

In Vitro Studies Toward the Use of Chitin as Nutraceutical: Impact on the Intestinal Epithelium, Macrophages, and Microbiota

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Scope: Chitin, the most abundant polysaccharide found in nature after cellulose, is known for its ability to support wound healing and to lower plasma-oxidized low-density lipoprotein (LDL) levels. Studies have also revealed immunomodulatory potential but contradicting results are often impossible to coalesce through usage of chitin of different or unknown physicochemical consistency. In addition, only a limited set of cellular models have been used to test the bioactivity of chitin.

Methods and Results: Chitin is investigated with well-defined physicochemical consistency for its immunomodulatory potency using THP-1 macrophages, impact on intestinal epithelial barrier using Caco-2 cells, and fermentation by fecal-derived microbiota. Results show that chitin with a degree of acetylation (DA) of $\approx 83\%$, regardless of size, does not affect the intestinal epithelial barrier integrity. Large-sized chitin significantly increases acetic acid production by gut microbiota without altering the composition. Exposure of small-sized chitin to THP-1 macrophages lead to significantly increased secretion of IL-1 β , IL-8, IL-10, and CXCL10 in a multi-receptor and clathrin-mediated endocytosis dependent manner.

Conclusions: These findings indicate that small-sized chitin does not harm the intestinal barrier nor affects SCFA secretion and microbiota composition, but does impact immune activity which could be beneficial to subjects in need of immune support or activation.

1. Introduction


Diseases that are associated with a disbalance in immune homeostasis are increasing in frequency over the past decades.^[1] In cases of autoimmune disorders and allergy, the immune system is overreacting to harmless antigens.^[2] Whereas in case of inflammatory bowel disease, the inflammation is of chronic nature with a failed feedback loop to resolve it.^[3] In contrast, the immune system is not sufficiently reactive in case of cancer^[4] or secondary opportunistic disease.^[5]

The immune balance is a result of genetic activity, stress, nutrition, and other environmental factors. Many studies focus on nutrition to skew immune responses into a supposedly beneficial direction.^[6] An example of immunomodulatory food components or nutraceuticals are dietary fibers. In particular non-digestible dietary fibers were shown to support health by strengthening the intestinal barrier, modifying microbiota composition and short-chain fatty acid (SCFA) production, and supporting immune function. Research toward these effects mainly

include in vitro and animal studies, but also clinical trials. For example, a clinical study reported that a four week intervention with arabinoxylan or resistant starch type 2 in adults with metabolic syndrome supported a healthy microbial diversity. They observed significantly increased acetate and butyrate concentrations and levels of *Bifidobacterium*, while simultaneously reducing the proportion of species that are associated with a dysbiosis.^[7] Similarly, a 16-week intervention with inulin in obese children resulted in reduced IL-6 levels in serum and increased *Bifidobacterium spp.* and decreased *Bacteroides vulgatus* levels.^[8] The observed effects on microbiota are possibly directly related to the type of dietary fiber, which can vary greatly based on source and extraction method.^[9] In general, however, dietary fibers increase the abundance of bifidobacteria and lactobacilli, which appear to be beneficial for health and reduce infections with pathogenic bacteria, such as *Escherichia coli*.^[10,11] Next to increasing beneficial and reducing harmful bacteria concentrations,

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changes in intestinal metabolite concentrations also appear to support beneficial health effects.^[12] The observed increases in SCFA, and butyrate in particular, have been related to improved health as they beneficially impact host metabolism and intestinal immunity.^[13] Dietary fibers have also been shown to impact peripheral immunity by increasing frequency and activity of circulating B cells, NK cells, and/or monocytes.^[14] Furthermore, there might be a direct interaction between dietary fibers or SCFAs and the gut-associated-lymphoid tissue as there are many receptors described to bind both.^[15]

A compound similar to commonly consumed or tested non-digestible dietary fibers, with potency to improve intestinal health, is chitin. Chitin is a linear polymer of β -(1-4)-linked *N*-acetyl-glucosamine and is the most abundant polysaccharide found in nature after cellulose. So far, clinical trials have reported that orally consumed chitin lowers plasma oxidized LDL levels and therefore reduces the risk of cardiovascular diseases.^[16] Furthermore, many studies described the potency of chitin to impact the immune system.^[16] Results have been contrasting with studies on the one hand demonstrating inflammatory effects, such as chitin-mediated release of IL-12, TNF α , IFN γ by spleen cells, and the induction of an oxidative burst in macrophages.^[17–19] On the other hand, studies report on anti-inflammatory properties, such as reduced secretion of IL-1 β , IL-6, TNF α , nitric oxide, and prostaglandin E2 by microglia cells, but also increased IL-10 secretion by macrophages.^[20,21] The conflicting results may stem from the differences in physicochemical properties of chitin fractions used in these studies. Despite this, these studies do demonstrate the potency for chitin to support intestinal health. Furthermore, chitin is readily available from large side-streams of shrimp and insect production securing practical availability. To consider chitin as nutraceutical to support intestinal health similar to dietary fibers, we need to improve the understanding of its functional impact. This requires analysis of parameters that are relevant to intestinal health and immune balance (i.e., the intestinal epithelial barrier, immune system, and microbiota), but also the need for detailed description of chitin characteristics, which is lacking in many studies.

In this study, we used chitin particles with a known DA and size fragmentation and evaluated the interaction with several models of the intestinal immune barrier and with microbiota.

2. Experimental Section

2.1. Chitin Preparation and Characterization

Fine chitin powders were prepared from a commercial chitin from shrimp shells in coarse flakes form (Sigma-Aldrich, Zwijndrecht, The Netherlands) by two continuous milling processes. First, chitin coarse flakes were cracked with the IKA mill (model A11 B, IKA-Werke GmbH and Co. KG, Staufen, Germany) while frozen by liquid nitrogen to obtain chitin particles followed by another milling process with a PM100 planetary ball mill (Retsch, Haan, Germany) in a 50 mL zirconium dioxide jar containing 17 ϕ 10 mm zirconium dioxide balls at a frequency of 500 rpm for 1.5 h. A 10 min interval was set among every 15 min ball milling to prevent overheating to obtain a fine chitin powder. Next, the chitin powder was separated based on particle size using the Air-jet Sieve (model e200LS, Hosokawa-Alpin, Augsburg, Germany)

with 100, 50, and 20 μ m sieves. Finally, the sample size was validated by the Mastersizer 3000 equipped with a laser diffraction practice analyzer (Malvern Panalytical, Worcestershire, UK). In addition to size, the DA of chitin fractions was determined by using the first derivative UV method and the formula: DA (%) = $((m1/203.21) \times 100) / ((m1/203.21) + ((M-m1)/161.17))$.^[22] In summary, glucosamine hydrochloride (Glc) (Sigma-Aldrich) and *N*-acetyl-D-glucosamine (GlcNAc) (Sigma-Aldrich) were dissolved in 0.85% phosphoric acid (Sigma-Aldrich) at a concentration of 0, 10, 20, 30, 40, 50 μ g mL⁻¹, and the first derivative of UV value at 203 nm (H203) was measured by a UV-vis spectrophotometer (model Shimadzu 1800, Shimadzu Corporation, Kyoto, Japan) using a far UV cuvette with a 10 mm pathway. A calibration curve was constructed by plotting the H203 as a function of different concentrations of GlcNAc and Glc. To evaluate the DA of chitin, the amount of GlcNAc in chitin samples was identified from the calibration curve. Specifically, 100 mg (*M*) chitin samples were suspended in 20 mL of 85% phosphoric acid and incubated at 60 °C for 40 min to fully dissolve the chitin. Next, 1 mL of this solution was diluted with 99 mL of MilliQ water and heated for another 2 h at 60 °C. After this, the H203 of the solution was measured immediately in technical triplicates and the mass of GlcNAc in 1 mL solution (*m1*) was calculated according to the calibration curve described above.

2.2. Endotoxin Measurements

HEK-Blue human TLR4 (Invitrogen, Bleiswijk, The Netherlands) cells were used for testing the endotoxin content in chitin. HEK-Blue hTLR4 cells were seeded on a poly-D-lysine-coated 96-well flat bottom plate (Greiner Bio-one, Frickenhausen, Germany) at a concentration of 0.5×10^6 cells mL⁻¹ (200 μ L) and incubated at 37 °C for 24 h. The cells were next exposed to medium, lipopolysaccharide (LPS) (titration of 0.0001–100 ng mL⁻¹) (Figure S1, Supporting Information) dissolved in medium and a homogeneous chitin suspension (0.1 mg mL⁻¹) in medium, achieved by thorough mixing through vortexing and resuspending, and incubated for 24 h at 37 °C. After incubation, 20 μ L of the supernatant was mixed with 180 μ L of Quanti-Blue (InvivoGen, Toulouse, France) in a 96-well flat bottom plate and incubated at 37 °C for 2 h after which the absorbance was measured at 655 nm using a spectrophotometer (model Infinite 200 PRO, TECAN, Giessen, The Netherlands).

2.3. THP-1 Cell Culture and Treatment with Chitin Fractions

The human monocytic leukemia cell line (THP-1; American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) containing 10% of fetal bovine serum (FBS; Hyclone PerBio, Etten-Leur, The Netherlands) and 1% of penicillin/streptomycin (Invitrogen) at 37 °C under 5% of CO₂. Cells were sub-cultured twice per week and set at 0.25×10^6 mL⁻¹ in 20 mL medium in a T75 culture flask (Corning, Amsterdam, The Netherlands). To differentiate the cells into macrophages, 0.5×10^6 cells were exposed to 100 ng mL⁻¹ phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) in 0.5 mL RPMI medium for 72 h in 24-well cell culture plate (Greiner

Bio-one). Following extensive washing to remove residual PMA, the cells were rested for 72 h. Rested THP-1 macrophages were exposed to medium (supplemented RPMI) as control or a homogeneous chitin suspension in medium, achieved by thorough mixing through vortexing and resuspending, at a concentration of 0.1 mg mL^{-1} and incubated at 37°C for 24 h.

2.4. Caco-2 Cell Culture and Treatment with Chitin Fractions

Caco-2 cells (American Type Culture Collection) from passage 30 to 40 were cultured in DMEM (Gibco, Bleiswijk, The Netherlands) containing 4.5 g L^{-1} D-glucose, L-glutamine, 25 mM HEPES, and supplemented with 10% of heat-inactivated FBS at 37°C with 5% CO_2 . Transwell inserts of 33.6 mm^2 , $0.4 \mu\text{m}$ pore size and 1×10^8 pores cm^{-2} (Greiner Bio-one) were seeded on the apical side with 3.375×10^4 cells in 150 μL and suspended in a 24-well flat bottom plate (Greiner Bio-one) with 700 μL basolateral medium and cultured for 7 or 21 days at 37°C with 5% of CO_2 to differentiate in colon-like or small-intestinal-like epithelial cells, respectively.^[23] Apical and basolateral medium was replaced three times a week and one day prior to the addition of samples. Differentiated Caco-2 were exposed to medium or a homogeneous chitin suspension in medium, achieved by thorough mixing through vortexing and resuspending, of 0.1 mg mL^{-1} and incubated at 37°C for 24 h. The transepithelial electrical resistance (TEER) was measured by using a MilliCell-ERS (Millipore, Amsterdam, The Netherlands) apparatus directly before treatment of the Caco-2 to verify viability of the cells and directly after treatment of the Caco-2 and at 1, 3, 6, and 24 h, and normalized to the starting time point (set at 100%).

2.5. RNA Isolation, cDNA Synthesis, and Real-Time Quantitative PCR

RNA isolation, cDNA synthesis, and RT-qPCR were performed as described previously.^[24] Briefly, both THP-1 and Caco-2 cells were lysed with 0.2 mL TRIzol (Invitrogen) after 24 h and RNA was isolated using the RNase mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. Subsequently, cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, CA, Hercules, USA) and 5 μL of cDNA was mixed with 10 μL of IQ SYBR Green Supermix (Bio-Rad) and 5 μL of primer pairs. The RT-qPCR program consisted of 90 s preheating at 95°C , 10 s denaturing at 95°C , 10 s annealing at 58°C and 15 s elongation at 72°C for 40 cycles and was run on a CFX96 touch Real-Time PCR detection system (Bio-Rad). Reference genes beta-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalization.

2.6. Measurements of Cytokine Secretion

In the collected supernatants from THP-1 macrophages the secretion of Arginase, CXCL10, IL-1 β , IL-1RA, IL-4, IL-6, IL-10, IL-

12p40, IL-12p70, IL-23, IFN γ , TARC, and TNF α was measured using the LEGENDplex kit (BioLegend, Koblenz, Germany). This is a fluorescent bead-based immunoassay that shares the same principle with sandwich ELISA and allows measuring multiple analytes simultaneously using flow cytometry for detection. The data was analyzed using the CytoFlex flow cytometer (Beckman Coulter, Woerden, The Netherlands) with CytoExpert software. IL-8 and IL-10 ELISA kits (BioLegend) were used to measure the individual cytokine levels according to the manufacturer's instruction.

2.7. Endocytosis Assay

The endocytosis assay was based on a method described elsewhere^[25] with a slight modification. Briefly, chitin fractions at a concentration of 10 mg mL^{-1} were incubated with 1 mg mL^{-1} fluorescein isothiocyanate (FITC; Sigma-Aldrich) in 0.1 M sodium bicarbonate (Sigma-Aldrich) for 1.5 h at RT in the dark. Next, the chitin was washed five times with MilliQ water through centrifugation at $20\,800 \times g$ for 5 min and dialyzed using a membrane with a pore size of 12–14 kDa (Medicell Membranes Ltd., London, UK) and 5 L MilliQ for 24 h, with the dialysate solution being refreshed after 8 h. The FITC-labelled chitin was lyophilized and stored at RT in the dark. FITC-labelled chitin fractions were suspended in medium and a homogeneous mixture was ensured through vortexing and resuspending and incubated with THP-1 macrophages at a concentration of 0.1 mg mL^{-1} for 1 h at 37°C after which the cells were washed with PBS (Gibco). Subsequently, THP-1 macrophages were incubated with 0.2 mL of trypsin (Gibco) at 37°C for 10 min and upon collection 0.8 mL of FBS was added to the cells and they were centrifuged at $450 \times g$ for 5 min. Cells were re-suspended and stored in 0.2 mL paraformaldehyde (PFA; Sigma-Aldrich) at 4°C . Before measurement the fluorescence from particles attached to the outer cell membrane was quenched by adding 40 μL of 0.25% trypan-blue (Gibco). To block specific endocytic routes of chitin uptake inhibitors were added for 2 h at 37°C prior to chitin addition: Cytochalasin B (CB) ($10 \mu\text{g mL}^{-1}$; Sigma-Aldrich) to block phagocytosis, nystatin (NYS) ($25 \mu\text{g mL}^{-1}$; Sigma-Aldrich) to block caveolin-mediated endocytosis, and chlorpromazine ($10 \mu\text{g mL}^{-1}$; Sigma-Aldrich) to block clathrin-mediated endocytosis. Similarly, to determine involvement of receptors in uptake THP-1 macrophages were exposed to $15 \mu\text{g mL}^{-1}$ of anti-TLR2 antibody (Clone: TL2.1, BioLegend), $20 \mu\text{g mL}^{-1}$ of anti-human CD206 antibody (Clone: 19.2, BD Bioscience, Vianen, The Netherlands), 1 mg mL^{-1} of laminarin (Sigma-Aldrich), or 10 mM lactose (Sigma-Aldrich) for 30 min at 37°C prior to chitin incubation, to block TLR2, mannose receptor, dectin-1, or galectin-3, respectively. Cells were washed with medium after blocking routes of endocytosis or receptors before addition of FITC-labelled chitin as described above. Intracellular FITC-signal was measured using the Accuri flow cytometer (BD Biosciences) and Accuri C6 software. The relative uptake of FITC-labelled chitin was determined by subtracting the mean fluorescence intensity (MFI) levels of the background fluorescence of cells treated with RPMI medium from the MFI of cells exposed to FITC-labelled chitin.

2.8. In Vitro Fermentation

The batch in vitro fermentation was conducted in a biological duplicate using two healthy adult microbiome donors. Each fermentation vessel contained 43 mL of autoclaved medium (containing per liter: 2 g peptone (Duchefa Biochemie, Haarlem, The Netherlands), 2 g yeast (Sigma-Aldrich), 0.5 g L-cysteine (Sigma-Aldrich), 5.22 g K₂HPO₄ (Merck, Darmstadt, Germany), 16.32 g KH₂PO₄ (Merck), 2 g NaHCO₃ (Merck), 1 g mucin (Sigma-Aldrich), and 2 mL Tween 80 (Sigma-Aldrich) to which 20 mL PBS (control) or PBS containing 350 mg chitin was added. Continuous sparging with O₂-free N₂ resulted in anaerobic fermentation vessels to which 7 mL of microbiota was added. The microbiota was sampled from the distal colon vessel reactor of the Simulator of Human Intestinal Microbial Ecosystem (SHIME) reactor (ProDigest, Gent, Belgium) after 2 weeks of stabilization.^[26] After adding microbiota, the vessels were incubated at 37 °C at constant shaking (200 rpm) and samples (3 mL) were taken after 0 and 24 h for analysis. The samples were centrifuged at 10 000 rpm for 3 min at 4 °C and both the supernatant and the pellet were collected and stored at -80 °C. Pellets obtained after 0 h (control) or 24 h (control and chitin fractions) incubation were mixed with 1 mL DNA/RNA shield (Zymo Research, Freiburg im Breisgau, Germany) and sent to Baseclear B.V. (Leiden, The Netherlands) for microbiota composition analysis.

2.9. SCFA Analysis

The production of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and lactic acid was determined by high performance liquid chromatography (HPLC) (model Acquity Arc, Waters, Eschborn, Germany) equipped with an AMINEX HPX-87H column (Aminex HPX-87H, 300 × 7.8 mm, Bio-Rad Laboratories, Richmond, VA, USA) and the column was maintained at 35 °C using an integral column heater (Waters). The target compounds were detected by using a refractive index detector (model R2414, Waters) and the eluent for analysis was 8.3 mM sulfuric acid (Sigma-Aldrich) at a flow rate of 0.5 mL min⁻¹. Standards were prepared at a concentration of 11.47 mM for lactic acid (Sigma-Aldrich), 16.55 mM for acetic acid (Merck), 13.96 mM for propionic acid (Sigma-Aldrich), 4.24 mM for isobutyric acid (Fluka, Cheniou, Gmbh, Germany), 11.53 mM for butyrate acid (Sigma-Aldrich), 10.51 mM for isovaleric acid (Fluka), and 10.44 mM for valeric acid (Acros Organic, Geel, Belgium) in 1 L of 8.3 mM sulfuric acid (Sigma-Aldrich). The standard samples were injected repeatedly every five measurements. The calibration curve was constructed by plotting the peak area against the molarity of standard solutions. Tested samples for HPLC were prepared by diluting the collected supernatant with 16.6 mM sulfuric acid at a ratio of 1:1 v/v.

2.10. Microbiota Composition Analysis

Bacterial genomic DNA was extracted from each pellet and the 16S rRNA gene sequencing was performed by Baseclear B.V.

Table 1. Physicochemical and biochemical properties of chitin.

	SC	IC	LC
Size distribution (80%) ^{a)}	4.8–25.5 μm	19.8–71.6 μm	50.4–140.2 μm
Degree of acetylation	80 ± 0.7%	81.3 ± 2.7%	87.5 ± 3.9%
LPS contamination ^{b)}	n.d	n.d	n.d

The degree of acetylation of chitin fractions was described as the mean ($n = 2$) ± SD. ^{a)} 10% of the sample was smaller and 10% larger than the provided size range; ^{b)} Detection limit of the assay was 1 pg mL⁻¹ as determined with a titration experiment (Figure S1, Supporting Information) and the applied chitin concentration 0.1 mg mL⁻¹. Statistical analysis on the DA among chitin fractions was performed by one way ANOVA but no significant differences were observed. Abbreviations: IC: intermediated chitin; LC: large chitin; LPS: lipopolysaccharide; n.d: not detected; SC: small chitin.

(L457; NEN-EN-ISO/IEC 17025). Briefly, the microbial DNA was extracted and the V3-V4 region of the 16S rRNA gene was amplified and sequenced by using MiSeq System (Illumina, San Diego, CA, USA). Subsequently, the raw paired-ends FASTQ files were trimmed and converted by bcl2fastq2 Conversion Software (version 2.18, Illumina). The analysis on the resulting data was conducted using CLC Genomics Workbench (Microbial Genomics toolbox version 20.0, Qiagen). 16S rRNA gene paired read sequences were used to prepare an operational taxonomic units (OTUs) table at 99% reference of sequence similarity from Silva 16S/18S gene database (version 132). To evaluate the alpha diversity or beta diversity of each microbiota community, a phylogenetic tree was created by using MUSCLE (version 3.8.425). The phylogenetic tree together with the OTU abundance table were used for alpha and beta diversity clustering.

2.11. Statistics

Microbiota data were presented as the mean of two independent experiments with two different donors and statistical analysis between parameters was performed using the Kruskal–Wallis test (Figure 2B,C) or the Wald test (Figure 2D). Other data were presented as mean + SD and statistically significant differences between parameters were analyzed by one way ANOVA (Graphpad Prism 8, La Jolla, CA, USA).

3. Results

3.1. Physicochemical Characterization of Size-Separated Chitin Fractions

Fine chitin powder was fractionated by size, resulting in fractions with 80% of the size distributed between 4.8 to 25.5 μm termed small chitin (SC), 19.8 to 71.6 μm termed intermediate chitin (IC), and 50.4 to 140.2 μm termed large chitin (LC) (Table 1). Next to size, the DA was determined and was found to be 80 ± 0.7%, 81.3 ± 2.7%, and 87.5 ± 3.9% for SC, IC, and LC, respectively (Table 1). In addition, chitin fractions were analyzed for contamination with the strongly immunomodulatory lipopolysaccharide (LPS), which was not detected in any of the samples (<1 pg/0.1mg; Table 1).

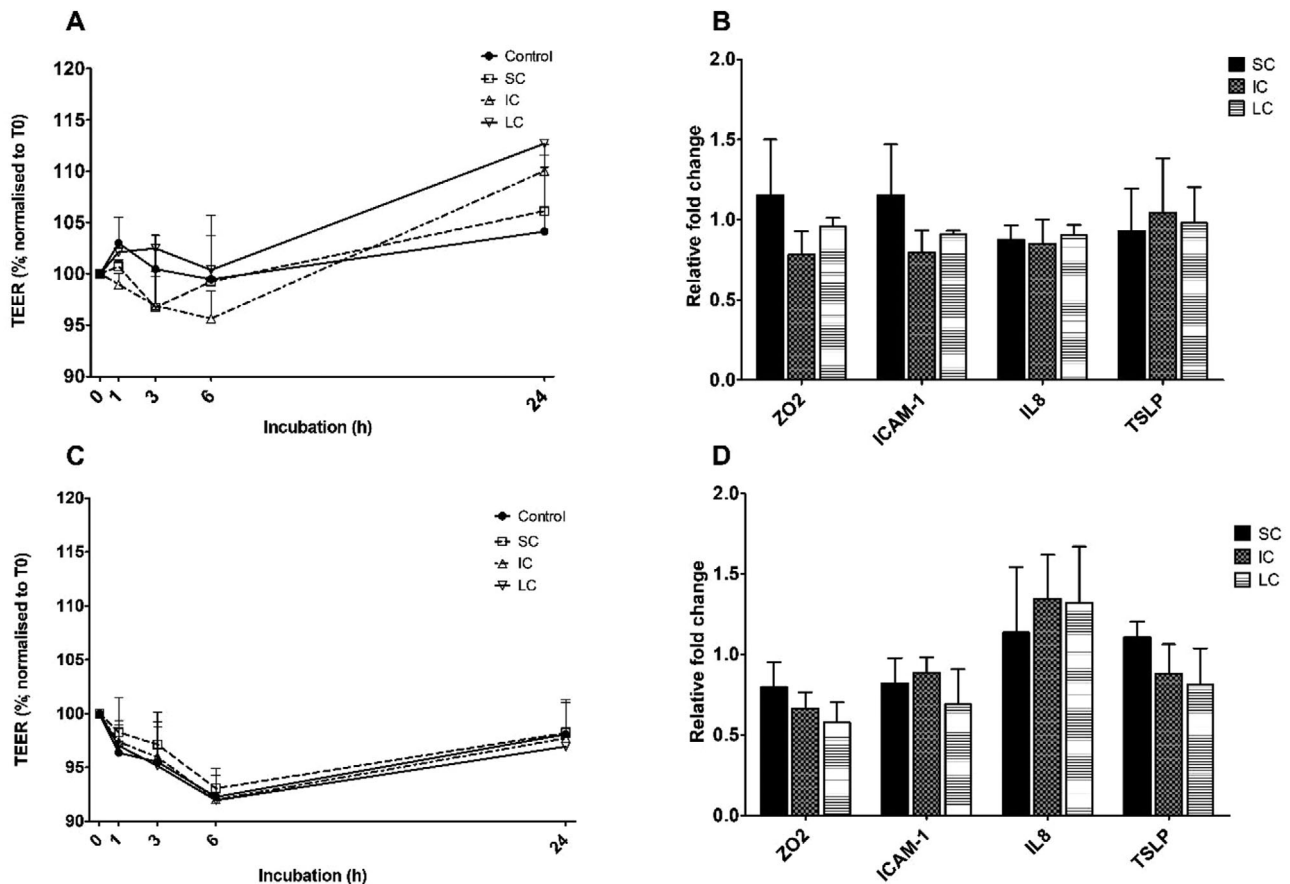


Figure 1. Effect of chitin fractions on the barrier integrity of small- and large-intestinal epithelial-like Caco-2 cells models. Caco-2 cells differentiated into A,B) colonic (7 day culture on transwell inserts) and C,D) small-intestinal-like (21 day culture on transwell inserts) epithelial cells were exposed to 0.1 mg mL⁻¹ SC, IC, and LC chitin for 24 h. Changes in barrier integrity were monitored using TEER analysis (A,C) in percentages relative to the starting values (set at 100%) during a period of 24 h and gene expression analysis with qPCR (B,D) after 24 h in fold change to medium (set at 1). Line charts and bar charts show the mean of 3 independent experiments + SD. Statistical analysis was performed by one way ANOVA but no significant differences were observed. IC: intermediate chitin; LC: large chitin; SC: small chitin.

3.2. Chitin Fractions Did Not Affect the Barrier Integrity of Caco-2 in a Transwell System

Caco-2 cells, mimicking small- or large-intestinal epithelial cells, were exposed to the different chitin fractions and the barrier integrity and transcriptional changes of both tight junction and adherence junction proteins were measured using TEER and qPCR. SC, IC, and LC fractions increased the TEER levels of colonic epithelial-like Caco-2 cells, albeit non-significantly, following 24 h of exposure (Figure 1A). Using qPCR, the transcription levels of tight junction proteins zona occludens 2 (ZO2) and intercellular adhesion molecule 1 (ICAM-1) were measured. None of the chitin fractions induced significant changes in the transcriptional profile of these genes (Figure 1B).

Similar as for colonic epithelial-like Caco-2 cells, the barrier integrity and transcription of tight junction and adherence junction proteins were investigated of small intestinal epithelial-like Caco-2 cells following exposure to the chitin fractions. Again, no significant effects were observed on TEER (Figure 1C) nor on transcription levels of ZO2 and ICAM-1 (Figure 1D).

3.3. Incubation of Microbiota with Large Chitin Resulted in Increased Levels of Acetate but No Change in Composition

To investigate whether chitin interacts with gut microbiota an *in vitro* fermentation with the different chitin fractions was conducted. Following 24 h of anaerobic incubation of microbiota with control medium containing only PBS or PBS with SC, IC, or LC chitin fractions, the production of lactic acid and SCFAs acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid were assessed (Figure 2A). There was a significant increase in the production of acetic acid following microbiota incubation with LC when compared to the control. To follow up on this finding, we investigated the impact of chitin on gut microbiota composition after 24 h incubation. This revealed that chitin fractions did not significantly change the total number of species (Figure 2B) or the abundance of gut microbiota (Figure 2C) when compared to control. Of note, 24 h incubation of microbiota with the control medium also did not significantly change the microbiota composition (Figure S2, Supporting Information). In addition, we examined the relative abundance of different bacterial groups of total microbiota at the class (Figure 2D)

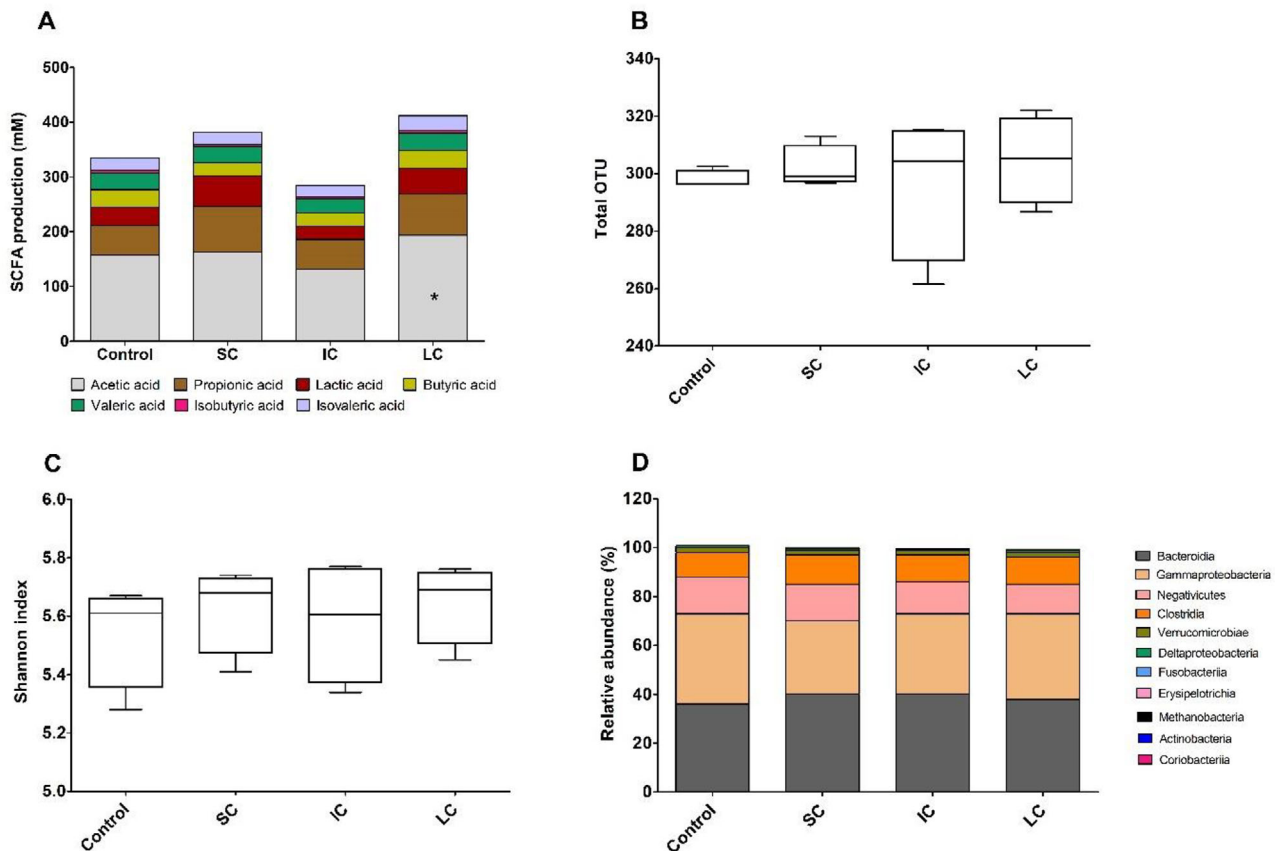


Figure 2. SCFAs, lactic acid, and gut microbiota composition changes following exposure to chitin fractions. A) Gut microbiota cultures were incubated for 24 h with PBS (control), SC, IC, or LC after which lactic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid production were measured. The alpha diversity of the gut microbiota was determined and indicated as B) total OTU count and C) Shannon index. D) The relative abundance of different bacterial groups of the total microbiota were tested and shown at the class level. Stacked bar and box plots show the mean of 2 independent experiments using 2 different donors. Statistical analysis was performed by Kruskal–Wallis test (B,C) and the Wald test (D) but no significant differences were found, and one way ANOVA (A) with $*p < 0.05$; IC: intermediate chitin; LC: large chitin; SC: small chitin; OTU: operational taxonomic unit.

and genus level (data not shown) following 24 h of incubation which again revealed no significant differences.

3.4. Immunomodulatory Effects of Chitin Fractions on Caco-2 and THP-1 Macrophages

The intestinal epithelium represents an important mediator for immune function.^[27] To investigate the effects of chitin fractions on the intestinal immune system, we analyzed the transcription levels of IL-8 and TSLP in Caco-2 cells. Neither colonic nor small-intestinal-like Caco-2 cells demonstrated altered transcription levels of IL-8 or TSLP following exposure to any of the chitin fractions (Figure 1B,D).

Next, THP-1 macrophages were tested for their response to chitin by analyzing transcriptional changes and protein secretion of various signaling molecules. Using qPCR, the differential transcription of the cytokines IL-1 β , IL-8, TNF α , IL-10, and TGF β and chemokines CCL1, CCL5, CCL15, CCL18, CCL22, CCL24 were determined (Figure 3A,B). This revealed that SC significantly increased the transcription of IL-1 β , IL-8, TNF α , IL-10,

CCL1, CCL15, CCL18, and CCL24 compared to the medium control. Furthermore, IC significantly increased transcription of IL-1 β and CCL1 whereas exposure of LC only resulted in a significantly increased transcription of CCL1. The THP-1 macrophages were also investigated for their secretion of IL-8 and IL-10 following exposure to the chitin fractions. In line with the transcription data, IL-8 and IL-10 secretion was chitin-size dependent and only SC significantly stimulated the production of both IL-8 and IL-10 (Figure 3C). To further explore the impact of SC on THP-1 macrophage activity, the secretion of IL-1 β , CXCL10, IL-1RA, IL-4, IL-6, IL-12p40, IL-12p70, IL-23, TNF α , TARC, and arginase were examined using a multiplex assay. This revealed that SC significantly increased IL-1 β and CXCL10 secretion by THP-1 macrophages in addition to IL-8 and IL-10 (Figure 3D).

3.5. THP-1 Macrophage Responses to Small Size Chitin was Mediated via Clathrin-Dependent Endocytosis

Receptor binding and/or endocytosis are the most likely pathways leading to secretion of signaling molecules by THP-1

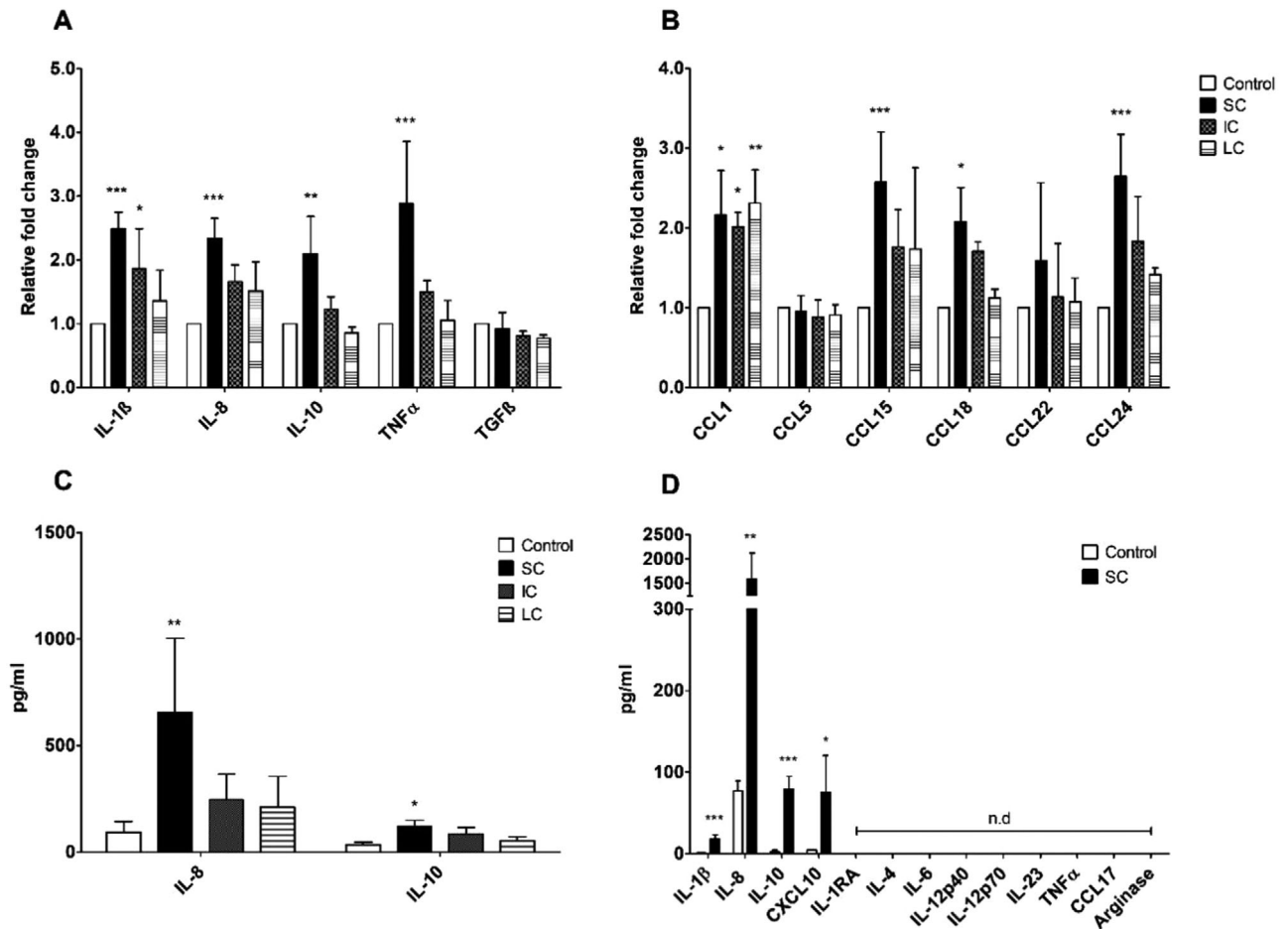


Figure 3. Transcription and secretion of signaling molecules by THP-1 macrophages following exposure to chitin fractions. A,B) THP-1 macrophages were exposed to medium (control) or to chitin fractions for 24 h after which the transcription of cytokines and chemokines was measured with qPCR, C) the secretion of IL-8 and IL-10 with ELISA, and D) the secretion of a panel of cytokines and chemokines with a bead-based multiplex assay to simultaneously detect 13 analytes using flow cytometry. Bar charts show the mean + SD of $n = 3$ independent experiments. IC: intermediate chitin; LC: large chitin; n.d.: not detected; SC: small chitin; Statistical analysis was performed by one way ANOVA with $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

macrophages following exposure to chitin. To determine whether the chitin fractions were endocytosed, THP-1 macrophages were exposed to fluorescently labelled chitin fractions and analyzed using flow cytometry. The results showed an inverse correlation between chitin size and endocytosis efficiency (Figure 4A) with approximately one-third of the macrophages endocytosing SC (i.e., 37.7%) and a minor fraction endocytosing IC and LC (i.e., 13% and 1.7%, respectively).

The SC were further investigated for the receptors involved in the endocytosis. To examine this, TLR2, mannose receptor, dectin-1, and galectin-3, which are potentially involved in SC endocytosis, were blocked (Figure 4B). The results revealed that blocking either of these receptors significantly decreased the endocytosis of SC by approximately 20%, indicating that all tested receptors are involved in the endocytosis of SC.

Similar as for receptors, compounds can be endocytosed via different routes. To investigate which routes were used by THP-1 macrophages to endocytose SC, CB, NYS, and chlorpromazine (CP) were used to specifically inhibit phagocytosis, caveolin-dependent endocytosis, and clathrin-dependent endocytosis, re-

spectively. The inhibitors did not affect cell viability which could have led to false negative results (data not shown). CB and NYS did not affect SC endocytosis by THP-1 macrophages but chlorpromazine significantly inhibited the endocytosis by 74% (Figure 4C).

Finally, to verify that clathrin-dependent endocytosis was the dominant pathway for endocytosis of SC we analyzed whether inhibiting clathrin-mediated endocytosis of SC by macrophages affected the cytokine production (Figure 4D). THP-1 macrophage pre-incubation with chlorpromazine before SC exposure significantly lowered the secretion of all tested signaling molecules.

4. Discussion

The present study represents a comprehensive evaluation of activity of chitin with clearly characterized physicochemical properties toward in vitro intestinal immune models and microbiota. We demonstrated that chitin with a DA of $\approx 83\%$, regardless of size, does not affect the intestinal epithelial barrier integrity. Only LC was actively fermented by microbiota as determined

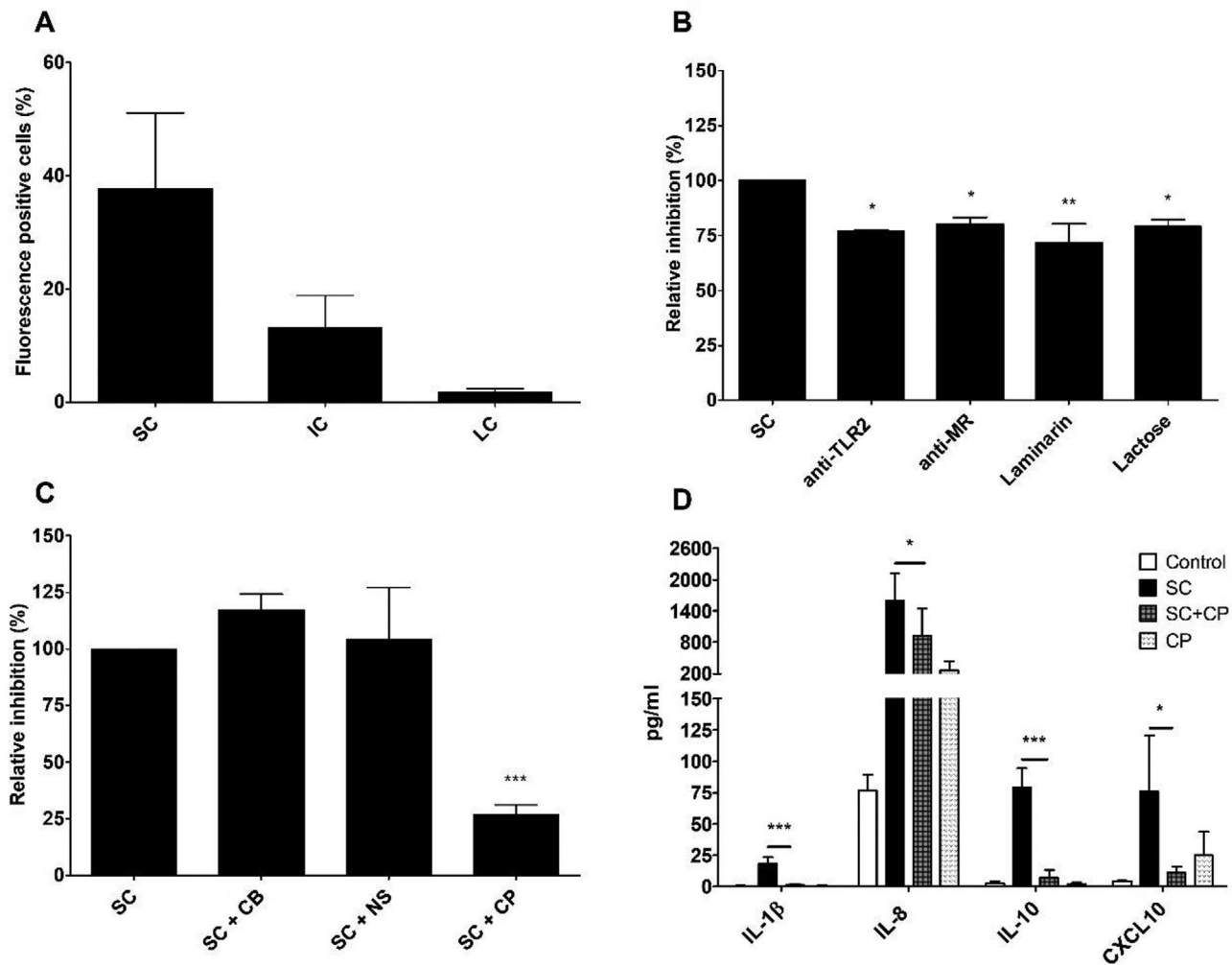


Figure 4. Endocytosis of chitin fractions by THP-1 macrophages. THP-1 macrophages were incubated for 24 h with FITC-labelled chitin fractions and analyzed for: A) endocytosis; B) endocytosis of SC in the presence of receptor blocking agents, or C) endocytosis blocking agents. D) THP-1 macrophages incubated with SC, CP, or SC + CP for 24 h were also analyzed for cytokine secretion. Bar charts show the mean + SD of $n = 3$ independent experiments. CB: cytochalasin B; CP: chlorpromazine; IC: intermediate chitin; LC: large chitin; MR: mannose receptor; NS: nystatin; SC: small chitin. Statistical analysis was performed by one way ANOVA with * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

by SCFA levels, but without significantly changing total or relative abundance of different bacterial groups of the total microbiota. Furthermore, small sized chitin strongly activates THP-1 macrophages to secrete IL-8, IL-10, IL-1 β , and CXCL10 in a clathrin- and multi-receptor-dependent manner.

The gastrointestinal tract has two main and opposing functions. It selectively absorbs nutrients released from the food matrix through digestion or fermentation, but prevents translocation of microbiota that are critical for fermentation or harmful pathogens that can enter the body with the food.^[28] This is achieved through maintaining intestinal barrier function and immune homeostasis. Two important tissues in this respect are the lamina propria which supports the immune system and the intestinal epithelium. The epithelium forms a physical barrier against pathogen entry and is composed mainly of absorptive intestinal epithelial cells (80%), but also mucus producing goblet cells, antimicrobial peptide secreting Paneth cells, and antigen sampling microfold cells.^[29] Many studies have investigated

nutraceutical components for their capacity to strengthen the epithelial barrier against pathogens or reduce pathogen-induced damage. Inulin and oligofructose were found to improve the intestinal barrier function in pigs by increasing the depth of mucosal crypts and the density of intestinal epithelial cells.^[30] Similarly, Nofrarias and colleagues revealed that long-term ingestion of resistant-starch enhanced the colonic mucosal integrity in pigs through decreasing apoptosis of colonocytes and colonic immune cells.^[31] The underlying mechanisms for these beneficial effects of dietary fibers were attributed to their ability to alter the microbiota composition and microbiome metabolome. Intestinal microbiota ferment dietary fibers for growth, which releases energy and metabolites such as SCFAs. SCFAs have important roles in maintaining intestinal health. Here, we demonstrated that chitin does not directly affect the integrity of small intestinal-like or colonic epithelial cells in a Caco-2 model, but large chitin significantly increased the production of acetate in an in vitro microbiota fermentation model (Figures 1 and 2). So far,

descriptions of SCFA production upon chitin fermentation are limited to acetate, propionate, and butyrate production upon chitin-glucan^[32] or chitosan oligosaccharide fermentation (the deacetylated [$<50\%$] form of chitin^[33,34]); or acetate, butyrate, propionate, isobutyrate, isovalerate, and valerate production following whole insect fermentation.^[35] In contrast to these experiments, our findings are based solely upon chitin more clearly demonstrating its specific prebiotic potential. So far, deacetylase activity has only been identified in fungi and bacteria,^[36] but if present in the human microbiome this could account for the observed acetate levels. However, acetate production is only observed following microbiota exposure to large chitin, and deacetylation would also occur with intermediate or small chitin, making fermentation the more likely source of acetate. Microbiota composition analysis following 24 h of chitin incubation revealed no significant changes in diversity, richness or relative abundance of specific classes of the total microbiota. In line with our findings, in a similar *in vitro* fermentation setting Sasaki and colleagues found that the prebiotics Fibersol-2 and Dextran 40 kDa increased the levels of acetate and propionate, without changing the microbiota composition or diversity.^[37] Potentially, the incubation period (i.e., 24 h in our setting and 30 h in Sasaki and colleagues) is sufficient to significantly change metabolite concentrations, but not to significantly induce compositional changes. Taken together, our results show that chitin is not harmful to the intestinal epithelial barrier nor affects the gut microbiota composition and only to a limited extent supports production of SCFAs.

The second tissue of the intestinal immune barrier is the lamina propria. The lamina propria is home to a large collection of immune cells of which macrophages represent an important subset.^[38] Gut macrophages are important for maintaining intestinal homeostasis as they regulate the inflammatory response to microbes that breach the epithelium, scavenge dead cells and their metabolites, and protect the mucosa against harmful pathogens.^[39,40] Of note, we did not observe any transport of SC to the basolateral medium in small-intestinal-like transwell culture (Figure S3, Supporting Information). This makes the alternative routes of exposure of luminal content to macrophages, via microfold cells or by directly sampling the lumen, the most likely mechanism of macrophage interaction with chitin.^[41] Microfold cells are specialized intestinal epithelial cells that are associated with Peyer's patches and transport luminal antigens or microbes to this Peyer's patch in which, among other immune cells, macrophages reside.^[42] Alternatively, macrophages, but arguably also a subset of dendritic cells, can directly sample luminal antigens via sending their protrusions out into the lumen by means of the CX3CR1 receptor.^[41] The interaction between chitin and macrophages has been extensively investigated, however, the response of macrophages to chitin varied in many studies.^[16] The different responses of macrophages to chitin putatively resulted from variation in the physicochemical properties of chitin including size, DA, source, and contamination.^[43] Correlative analysis of these studies is hampered by incomplete physicochemical characterization of the chitin preparations. Here, we used chitin that we defined according to the above mentioned physicochemical parameters (Table 1) and separated on size as this was previously shown to be important for immunological responses.^[44] Indeed, results showed that the immunomodulatory effect of chitin on THP-1

macrophages was inversely linked to its size. Small sized chitin (SC) significantly increased the transcription of chemokine genes (CCL1, CCL15, CCL18, and CCL24) and cytokine genes (TNF- α , IL-10, IL-8, and IL-1 β) and cytokine secretion (IL-8 and IL-10), which was less pronounced upon increasing chitin size (Figure 3). This is in line with previous studies in which murine peritoneal macrophages were exposed to SC (2–10 μm) and IC (40–70 μm) chitin resulting in increased production of TNF α by both and IL-10 by only SC.^[44] To explore a broader spectrum of the impact of SC on THP-1 macrophage activation, we measured secretion of 11 other signaling molecules. The results showed that SC also induced the secretion of IL-1 β and CXCL10 by THP-1 macrophages but not IL-1RA, IL-4, IL-6, IL-12p40, IL-12p70, IL-23, TNF- α , TARC, or arginase (Figure 3D). Notably, this is the first report of IL-8 and CXCL10 secretion by THP-1 macrophages upon exposure to SC. The secreted signaling molecules are linked to recruitment of innate immune cells such as monocytes/macrophages, neutrophils, and nature killer cells.^[45,46] Similar as for signaling molecule production, chitin size was also inversely related to endocytosis, with SC chitin being most efficiently endocytosed (Figure 4A). Chitin endocytosis therefore appeared to be at the basis of the responses by THP-1 macrophages. We inhibited internalization via phagocytosis, clathrin-dependent endocytosis, or caveolin-dependent endocytosis and found that secretion of IL-1 β , IL-8, IL-10, and CXCL10 were all mediated through clathrin-dependent endocytosis of SC (Figure 4C). In contrast, Da Silva and colleagues found that SC-mediated TNF α production was to some extent a result of phagocytosis and partially blocked by cytochalasin D and nocodazole.^[44] However, they did not investigate the effect of inhibiting clathrin-coated endocytosis. In general, cells employ phagocytosis to engulf large particles ($>1 \mu\text{m}$), and clathrin-mediated endocytosis to internalize small particles after binding to membrane receptors.^[47,48] It has also been demonstrated that clathrin-coated pits can be hijacked by bacteria up to 6 μm in length.^[49] A similar mechanism might apply to the internalization of chitin particles. Finally, we demonstrated that the surface receptors TLR-2, dectin-1, mannose receptor, and galectin-3 receptors were involved in this process (Figure 4B), which was also in line with previous findings.^[17,44,50] The present study focused on multiple *in vitro* models to investigate the effect of chitin particles on intestinal immunity and microbiota. The epithelial cells model and microbiota assays provide novel insight into potential interaction and suitability of chitin as dietary supplement. Using the THP-1 macrophage cell model we extended our understanding of the immunomodulatory potential of chitin. Together the results indicate that large-sized chitin could act as prebiotic and increase intestinal acetate levels, which has shown to beneficially impact the host energy and substrate metabolism.^[51] Furthermore, shrimp-derived small sized chitin with a DA of $\approx 83\%$ does not impede the intestinal epithelial barrier, but activates macrophages to produce pro-inflammatory signaling molecules and putatively recruit a wide array of innate immune cells. This activity could be beneficial to subjects suffering from parasitic infections^[52] or allergic reactions^[53] as small-size chitin interventions in murine studies demonstrated to relief symptoms in such models. In contrast, caution should be taken with regard to subjects suffering from autoimmunity^[54] or IBD.^[55] Taken together, the immune activating potency of chitin warrants further *in vivo*

analysis and be tailored to the immune status of the subjects. Further (mechanistical) research and animal studies are required to better specify the nature of the immunomodulatory potential, but moreover unified methods and description of physicochemical parameters of chitin are required to effectively correlate findings.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

L.D., C.G., and H.J.W. conceived and designed the experiments. L.D., M.T., and R.A. performed the experiments. L.D. and R.A. performed data analysis and interpretation. L.D. and C.G. wrote the manuscript. All authors read and approved the final manuscript.

Keywords

chitin, intestinal epithelium, immunomodulation, microbiota, SCFAs

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