production by MDMs upon 1 h pre-incubation with pectins followed by 23 h treatment with PAM3CSK4 (100 ng/mL) in the presence of pectins. Data are means ± SEM of 3 independent experiments. Significance was declared when $P < 0.05$ (*).

labelled PAM3CSK4 to HD11 cells after pre-incubation with pectins.

An alternative explanation for these observations could be that the pectins are not competing for TLR2 binding but actually bind PAM3CSK4 itself and thereby partially inhibit PAM3CSK4 binding to TLR2. The ITC results showed that SPE8 indeed binds to PAM3CSK4 and that this may contribute to the described effects on PAM3SCK4 activation of HD11 cells, but it is difficult to predict exactly how much this direct binding contributes to inhibition of PAM3CSK4 induced activation. More importantly, no binding to PAM3CSK4 was observed for SPE6 and SPE7 suggesting that for these HM pectins binding to TLR2 can be a way by which these pectins reduced PAM3CSK4 activation of HD11 macrophages. The difference in the binding to PAM3CSK4 between LMP SPE8 and HMP SPE6/SPE7 was striking and very likely related to the difference in structure between these pectins. In line with this explanation, previous literature has shown that (low DM) lemon pectins blocked TLR2 on HEK-Blue TLR2 cells [72,73]. The same was described for an orange pectin with high DM and degree of blockiness (DB) which inhibited TLR2/1. Although it is difficult to directly compare the effect of different pectins used in different experimental setups, it is interesting to note that despite the differences in TLR2 structure and dimerization characteristics between chicken TLR2 and mammalian TLR2 [74,75], the general concept of pectin mediated activation may be comparable.

5. Conclusions

The current study demonstrates that the studied pectins have direct effects on chicken macrophages and we hypothesize that this may occur *via* their interaction with TLRs in a pectin structure-dependent manner. Specifically, we hypothesize that SPE8 binds and activates TLR4, while all three pectins, to some extent, can interact (but not activate) TLR2, thereby hindering activation of TLR2 by other ligands. The direct immunomodulatory influence is a significant factor for feed additives like pectins, as they may come into contact with immune cells in the intestinal tract. It is of high importance to determine these effects in the target animal for which the pectins are intended to be used, in this case chicken, since immune receptors and immune regulation can differ considerably between species. It has been described that pectins may pass the intestinal barrier to directly interact with dendritic cells in humans and mice [28,76,77], indicative that this could happen in chicken as well. Although this study did not set up to determine a correlation between structural characteristics of pectins and their interaction with macrophages, the differences in functional immunological properties of SPE6/SPE7 and SPE8 can likely be contributed to differences in their methyl ester level and distribution. For a more fundamental understanding of how these pectins interact with TLRs a systematic approach with many more different pectins could be evaluated. In addition, further *in vivo* studies are required to determine more clearly how *in vitro* results correlate to immunomodulatory properties of pectins in disease models in chickens.

CRediT authorship contribution statement

Adil Ijaz: Writing – original draft, Investigation, Conceptualization. **Noah Pols:** Investigation, Formal analysis. **Kahlile Youssef Abboud:** Investigation, Formal analysis. **Victor P.M.G. Rutten:** Writing – review & editing, Supervision. **Femke Broere:** Supervision, Project administration, Funding acquisition. **Henk Schols:** Writing – review & editing. **Edwin J.A. Veldhuizen:** Writing – review & editing, Supervision, Conceptualization. **Christine A. Jansen:** Writing – review & editing, Supervision, Conceptualization.

Funding

This research was funded by public-private partnership "carbobiotics" project number LWCC.2017.010. Carbobiotics was financed by participating industrial partners Cooperative Avebe U.A, Friesland Campina Nederland B.V., Nutrition Sciences N.V., and allowances of the Dutch Research Council (NWO). Adil Ijaz is the recipient of an international PhD fellowship from the Punjab Educational Endowment Fund, Punjab, Pakistan.

Declaration of competing interest

The authors declare no competing interest.

Acknowledgements

The authors would like to thank Prof. Koen Venema for the help in the characterization of the pectins. We thank the Flow Cytometry and Cell Sorting Facility of The Faculty of Veterinary Medicine at Utrecht University for support.

Data availability

Data will be made available on request.

References

[1] H.D. Hedman, K.A. Vasco, L. Zhang, A review of antimicrobial resistance in poultry farming within low-resource settings, Animals 10 (8) (2020) 1264.
[2] L. Pohjola, S. Nykäsenoja, R. Kivistö, T. Soveri, A. Huovilainen, M. Hänninen, M. Fredriksson-Ahomaa, Zoonotic public health hazards in backyard chickens, Zoonoses Public Health 63 (5) (2016) 420–430.
[3] Anon., Commission Regulation (EC) No 1177/2006 of 1 August 2006 Implementing regulation (EC) no 2160/2003 of the European Parliament and of the council as regards requirements for the use of specific control methods in the framework of the national programmes for the control of salmonella in poultry, Off. J. Eur. Union 212 (2006) 3–5.
[4] E.E. team, Reducing salmonella in European egg-laying hens: EU targets now set, Weekly Releases (1997–2007) 11(32) (2006) 3021.
[5] G. Suresh, R.K. Das, S. Kaur Brar, T. Rouissi, A. Avalos Ramirez, Y. Chorfi, S. Godbout, Alternatives to antibiotics in poultry feed: molecular perspectives, Crit. Rev. Microbiol. 44 (3) (2018) 318–335.
[6] Y. Yin, F. Lei, L. Zhu, S. Li, Z. Wu, R. Zhang, G.F. Gao, B. Zhu, X. Wang, Exposure of different bacterial inocula to newborn chicken affects gut microbiota development and ileum gene expression, ISME J. 4 (3) (2010) 367–376.
[7] S. Kim, R. Jha, Nutritional Intervention for the Intestinal, (2021).
[8] A.L. Smith, C. Powers, R. Beal, The Avian Enteric Immune System in Health and Disease, Avian immunology, Elsevier2022, pp. 303–326.
[9] A. Ijaz, E.J. Veldhuizen, F. Broere, V.P. Rutten, C.A. Jansen, The interplay between Salmonella and intestinal innate immune cells in chickens, Pathogens 10 (11) (2021) 1512.
[10] M.P. Ariaans, M.G. Matthijs, D. van Haarlem, P. van de Haar, J.H. van Eck, E. J. Hensen, L. Vervelde, The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after infectious bronchitis virus infection, Vet. Immunol. Immunopathol. 123 (3–4) (2008) 240–250.
[11] R.H. Van den Biggelaar, W. Van Eden, V.P. Rutten, C.A. Jansen, Nitric oxide production and fc receptor-mediated phagocytosis as functional readouts of macrophage activity upon stimulation with inactivated poultry vaccines in vitro, Vaccines 8 (2) (2020) 332.
[12] F.O. Martinez, A. Sica, A. Mantovani, M. Locati, Macrophage activation and polarization, Frontiers in Bioscience-Landmark 13 (2) (2008) 453–461.
[13] S. Gordon, Pattern recognition receptors: doubling up for the innate immune response, Cell 111 (7) (2002) 927–930.
[14] T. Kawasaki, T. Kawai, Toll-like receptor signaling pathways, Front. Immunol. 5 (2014) 461.
[15] M.A. Armant, M.J. Fenton, Toll-like receptors: a family of pattern-recognition receptors in mammals, Genome Biol. 3 (2002) 1–6.
[16] M.H. Kogut, M. Iqbal, H. He, V. Philbin, P. Kaiser, A. Smith, Expression and function of toll-like receptors in chicken heterophils, Dev. Comp. Immunol. 29 (9) (2005) 791–807.
[17] A. Fukui, N. Inoue, M. Matsumoto, M. Nomura, K. Yamada, Y. Matsuda, K. Toyoshima, T. Seya, Molecular cloning and functional characterization of chicken toll-like receptors: a single chicken toll covers multiple molecular patterns, J. Biol. Chem. 276 (50) (2001) 47143–47149.
[18] D.J. Lynn, A.T. Lloyd, C. O'Farrelly, In silico identification of components of the toll-like receptor (TLR) signaling pathway in clustered chicken expressed sequence tags (ESTs), Vet. Immunol. Immunopathol. 93 (3–4) (2003) 177–184.
[19] M. Iqbal, V.J. Philbin, A.L. Smith, Expression patterns of chicken toll-like receptor mRNA in tissues, immune cell subsets and cell lines, Vet. Immunol. Immunopathol. 104 (1–2) (2005) 117–127.
[20] R. Higgs, P. Cormican, S. Cahalane, B. Allan, A.T. Lloyd, K. Meade, T. James, D. J. Lynn, L.A. Babiuk, C. O'farrelly, Induction of a novel chicken toll-like receptor following Salmonella enterica serovar typhimurium infection, Infect. Immun. 74 (3) (2006) 1692–1698.
[21] M.S.-u. Rehman, S.U. Rehman, W. Yousaf, F.-u. Hassan, W. Ahmad, Q. Liu, H. Pan, The potential of toll-like receptors to modulate avian immune system: exploring the effects of genetic variants and phytonutrients, Front. Genet. 12 (2021) 671235.
[22] Y. Lu, A.J. Sarson, J. Gong, H. Zhou, W. Zhu, Z. Kang, H. Yu, S. Sharif, Y. Han, Expression profiles of genes in toll-like receptor-mediated signaling of broilers infected with Clostridium perfringens, Clin. Vaccine Immunol. 16 (11) (2009) 1639–1647.
[23] A.M. Keestra, M.R. de Zoete, L.I. Bouwman, M.M. Vaezirad, J.P. van Putten, Unique features of chicken toll-like receptors, Dev. Comp. Immunol. 41 (3) (2013) 316–323.
[24] Y. Cai, J. Folkerts, G. Folkerts, M. Maurer, S. Braber, Microbiota-dependent and-independent effects of dietary fibre on human health, Br. J. Pharmacol. 177 (6) (2020) 1363–1381.
[25] Z. Ao, A. Kocher, M. Choct, Effects of dietary additives and early feeding on performance, gut development and immune status of broiler chickens challenged with Clostridium perfringens, Asian Australas. J. Anim. Sci. 25 (4) (2012) 541.
[26] F. Rostami, H.A. Ghasemi, K. Taherpour, Effect of Scrophularia striata and Ferulago angulata, as alternatives to virginiamycin, on growth performance, intestinal microbial population, immune response, and blood constituents of broiler chickens, Poult. Sci. 94 (9) (2015) 2202–2209.
[27] K. Taha-Abdelaziz, D.C. Hodgins, A. Lammers, T.N. Alkie, S. Sharif, Effects of early feeding and dietary interventions on development of lymphoid organs and immune competence in neonatal chickens: a review, Vet. Immunol. Immunopathol. 201 (2018) 1–11.
[28] T. Eiwegger, B. Stahl, P. Haidl, J. Schmitt, G. Boehm, E. Dehlink, R. Urbanek, Z. Szépfalusi, Prebiotic oligosaccharides: in vitro evidence for gastrointestinal epithelial transfer and immunomodulatory properties, Pediatr. Allergy Immunol. 21 (8) (2010) 1179–1188.
[29] M. Beukema, M.M. Faas, P. de Vos, The effects of different dietary fiber pectin structures on the gastrointestinal immune barrier: impact via gut microbiota and direct effects on immune cells, Exp. Mol. Med. 52 (9) (2020) 1364–1376.
[30] R. Jha, P. Mishra, Dietary fiber in poultry nutrition and their effects on nutrient utilization, performance, gut health, and on the environment: a review, J. Anim. Sci. Biotechnol. 12 (2021) 1–16.
[31] M. Alizadeh, A. Rogiewicz, E. McMillan, J. Rodriguez-Lecompte, R. Patterson, B. Slominski, Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance and local innate immune response of broiler chickens challenged with Clostridium perfringens, Avian Pathol. 45 (3) (2016) 334–345.
[32] R. Shashidhara, G. Devegowda, Effect of dietary mannan oligosaccharide on broiler breeder production traits and immunity, Poult. Sci. 82 (8) (2003) 1319–1325.
[33] B. Baurhoo, P. Ferket, C.M. Ashwell, J. de Oliviera, X. Zhao, Cell walls of Saccharomyces cerevisiae differentially modulated innate immunity and glucose metabolism during late systemic inflammation, PLoS One 7 (1) (2012) e30323.
[34] N. Meijerink, J.E. De Oliveira, D.A. Van Haarlem, G. Hosotani, D.M. Lamot, J. A. Stegeman, V.P. Rutten, C.A. Jansen, Glucose oligosaccharide and long-chain glucomannan feed additives induce enhanced activation of intraepithelial NK cells and relative abundance of commensal lactic acid bacteria in broiler chickens, Vet. Sci. 8 (6) (2021) 110.
[35] N. Meijerink, J.E. De Oliveira, D.A. Van Haarlem, D.M. Lamot, F.C. Velkers, H. Smidt, J.A. Stegeman, V.P. Rutten, C.A. Jansen, Long-chain glucomannan supplementation modulates immune responsiveness, as well as intestinal microbiota, and impacts infection of broiler chickens with Salmonella enterica serotype Enteritidis, Vet. Res. 53 (1) (2022) 1–20.
[36] W.G. Willats, L. McCartney, W. Mackie, J.P. Knox, Pectin: cell biology and prospects for functional analysis, Plant Mol. Biol. 47 (2001) 9–27.
[37] B.L. Ridley, M.A. O'Neill, D. Mohnen, Pectins: structure, biosynthesis, and oligogalacturonide-related signaling, Phytochemistry 57 (6) (2001) 929–967.
[38] D. Aune, D.S. Chan, R. Lau, R. Vieira, D.C. Greenwood, E. Kampman, T. Norat, Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies, BMJ 343 (2011).
[39] R.-M. Catalioto, C.A. Maggi, S. Giuliani, Intestinal epithelial barrier dysfunction in disease and possible therapeutical interventions, Curr. Med. Chem. 18 (3) (2011) 398–426.
[40] D. Mohnen, Pectin structure and biosynthesis, Curr. Opin. Plant Biol. 11 (3) (2008) 266–277.
[41] K. Ishisono, T. Mano, T. Yabe, K. Kitaguchi, Dietary fiber pectin ameliorates experimental colitis in a neutral sugar side chain-dependent manner, Front. Immunol. 10 (2019) 2979.
[42] M. Beukema, É. Jermendi, M.A. van den Berg, M. Faas, H.A. Schols, P. De Vos, The impact of the level and distribution of methyl-esters of pectins on TLR2-1 dependent anti-inflammatory responses, Carbohydr. Polym. 251 (2021) 117093.
[43] N.M. Sahasrabudhe, M. Beukema, L. Tian, B. Troost, J. Scholte, E. Bruininx, G. Bruggeman, M. Van den Berg, A. Scheurink, H.A. Schols, Dietary fiber pectin directly blocks toll-like receptor 2–1 and prevents doxorubicin-induced ileitis, Front. Immunol. 9 (2018) 383.
[44] M. Beukema, É. Jermendi, T. Koster, K. Kitaguchi, B.J. de Haan, M.A. van den Berg, M.M. Faas, H.A. Schols, P. de Vos, Attenuation of doxorubicin-induced small intestinal mucositis by Pectins is dependent on Pectin's methyl-Ester number and distribution, Mol. Nutr. Food Res. 65 (18) (2021) 2100222.
[45] G.E. do Nascimento, S.M.B. Winnischofer, M.I. Ramirez, M. Iacomini, L.M.C. Cordeiro, The influence of sweet pepper pectin structural characteristics on cytokine secretion by THP-1 macrophages, Food Res. Int. 102 (2017) 588–594.
[46] L.M. Vogt, N.M. Sahasrabudhe, U. Ramasamy, D. Meyer, G. Pullens, M.M. Faas, K. Venema, H.A. Schols, P. de Vos, The impact of lemon pectin characteristics on TLR activation and T84 intestinal epithelial cell barrier function, J. Funct. Foods 22 (2016) 398–407.
[47] N. Guriec, F. Bussy, C. Gouin, O. Mathiaud, B. Quero, M. Le Goff, P.N. Collén, Ulvan activates chicken heterophils and monocytes through toll-like receptor 2 and toll-like receptor 4, Front. Immunol. 9 (2018) 2725.
[48] N. Guriec, F. Bussy, C. Gouin, O. Mathiaud, M. Le Goff, J. Delarue, P.N. Collén, Activation of chicken gamma-delta T lymphocytes by a purified ulvan extract, Vet. Immunol. Immunopathol. 237 (2021) 110255.
[49] A. Ijaz, F. Broere, V.P. Rutten, C.A. Jansen, E.J. Veldhuizen, Perforin and granzyme a release as novel tool to measure NK cell activation in chickens, Dev. Comp. Immunol. 105047 (2023).
[50] G. Ávila, D. De Leonardis, G. Grilli, C. Lecchi, F. Ceciliani, Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response, Vet. Immunol. Immunopathol. 237 (2021) 110269.
[51] B. Kaspers, P. Kaiser, Avian antigen-presenting cells, Avian Immunology, Elsevier 2014, pp. 169–188.
[52] N. Blumenkrantz, G. Asboe-Hansen, New method for quantitative determination of uronic acids, Anal. Biochem. 54 (2) (1973) 484–489.
[53] T. JF, Automatisation du dosage des substances pectiques par la méthode au métahydroxydiphényle, Lebensm. Wiss. Technol. 12 (1979) 247–251.
[54] M. Huisman, A. Oosterveld, H. Schols, Fast determination of the degree of methyl esterification of pectins by head-space GC, Food Hydrocoll. 18 (4) (2004) 665–668.
[55] H. Beug, A. von Kirchbach, G. Döderlein, J.-F. Conscience, T. Graf, Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation, Cell 18 (2) (1979) 375–390.

[56] L. Peng, R.H. van den Biggelaar, C.A. Jansen, H.P. Haagsman, E.J. Veldhuizen, A method to differentiate chicken monocytes into macrophages with proinflammatory properties, Immunobiology 225 (6) (2020) 152004.
[57] L. Rothwell, J.R. Young, R. Zoorob, C.A. Whittaker, P. Hesketh, A. Archer, A. L. Smith, P. Kaiser, Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima, J. Immunol. 173 (4) (2004) 2675–2682.
[58] R.A. Dalloul, T.W. Bliss, Y.-H. Hong, I. Ben-Chouikha, D.W. Park, C.L. Keeler, H. S. Lillehoj, Unique responses of the avian macrophage to different species of Eimeria, Mol. Immunol. 44 (4) (2007) 558–566.
[59] F. Rohde, B. Schusser, T. Hron, H. Farkašová, J. Plachý, S. Härtle, J. Hejnar, D. Elleder, B. Kaspers, Characterization of chicken tumor necrosis factor-α, a long missed cytokine in birds, Front. Immunol. 9 (2018) 605.
[60] W.G. Degen, J. Smith, B. Simmelink, E.J. Glass, D.W. Burt, V.E. Schijns, Molecular immunophenotyping of lungs and spleens in naive and vaccinated chickens early after pulmonary avian influenza a (H9N2) virus infection, Vaccine 24 (35–36) (2006) 6096–6109.
[61] M. Coorens, V.A. Schneider, A.M. de Groot, A. van Dijk, M. Meijerink, J.M. Wells, M.R. Scheenstra, E.J. Veldhuizen, H.P. Haagsman, Cathelicidins inhibit escherichia coli–induced tlr2 and tlr4 activation in a viability-dependent manner, J. Immunol. 199 (4) (2017) 1418–1428.
[62] É. Jermendi, M. Beukema, M.A. van den Berg, P. de Vos, H.A. Schols, Revealing methyl-esterification patterns of pectins by enzymatic fingerprinting: beyond the degree of blockiness, Carbohydr. Polym. 277 (2022) 118813.
[63] E.M. Longhin, N. El Yamani, E. Rundén-Pran, M. Dusinska, The alamar blue assay in the context of safety testing of nanomaterials, Front. Toxicol. 4 (2022) 981701.
[64] G. Dongowski, A. Lorenz, H. Anger, Degradation of pectins with different degrees of esterification by Bacteroides thetaiotaomicron isolated from human gut flora, Appl. Environ. Microbiol. 66 (4) (2000) 1321–1327.
[65] G.H. Hyun, I.H. Cho, Y.Y. Yang, D.-H. Jeong, Y.P. Kang, Y.-S. Kim, S.J. Lee, S. W. Kwon, Mechanisms of interactions in pattern-recognition of common glycostructures across pectin-derived heteropolysaccharides by toll-like receptor 4, Carbohydr. Polym. 314 (2023) 120921.
[66] N.M. Sahasrabudhe, L. Tian, B. Troost, M. Beukema, J. Scholte, E. Bruininx, G. Bruggeman, M. van den Berg, A. Scheurink, H.A. Schols, Pectin attenuates immune responses by directly blocking toll-like receptor 2, Experimental studies on dietary fibers 99 (2016).
[67] S.B. Prado, M. Beukema, E. Jermendi, H.A. Schols, P. de Vos, J.P. Fabi, Pectin interaction with immune receptors is modulated by ripening process in papayas, Sci. Rep. 10 (1) (2020) 1690.
[68] R. Zhou, X. Shi, Y. Gao, N. Cai, Z. Jiang, X. Xu, Anti-inflammatory activity of guluronate oligosaccharides obtained by oxidative degradation from alginate in lipopolysaccharide-activated murine macrophage RAW 264.7 cells, J. Agric. Food Chem. 63 (1) (2015) 160–168.
[69] J.C. Amorim, L.C. Vriesmann, C.L. Petkowicz, G.R. Martinez, G.R. Noleto, Modified pectin from Theobroma cacao induces potent pro-inflammatory activity in murine peritoneal macrophage, Int. J. Biol. Macromol. 92 (2016) 1040–1048.
[70] C. Song, F. Huang, L. Liu, Q. Zhou, D. Zhang, Q. Fang, H. Lei, H. Niu, Characterization and prebiotic properties of pectin polysaccharide from Clausena lansium (Lour.) Skeels fruit, Int. J. Biol. Macromol. 194 (2022) 412–421.
[71] S. McKay, P. Oranje, J. Helin, J.H. Koek, E. Kreijveld, P. van den Abbeele, U. Pohl, G. Bothe, M. Tzoumaki, M. Aparicio-Vergara, Development of an affordable, sustainable and efficacious plant-based immunomodulatory food ingredient based on bell pepper or carrot RG-I pectic polysaccharides, Nutrients 13 (3) (2021) 963.
[72] M. Beukema, É. Jermendi, H.A. Schols, P. de Vos, The influence of calcium on pectin's impact on TLR2 signalling, Food Funct. 11 (9) (2020) 7427–7432.
[73] É. Jermendi, C. Fernández-Lainez, M. Beukema, G. López-Velázquez, M.A. van den Berg, P. de Vos, H.A. Schols, TLR 2/1 interaction of pectin depends on its chemical structure and conformation, Carbohydr. Polym. 303 (2023) 120444.
[74] Z. Świderská, A. Šmídová, L. Buchtová, A. Bryjová, A. Fabiánová, P. Munclinger, M. Vinkler, Avian toll-like receptor allelic diversity far exceeds human polymorphism: an insight from domestic chicken breeds, Sci. Rep. 8 (1) (2018) 17878.
[75] A. Yilmaz, S. Shen, D.L. Adelson, S. Xavier, J.J. Zhu, Identification and sequence analysis of chicken toll-like receptors, Immunogenetics 56 (2005) 743–753.
[76] M.L.A. De Leoz, S. Wu, J.S. Strum, M.R. Niñonuevo, S.C. Gaerlan, M. Mirmiran, J. B. German, D.A. Mills, C.B. Lebrilla, M.A. Underwood, A quantitative and comprehensive method to analyze human milk oligosaccharide structures in the urine and feces of infants, Anal. Bioanal. Chem. 405 (2013) 4089–4105.
[77] F. Hong, J. Yan, J.T. Baran, D.J. Allendorf, R.D. Hansen, G.R. Ostroff, P.X. Xing, N.-K.V. Cheung, G.D. Ross, Mechanism by which orally administered β-1, 3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models, J. Immunol. 173 (2) (2004) 797–806.